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*M. tuberculosis* continues to be one of the most dreaded pathogens around the world and its interaction with HIV fuels both the epidemics. Of the estimated 39 million people living with HIV, about one third are estimated to have concomitant latent infection with *M. tuberculosis*. In 2010, of 8.8 million new TB cases reported worldwide, 1.1 million were co-infected with HIV. From these, India and China account for approximately 40% of all notified cases of TB (WHO, 2011a). With an estimated 350,000 deaths (320,000-390,000) due to TB co-infection, makes it a leading cause of mortality in people living with HIV/AIDS. Depending on the prevalence of HIV in the population, the risk for active tuberculosis becomes 20–37 times higher in individuals living with HIV than in the general population (Getahun et al., 2010). HIV-related tuberculosis remains a serious challenge for the health sector’s response to HIV. In this study, we looked at few of the mechanisms to explain, why HIV-1 patients are more vulnerable to *M. tuberculosis* and how TB accelerates course of HIV infection.

The role and frequency of T-regulatory cells in HIV-1 infected individuals has long been controversial/debatable. In the present study, we have analyzed the frequency and suppressive function of Treg cells in HIV-1 infected subjects at different stages of the disease and shown how pulmonary tuberculosis affects this cell population. Our study demonstrates an initial decrease in Treg population, expression of FoxP3 and suppressive function in asymptomatic phase of HIV-1 infection, followed by a constant rise in these parameters as the disease progresses. The increase in the Treg frequency and FoxP3 expression correlated negatively with absolute CD4 count, indicating an onset of immuno-compromised state making these subjects more vulnerable to opportunistic infections including active tuberculosis. This immuno-compromised state was further substantiated with up-regulation in the expression of certain factors like HO-1 expression irrespective of the stage of the disease which are known to play a crucial role in promoting latency and survival of Mycobacteria inside the host. In this study, we observed that HO-1 (which in previous studies has been shown to have protective role against HIV-1 via its catabolic products) was significantly decreased and NF-κB (which induces HIV-1 replication via binding to the LTR region of integrated HIV-1 provirus) was significantly increased in HIV-PTB
co-infected subjects in comparison to HIV subjects (PTB negative) with similar disease state resulting in increased HIV-1 replication.

On estimating the Treg cell frequency and FoxP3 expression among CD4+ T cells we found a significant decrease in the frequency of CD4+Foxp3+ Treg cells (p=0.0003) in HIV-1 infected individuals in early stage of disease (with CD4 count > 500 cells/μl) correlating with significant decreased levels of Foxp3 expression per cell (MFI, p=0.0337). These findings suggest an initial preferential killing of Foxp3 expressing CD4+ T-cells from the peripheral blood in this disease condition or probable sequestration of these in the lymphoid tissue. At the same time, decreased FoxP3 expression makes the existing Treg cells functionally inactive thereby contributing to the high levels of immune activation that promote HIV-1 replication seen in this phase of the disease. Concomitantly we observed an increase in the frequency of CD4+FoxP3+ Treg cells and FoxP3 expression with HIV disease progression in terms of decline in the CD4 count. Some studies suggest that Treg cells get depleted by HIV infection (Kinter et al., 2004; Apoil et al., 2005; Eggena et al., 2005), while others indicate that the proportion of these cells may be rather increased (Weiss et al., 2004; Tsunemi et al., 2005). This difference in opinion could be due to difference in the stage of the disease at the time of sampling by the researchers or infection with different HIV-1 strains.

The level of FoxP3 gene expression has been positively linked with not only the maintenance but also the suppressive capacity of Treg cell population (Fontenot et al., 2003; Sakaguchi, 2005). We found that there was a significant positive relationship between FoxP3 expression vis-à-vis Treg frequency and their suppressive activity towards proliferative response to PHA stimulation in individuals infected with HIV-1. On the other hand the FoxP3 expression as well as Treg frequency was inversely related to CD4 counts in these individuals. Though, the hallmark of HIV infection is the depletion of CD4+ T-cells, there is further down modulation of CD4 T-cell activity with increase in Treg cell frequency making the individual severely immuno-compromised thereby increasing its susceptibility to M.tuberculosis. The increase in the frequency of these cells was also directly associated with the inhibition of IFN-γ secretion as the treatment of patients infected with M.tuberculosis results in a decrease in the number of regulatory T cells and restored production of IFN-γ.
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(Ribeiro-Rodrigues et al., 2006; Chen et al., 2007). On a similar note, in an experimental tuberculosis model, the depletion of Foxp3+ cells in infected C57BL/6 mice resulted in fewer bacteria in the lungs as compared to mice with Foxp3+ cells (Scott-Browne et al., 2007). While C57BL/6 mice exhibited an increased magnitude of their Th1 response and a lower effector function of their regulatory T cells, BALB/c mice had a lower magnitude of Th1 response and effector function of their regulatory T cells that suppressed IL-2 and IFN-γ secretion. These findings suggest that regulatory T cells may potentially represent a susceptibility factor in tuberculosis.

