Chapter 6

SUMMARY

Monocyte chemoattractant protein-1 (MCP-1) is an important chemokine having chemotactic properties for monoctes and T-lymphocytes. It plays a crucial role during M.tb infection by recruiting macrophages and T-cells to the site of infection. This chemokine plays a very important role in granuloma formation during M.tb infection and helps controlling the dissemination of M.tb. The importance of MCP-1 at protein level has been reported in several diseases, including tuberculosis. During tuberculosis MCP-1 also plays a very pivotal role in T cell response and polarization. The single nucleotide polymorphism -2518A/G was reported to be associated with several diseases, including tuberculosis, in many studies, while, being unrelated with the disease in some other studies. The results are found to be inconclusive. The polymorphism was reported to be modulating the transcription and translation of the MCP-1 gene in response to different stimulations. The -2518G allele was found to be associated with the increased production of MCP-1 at both transcriptional and protein level. While in some studies contrast findings were reported where they found the association of -2518A allele with increased production of MCP-1 at protein level. Meta analysis also explored the association of -2518A/G polymorphism in tuberculosis in Asian population. Another polymorphism at the regulatory region of MCP-1 gene, -362G/C, is also found to be associated with tuberculosis.

Considering the importance of MCP-1 and -2518A/G and -362G/C polymorphism, the present study was designed to understand the likely role of these polymorphisms in tuberculosis and leprosy in the populations of Agra and nearby regions of northern India. The major objectives of the study and the findings are summarized below.

1. MCP-1 genotyping was performed for -2518 A/G and -362G/C polymorphism in tuberculosis cases and healthy controls. Pulmonary tuberculosis cases of category I & II attended the State Tuberculosis Demonstration Centre, Agra, were included in the study. All TB cases were diagnosed by the physician and found to be suffering from tuberculosis. All TB cases included in the study were smear and culture positive. Healthy individuals associated with cases or residing in close contact of TB cases
were included in the study. Healthy student volunteers, who come for shorter duration for training from different regions, to National JALMA Institute for Leprosy and Other Mycobacterial Diseases were also included in the study as random healthy controls.

a) Genotyping for -2518A/G polymorphism was done in 373 PTB cases and 248 healthy controls by PCR-RFLP method. The frequency of G/G genotype in PTB cases (11.26%) was found to be higher as compared to controls (6.04%) and hence, statistically found to be associated with PTB (Odds ratio=1.7 and p=0.02). In contrast, the frequency of A/G genotype (43.15%) was higher in healthy controls as compared to PTB cases (31.37%). Thus the A/G genotype was statistically found to be associated with protection against PTB (Odds ratio=0.60 and p=0.002). No difference was observed in the frequency of alleles between PTB cases and healthy controls.

b) Genotyping for -362 G/C polymorphism was done in 330 PTB cases and 235 healthy controls by Real Time PCR using hybridization probe assay. Frequency of C/C genotype in PTB cases (12.73%) was found to be higher compared to controls (7.23%) and statistically found to be associated with PTB (Odds ratio=1.87 and p=0.03). In contrast, the frequency of G/C genotype (44.68%) was higher in healthy controls compared to PTB cases (34.55%). The G/C genotype was statistically found to be associated with protection against PTB (Odds ratio=0.65 and p=0.01). No difference was observed in the frequency of alleles between PTB cases and healthy controls.

c) Strong linkage disequilibrium between -2518A/G and -362G/C polymorphism was observed (D=0.96 and p=0.00).

d) Haplotype A-C, A allele of -2518A/G polymorphism and C allele of -362G/C polymorphism showed very strong association with PTB (Odds ratio=7.23 and p=0.006).

2. The levels of serum MCP-1 and cytokines IL-12p70, IFN-γ, TNF-α, TGF-β were measured in 120 PTB cases and 54 healthy controls. The results were also analysed on the basis of -2518A/G polymorphism.
a) MCP-1, IL-12p70 and TNF-α levels in serum were found to be higher in PTB cases compared to healthy controls. The difference was found to be significant for MCP-1 and IL-12p70 (p<0.05). TGF-β level was found significantly higher in healthy controls than in PTB cases (p<0.05), while no difference was observed in IFN-γ level between PTB cases and controls.
b) The highest level of MCP-1, IL-12p70 and IFN-γ was found in A/A genotype PTB cases, followed by A/G and lowest in G/G genotype PTB cases. The difference was significant between A/A & G/G and A/G & G/G genotype PTB cases for IL-12p70 and IFN-γ (p<0.05) and between A/A & A/G and A/A & G/G genotype PTB cases for MCP-1.
c) The highest TNF-α level was found in G/G genotype PTB cases, followed by A/G and lowest in A/A genotype PTB cases. The difference was significant between A/A & G/G and A/G & G/G genotype PTB cases. Equal level of TGF-β was found in all genotype groups of PTB cases.
d) In healthy controls, highest MCP-1 level was also found in A/A genotype, followed by A/G genotype and lower in G/G genotype healthy controls. However, the difference was significant only between A/A and G/G genotype controls.
e) Significant positive correlation was found between MCP-1 and all studied cytokines in PTB cases and healthy controls except IFN-γ in healthy controls.

