Tuberculosis co-infection in HIV-1 patients constitutes the main burden of infectious disease and is the leading killer of HIV positive individuals. The severity of the co-infection has been a hot research topic for the past decade and has increased our understanding of how HIV increases susceptibility to TB. It is widely accepted that HIV causes depletion of CD4 T-cells, which is likely to contribute to the susceptibility of co-infection to *M. tuberculosis*, as this subset is important in the control of TB. However, our current knowledge about the mechanisms of interaction of the two pathogens still has many gaps that need to be bridged in order to develop preventive measures against the two diseases. As CD4+ T-regulatory cells are known to modulate the activity of CD4 T-cells, in the present study titled ‘Role of T-Regulatory Cells in HIV-1 Patients With and Without *Mycobacterium tuberculosis* Co-infection’, we have carried out investigations to unravel the possible role of Treg cells in HIV infected individuals at different stages of the disease along with the basis of the synergistic relationship of HIV with TB. The important leads provided by the results from our experimental findings are summarized below:

There was a significant decrease in the frequency of Treg cells in early phase of the HIV-1 infection correlating with significant decreased levels of Foxp3 expression as revealed by immunophenotyping. These findings indicate that at initial stage of infection there is preferential killing/decrease of Foxp3 expressing CD4 T cells in the peripheral blood. At the same time, decreased FoxP3 expression may contribute to the high levels of immune activation and HIV-1 replication that is seen in early phase of the disease. However, with HIV-1 disease progression, there was an up-regulation of both, frequency of these Treg cells as well as level of FoxP3 expression. We noticed a significant inverse relationship of CD4 count with respect to the frequency of Treg cells and FoxP3 expression making these subjects more vulnerable to active tuberculosis in this immuno-compromised state. These findings are correlating and in agreement with the Treg cell function. There was a significant negative correlation between percent Treg cell frequency and T-cell proliferation, exhibiting intact suppressive activity inherent in these cells. Although, the hallmark of
HIV infection results in immunodeficiency state due to loss of CD4$^+$ T cells, there was further down regulation of CD4 T cell activity with increase in Treg cell frequency making the infected individual severely immunodeficient and increasing its susceptibility to *M.tuberculosis*.

With the onset of PTB co-infection in dampened immune system of HIV-1 individuals, we observed further significant increase in Foxp3 expression in CD4 T-cells in comparison to individuals infected only with HIV-1 and similar CD4 count (less than 250 cells/$\mu$l). Though statistically non-significant, there was also an increase in the Treg frequency in HIV-1 patients with PTB co-infection when compared with HIV-1 individuals with compatible CD4 cell count (CD4 count less than 250 cells/$\mu$l) indicating a possible role of *M.tuberculosis* in inducing Foxp3 expression. To confirm the above findings, we examined the Treg cells in PTB subjects. We noticed that *M.tuberculosis* positively up-regulates Foxp3 expression per cell (MFI) in Treg cells and significantly increases the frequency of FoxP3 positive cells among CD4 T-cells when compared to healthy controls. On the contrary, in a few studies reported, Treg levels as measured by quantitative RT-PCR have shown a decreased level of FoxP3 mRNA in HIV-1 subjects. Taking these observations into consideration, we carried out relative mRNA expression study of FoxP3 gene in PBMCs of HIV-1 patients at different stages of the disease. There was no significant difference in relative FoxP3 mRNA expression within the HIV-1 groups with disease progression. Further, we found a decrease in FoxP3 mRNA expression in PTB subjects in comparison to healthy controls, though statistically non-significant. These results were in contrary to protein levels done by flowcytometry. Absence of correlation between FoxP3 protein and total mRNA levels raised a question and prompted us to look further into the splice variants for a possible answer. FoxP3 variant 1 mRNA expression was significantly higher in PTB individuals in comparison to healthy controls. Further, its expression was also significantly higher in the HIV-PTB co-infected individuals when compared to HIV-1 individuals with similar CD4 count. These results were in agreement with the flowcytometry results. On the contrary, there was no significant difference in the expression of FoxP3 variant 2 between the studied groups. These results indicate a direct relationship between
Chapter 8: Summary

FoxP3 mRNA expression and FoxP3 protein expression (MFI) for all the studied groups only with variant 1 and not with variant 2 or whole FoxP3 gene expression.

The FoxP3 expression and Treg function have been linked with HO-1, and with the hypothesis that HO-1 derived carbon monoxide can induce the DosR dormancy regulon in mycobacteria leading to latency and survival of the organism inside the host granuloma. Such a situation could contribute to the fact that people living with HIV have 20-30 times higher lifetime risk of developing active tuberculosis. Hence, we studied the HO-1 mRNA expression in patient groups. We were successful to show a significant increase in the expression of HO-1 in HIV patients. These results are in line with previous findings where HO-1 has been shown to be induced by oxidative stress, and pro-inflammatory cytokines that are characteristic of HIV-1 infection. Thus immuno-compromised state was further deteriorated with significant increase in HO-1 expression (via carbon monoxide) in HIV-1 (PTB negative) group irrespective of the stage of the disease which is known to play a role in promoting latency and survival of Mycobacteria inside the host granuloma. This increase in HO-1 makes the environment favourable for mycobacterial infection. On the other hand, in HIV-PTB co-infected individuals, we observed significantly lower HO-1 expression when compared to HIV-1 subjects with similar CD4 count (subject group with CD4 count less than 250 cells/μl). Such a decrease in HO-1 expression by M.tuberculosis in HIV co-infected subjects results in the increase in redox stress which has been shown to reactivate latent HIV-1 provirus (Pace and Leaf, 1995) resulting in high plasma viral load.

The above findings further prompted us to look at the redox sensitive NF-κB expression, which is known to bind to the Long Terminal Repeats of the integrated HIV-1 provirus and induces its replication. Interestingly, we also observed that M.tuberculosis infection in HIV-1 individuals significantly increases NF-κB expression thereby making the conditions favorable for HIV-1 replication. This is further supported by the fact that as in correspondence with a significant decrease in HO-1 in HIV-PTB individuals there was a significant increase in NF-κB in comparison to HIV-1 infected individuals with similar CD4 count (CD4 count less than 250 cells/μl). These data suggests that decreasing HO-1 activity by
Chapter 8: Summary

*M. tuberculosis* co-infection in HIV-1 individuals can reactivate latent HIV-1 which may contribute to high viral load.

In HIV-1 subjects, as stated earlier, we found a significant decrease in the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup> T-regulatory cells in subjects with CD4 count more than 500 cells/μl followed by an increase in its frequency with disease progression. These observations further prompted us to look at the chemokine receptors CCR5 and CxCR4, the main HIV-1 co-receptors, to find out if they have a role in the change seen in Treg cells frequency within CD4 T-cells concomitant with disease progression. Frequency of CD4 T cells expressing CCR5 was found to be up-regulated in HIV-1 infected individuals when compared to the healthy controls, irrespective of the stage of the disease which is in agreement with the previous findings (Bleul *et al.*, 1997; Ostrowski *et al.*, 1998; Giovannetti *et al.*, 1999). Looking at the Treg population, there was a higher percentage of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> Treg cells expressing CCR5 in comparison to CD25<sup>low/negative</sup> effector cells (p<0.0001) and could be preferential initial targets for HIV-1. We found a higher frequency of CCR5 positive Treg cells only in the HIV-1 group with CD4 counts more than 500 cells/μl (p=0.0021). This makes these cells more vulnerable to HIV infection in the initial stages of the disease. This further supports our observation of initial decrease in percent Treg cell frequency. On the other hand, in the advance stage of the disease with CD4 count less than 250 cells/μl, CCR5 positive Treg cells had significantly lower frequency when compared to HIV-1 individuals with CD4 count more than 500 cells/μl (p=0.0022). This change in CCR5 expression might be responsible for the increase seen in the frequency of Treg cells at advanced stage of the disease.