3. MCP-1, IL-12p70, IFN-γ, TNF-α and TGF-β were also measured in PBMCs in 28 PTB cases and 18 healthy controls. The cases and controls were stratified according to -2518A/G polymorphism during analysis.
a) MCP-1 level was significantly high in healthy controls compared to PTB cases in unstimulated and in response to stimulation with WCL and rIFN-γ. No significant effect of WCL and rIFN-γ was observed on the production of MCP-1 in both PTB cases and healthy controls. No difference was observed in MCP-1 level between different conditions when analysis was done between and within different genotypes of -2518A/G polymorphism in PTB cases. While in controls, the effect of WCL was variable in different genotypes.
b) The level of IL-12p70 was higher in PTB cases compared to healthy controls, but the differences were not significant. In PTB cases and healthy controls no significant difference was observed between unstimulated and different stimulated conditions with different genotypes of -2518A/G polymorphism. No significant effect of WCL antigen and rIFN-γ on the production of MCP-1 was observed in cases and controls.

c) IFN-γ level was found to be significantly higher in healthy controls compared to controls in all conditions. Both WCL antigen and rIFN-γ induced the secretion of IFN-γ in PTB cases and healthy controls, but the differences were not significant. No significant difference was observed in IFN-γ level when analysis was done between different genotypes and within the genotype in PTB cases. While in controls, in G/G genotype, the difference in IFN-γ level was significant but not in A/A and A/G genotype controls.

d) The TGF-β level was higher in healthy controls compared to PTB cases in all conditions. The concentration at different stimulated conditions was lower than the basal level (concentration at zero hour) in both PTB cases and healthy controls. No significant effect of WCL antigen and rIFN-γ was observed in PTB cases and controls. No difference was observed in TGF-β level, before and after stimulation, when the comparison was made among different genotypes in PTB cases. However in healthy controls with G/G genotype, production of TGF-β was found to be significantly lower in response to WCL and rhIFN-γ compared to the basal level. Whereas, no difference was found within A/A and A/G genotype controls. Between the genotype analysis not showed any difference.

e) At unstimulated condition, the TNF-α level was found higher in PTB cases compared to controls. WCL antigen decreased the level in PTB cases, but increased the level in healthy controls. The difference in the level of TNF-α between different conditions were found significant in healthy controls but not in PTB cases. In PTB cases no difference was found in TNF-α concentration between and within different genotypes in different studied conditions. In healthy controls, no difference was found between different genotypes. But, the level of TNF-α in different conditions was found significantly different among the A/A, A/G and G/G genotype controls.
4. The expression levels of MCP-1 and CCL5, CCL3, IL-8 chemokines at mRNA level were evaluated in 13 PTB cases and 15 healthy controls.

a) The expression of MCP-1, CCL5, CCL3 and IL-8 genes were found higher in PTB cases compared to healthy controls in unstimulated condition. In stimulated condition with WCL antigen the expression of CCL3 and IL-8 was observed higher in PTB cases compared to healthy controls, while the expressions of MCP-1 and CCL5 were lower than in healthy controls. The antigen WCL decreased the expression of MCP-1, CCL5, CCL3 and IL-8 in PTB cases, but increased the expression in healthy controls.

b) No difference was found in the expression of CCL5 between different genotypes in PTB cases when the comparison was made according to -2518 A/G polymorphism.

c) The difference in the CCL3 expression was significant between A/G & G/G and A/A & G/G genotypes in unstimulated condition in PTB cases, while in response to WCL, the difference was significant between A/G & G/G genotypes.

d) The difference in the expression of IL-8 was significant between A/A & G/G genotypes in unstimulated condition, while in response to WCL, the difference was significant between A/G & G/G and A/A & G/G genotypes in PTB cases.

e) The difference in the expression of MCP-1 was significant between A/A & A/G genotypes in unstimulated condition while in response to WCL the difference was significant between A/G & G/G and A/A & G/G genotypes in PTB cases.

f) In healthy controls, the expression of MCP-1 and CCL5, CCL3, IL-8 genes was found to be significantly different between A/A & AG, A/G & G/G and A/A & GG genotypes in unstimulated condition. The level of expression was also found significant between A/A & A/G and A/A & G/G genotypes in WCL stimulated condition.