In contrast to CCR5, CxCR4, the main co-receptor for HIV-1 entry in advance stage of the disease, showed lower frequency of CD4 T-cells expressing CxCR4 at a later stages of HIV-1 disease. This is in agreement with the previous observation in CD4 T-cells in HIV subjects (Bleul *et al.*, 1997; Ostrowski *et al.*, 1998; Giovannetti *et al.*, 1999). It is interesting to note that there is a reversal in the scenario when looking at CxCR4 expression. There was an increase in the CxCR4 expression in advance stages of HIV-1 when compared to asymptomatic HIV-1 subjects. This could explain the decrease in the frequency of CxCR4 positive CD4 T-cells in later stage of the...
disease due to CxCR4 tropic viral shift and killing of these cells. Again, there was a significant decrease in percentage of CD4 T-cells expressing CxCR4 (p=0.0033) and CxCR4 expression per cell (MFI, p=0.0004) in CD25\textsuperscript{high} Treg cells when compared with CD25\textsuperscript{low/negative} cells in healthy controls. This could be the possible explanation for the decrease in percent CxCR4 positive CD4 T-cells in CD4\textsuperscript{+}CD25\textsuperscript{low/negative} within HIV-1 groups with disease progression but with no effect on low expressing CxCR4 positive Treg cell number.

Further, to confirm these findings, we studied the mRNA expression of CxCR4 and its splice variant 2. We found a significant inverse relationship of CxCR4 expression with absolute CD4 count i.e. increase in its expression with progression of HIV-1 disease. There was a significant positive correlation between NF-κB and CxCR4 gene expression in HIV subjects. NF-κB has been shown to bind to the CxCR4 promoter region, and treatment with NF-κB inhibitor reduces CxCR4 expression in MDA-MB-231 breast cancer cells (Helbig \textit{et al.}, 2003) and maybe one of the possible reasons for increased CxCR4 expression.

As per recent reports, Rac1 activity is required to maintain and regulate the responsive conformation of CxCR4, and its inhibition prevents HIV-1 infection (Zoughlami \textit{et al.}, 2012). We found Rac1 expression was significantly low in patients with a CD4 count more than 500 cells/μl when compared to healthy controls. These results indicate and support the findings that initially there is R5 viral infection. Low Rac1 expression means CxCR4 co-receptor is un-useable by HIV-1. Though, Rac1 does not affect CCR5 receptor conformation or R5 viral infection (Zoughlami \textit{et al.}, 2012). In advanced stage of the disease, we found an increased Rac1 expression. This change in Rac1 expression would make CxCR4 conformation favorable to HIV-1 attachment and possibly was one of the reasons for R5 to X4 viral shift with HIV-1 disease progression. We found a significant positive correlation of Rac1 with NF-κB and CxCR4 in healthy controls. Although this correlation of Rac1 and CxCR4 was not noticed in HIV-1 patients, NF-κB retained significant positive correlation in HIV-1 subjects too.
Chapter 8: Summary

To summarize, the present study has provided the experimental basis which highlights the interaction and interplay between HIV and *M. tuberculosis*. It has aptly addressed the controversy regarding Treg number in HIV and showed different Treg frequency at different stages of the disease. Apparently, HIV-1 makes conditions favorable for TB via increasing Treg cells, FoxP3 expression and HO-1 expression, making the individual severely immunosuppressed. On the other hand, *M. tuberculosis* increases HIV-1 replication by increasing redox stress (reducing HO-1), FoxP3 and NF-κB expression. Our findings appear to strongly indicate that the CCR5 expression was selectively up-regulated in CD25\textsuperscript{high} and CD25\textsuperscript{intermediate} T cells while CxCR4 expression was up-regulated on CD25\textsuperscript{low/negative} effector cells during *M. tuberculosis* co-infection. Further, an increase in Rac1 expression in advanced stage of HIV-1 disease favors X4 viral shift but with *M. tuberculosis* co-infection, the Rac1 expression was significantly down-regulated, making CxCR4 non-usable by HIV-1. This observation is corroborated by the finding that R5 viral variants were preferentially recovered from HIV infected patients with active TB (Morris *et al.*, 2001). We have recognized inter-relationships of genes and their behavior in HIV-1 therapy naïve individuals, highlighting how the virus manipulates the host machinery during *M. tuberculosis* co-infection to its advantage.