Our observations from in vivo experiments indicated the higher levels of MCP-1 and Th1 type cytokines, IL-12, IFN-γ and TNF-α in PTB cases compared to healthy controls. MCP-1 showed positive correlation with all Th1 type cytokines, IL-12, IFN-γ and TNF-α in both in vitro and in vivo experiments in PTB cases while no such correlation was found in healthy controls. As all these Th1 cytokines play important role in the development of CMI against M.tb and MCP-1 showed a strong correlation, it indicates about some role of MCP-1 in the development of CMI against M.tb.
infection. This observation indicates the interaction of MCP-1 and Th1 type cytokines during the diseases process. But, studying other factors influencing this interaction was a limitation in our study. To our interest, we observed a significant positive correlation of serum MCP-1 with AA genotype in all study subjects. Possibly other immunological/environmental factors alongwith MCP-1 influencing the outcome of *M.tuberculosis* infection. However, this correlation of MCP-1 production with genotypes could not be observed in PBMC culture supernatants. This could be possibly due to the limited sample size belonging to each genotypic subjects. It could be inferred that although MCP-1 production is related to the specific genotype of -2518A/G MCP-1 polymorphism, the disease phenotype of TB may be dependent on various factors and may be multifactorial which need to be addressed in future studies.

5. MCP-1 genotyping was performed for -2518 A/G and -362G/C polymorphism in leprosy cases and healthy controls. Leprosy cases of all category TT, BT, BB, BL, LL attended the OPD of National JALMA Institute for Leprosy and Other Mycobacterial Diseases were included in the study. Healthy individuals associated with cases or residing in close contact of leprosy cases were included in the study. Healthy student volunteers of National JALMA Institute for Leprosy and Other Mycobacterial Diseases were also included in the study as random healthy controls.
   a) Genotyping for -2518A/G polymorphism was done in 577 leprosy cases and 280 healthy controls. No difference was observed in the frequency of G/G, A/G and A/A genotypes and alleles between leprosy cases and controls.
   b) Genotyping for -362 G/C polymorphism was done in 548 leprosy cases and 295 healthy controls. The frequency of C/C genotype in leprosy cases (10.40%) was found to be higher compared to controls (5.08%) and statistically found to be associated with leprosy disease (Odds ratio=2.08 and p=0.01). While frequency of G/C genotype (55.25%) was higher in healthy controls compared to leprosy cases (47.81%). The G/C genotype was statistically found to be associated with protection against Leprosy (Odds ratio=0.74 and p=0.03). No difference was observed in the frequency of alleles between leprosy cases and healthy controls.
c) Analysis between different categories of leprosy cases and controls showed significant difference in genotype frequency between BB & LL cases compared to healthy controls for -362 G/C polymorphism.

d) Again, no difference was found when analysis was done between reactional vs nonreactional leprosy cases and between different categories of leprosy cases for both -2518A/G and -362 G/C polymorphism.

e) Strong linkage disequilibrium between -2518A/G and -362G/C polymorphism was observed (D=0.88 and p=0.00).

f) Haplotype A-C (Odds ratio=0.57, p=0.03) and G-G (Odds ratio=0.26, p=0.00001) were found to be associated with healthy controls and provides protection against leprosy.

6. Serum MCP-1 and cytokines IL-12p70, IFN-γ, TNF-α, TGF-β level was measured in 120 Leprosy cases and 106 healthy controls. The results were also analysed on the basis of -2518A/G polymorphism.

a) MCP-1, IL-12p70, TNF-α and TGF-β level in serum was found significantly higher in leprosy cases compared to healthy controls (p<0.05).

b) Only IFN-γ level was significantly elevated in healthy controls as compared to leprosy cases (p<0.05).

c) The highest level of MCP-1 and TGF-β was found in A/A genotype leprosy cases, followed by A/G and lowest in G/G genotype leprosy cases. The difference was significant between A/A & G/G and A/G & G/G genotype leprosy cases (p<0.05).

d) IL-12p70 and TNF-α level was found highest in G/G genotype leprosy cases, followed by A/G and lowest in A/A genotype leprosy cases. For IL-12p70, the difference was significant between A/A & G/G and A/G & G/G genotype leprosy cases, while for TNF-α, the difference was significant between A/A & A/G and A/G & G/G genotype leprosy cases (p<0.05).

e) In healthy controls, slightly elevated level of MCP-1 was found in G/G genotype, compared to A/A and A/G genotype controls. No difference was observed in IFN-γ level between different genotypes in healthy controls.
f) Almost equal level of IL-12p70, TNF-α and TGF-β was found in A/A and A/G genotype controls, while in G/G genotype controls, the level was lower. The difference was significant between A/A & G/G and A/G & G/G genotype controls (p<0.05).

g) Significant negative correlation was found between MCP-1 and IL-12p70 and positive correlation was found with TGF-β.

7. PBMCs were isolated from 39 leprosy cases and 27 healthy controls and stimulated with WCS *M. leprae* antigen and rIFN-γ. MCP-1 and cytokines IL-12p70, IFN-γ, TNF-α, TGF-β were measured in the culture supernatant and analysed between cases and controls and as per -2518A/G genotypes.

a) MCP-1, TNF-α and TGF-β level was found to be higher in healthy controls compared to leprosy cases in both unstimulated and in response to WCS antigen. MCP-1 and TGF-β level was also higher in controls compared to cases in response to rIFN-γ, while reverse observation was found for TNF-α. But, the differences were significant only for MCP-1 and TGF-β.

b) In leprosy cases, the level of IL-12p70 and IFN-γ was higher compared to healthy controls in all studied conditions.

c) WCS antigen slightly decreased the production of MCP-1, IL-12p70 and IFN-γ, while induced the production of TNF-α and TGF-β in leprosy cases.

d) In healthy controls, no effect of WCS antigen was observed on the production of MCP-1, IL-12p70, IFN-γ, and TGF-β, but increased the production of TNF-α.

e) IL-12p70, TNF-α and TGF-β production was elevated in leprosy cases in response to rIFN-γ, while MCP-1 slightly decreased compared to unstimulated condition. In healthy controls, slightly higher amount of MCP-1, TNF-α and TGF-β was detected in response to rIFN-γ, but IL-12p70 level goes down in comparison to unstimulated condition.

f) We observed no significant difference in the level of any studied cytokines between different conditions after comparison was done between and within the A/A, A/G and G/G genotypes of -2518A/G polymorphism in both leprosy cases and controls.
g) MCP-1 and IFN-γ level was found to be higher in LL leprosy cases compared to BT and BB/BL leprosy cases. While no considerable variation in the level of IL-12p70, TNF-α and TGF-β was observed between the LL, BT and BB/BL leprosy cases.

8. The expression levels of MCP-1, CCL5, CCL3 and IL-8 chemokines at mRNA level were evaluated in 20 leprosy cases and 10 healthy controls.

a) The expression of CCL5 gene was found significantly higher in healthy controls, compared to leprosy cases in both unstimulated and in response to WCS antigen of *M. leprae*.

b) No significant difference was observed in the expression level of MCP-1, CCL3 and IL-8 between leprosy cases and controls in both unstimulated and in response to *M. leprae* WCS antigen conditions.

c) WCS showed negative impact on the expression of MCP-1 and IL-8 and reduced their level compared to unstimulated condition in both leprosy cases and controls, but the differences was not significant. While WCS showed no impact on the expression of CCL5 and increased the CCL3 expression in leprosy cases, but decreased in healthy controls.

d) Analysis of the results on the basis of -2518A/G polymorphism revealed that the expression level of MCP-1 was found to be higher in A/A genotype, followed by A/G and lower in G/G genotype in both leprosy cases and healthy controls. Significant difference in the expression of MCP-1 between A/A & A/G, A/G & G/G and A/A & G/G genotypes was observed in unstimulated condition, while in stimulated condition, no difference was observed between genotypes in PTB cases.

e) In healthy controls no significant difference was observed in the expression of MCP-1 and CCL5, CCL3 and IL-8 chemokine genes between A/A, A/G and G/G genotypes in both stimulated and unstimulated conditions.

Overall our studies for the first time report MCP-1 gene polymorphisms in leprosy. These studies, however, could not establish definitive role of MCP-1 in immune response against leprosy, because the level was higher in leprosy cases in invivo studies. But the results of in vitro studies did not match with in vivo findings, due to which it is difficult to establish any correlation of MCP-1 immunologically against
leprosy. In vitro studies are more informative than in vivo studies, because these show specific effect of particular stimulation, whereas in vivo responses may be the result of multiple factors, are dynamic and change time to time. Similarly, no clear role of -2518A/G gene polymorphism was observed immunologically. Although differences were found between different genotypes. A similar pattern was also found in healthy controls, immunologically. So, it is not possible to establish a correlation of -2518A/G polymorphism and the immune response against leprosy.

9. Nuclear extract was isolated from THP-1 cell line, stimulated with WCL and WCS antigens of *M.tb* and *M.leprae*, respectively, and incubated with oligonucleotides designed against -2518A/G polymorphic site.

   a) EMSA experiments indicates that a protein binds to both the oligonucleotides in all studied condition.

   b) The binding protein was identified as transcription factor, Zinc finger protein, by MALDI-TOF.