With the onset of *M. tuberculosis* co-infection in HIV-1 infected individuals, we observed significant increase in the Foxp3 expression and Treg number in comparison to HIV-1 infected individuals with similar CD4 count (i.e. similar HIV disease status). These results indicate a possible role of *M. tuberculosis* in inducing Foxp3 expression. To confirm this we examined PTB individuals and found significantly higher FoxP3 expression in these individuals as compared to healthy controls.

Also there was a significantly low frequency of FoxP3+CD4 cells in early stage of the disease followed by a gradual increase with the progression of disease. These observations prompted us to look at the expression of CCR5 and CxCR4 on Treg cell sub population for their possible role in influencing the change in Treg cell frequency with HIV disease progression. Frequency of CD4+ T-cells expressing CCR5 was found to be increased in HIV-1 infected individuals when compared to the healthy controls, irrespective of the stage of the disease, which is in concordance with the previous findings (Bleul et al., 1997; Ostrowski et al., 1998; Giovannetti et al., 1999). With previous reports in non-subtype C infections, the depletion of CCR5 expressing memory cells in acute infection may drive viral evolution towards CxCR4 (Cilliers et al., 2003; Moore et al., 2004), and recent reports showing about 30% of CxCR4-utilising viruses in untreated and treated HIV-1 subtype C infected adults from South Africa, Malawi, and Zimbabwe, suggesting a shift in viral properties (Johnston et al., 2003; Connell et al., 2008; Raymond et al., 2010). This change in CCR5 expression might be responsible for increase in the frequency of Treg cells in the advanced stages of the disease. In order to address this question we selected three different sub-populations of CD4+ cells based on the intensity of CD25 staining viz:
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CD4^+CD25^{high}, CD4^+CD25^{intermediate}, CD4^+CD25^{low/negative}. There was higher percentage of CD25^{high} Treg cells expressing CCR5 in comparison to CD25^{low/negative} effector cells (p<0.0001) and which could make them preferential targets for R5 viral attack in the initial stages of disease. On the other hand, in advanced stage of the disease (CD4 count <250 cells/μl), the number of CCR5^+ Treg cells were significantly lower when compared to HIV-1 infected individuals in early stage of disease (p=0.0022).

On the contrary we observed a lower frequency of CD4 T-cells expressing chemokine receptor CxCR4, in the later stages of HIV disease when compared to healthy controls, although statistically non-significant. This is in concordance with previously reported observation in HIV subjects (Bleul et al., 1997; Ostrowski et al., 1998; Giovannetti et al., 1999). It has been suggested and hypothesized that there is relative abundance of R5-tropic virus in early stage of the disease due to CCR5 expressing macrophages at the site of primary infection (Davenport et al., 2002; Ribeiro et al., 2006) and higher turnover rate of CCR5 expressing memory/activated T-cells in comparison to slow-dividing CxCR4 expressing naive-cells (Macallan et al., 2003; Macallan et al., 2004; Zhang et al., 2013). Why then emergence of X4-tropic strains are associated with disease progression? Working on this line, we observed an increase in the CxCR4 expression in advanced stages of HIV-1 when compared to asymptomatic early stage HIV-1 subjects. This could explain the decrease in the frequency of CxCR4^+ CD4 T-cells in later stages of the disease and increase in the production of X4 tropic virus i.e. the viral shift and killing of these cells.

In contrast to CCR5, there was a significantly lower frequency of CD4^+T-cells expressing CxCR4 (HIV co-receptor) in CD25^{high} cells (Tregs) when compared to CD25^{low/negative} cells in healthy controls. Further, the expression of CxCR4 was also observed to be significantly lower in these Treg cells. This could be a possible explanation for the decrease in the frequency of CD4^+T-cells expressing higher CxCR4 only in CD25^{low/negative} subpopulation within HIV-1 infected individuals with disease progression (possibly because of HIV co-receptor switching). This decrease was not observed in low CxCR4 expressing Treg cell in HIV-1 infected individuals.
However the picture was totally different on perusing the data from patients infected with only *M. tuberculosis* or co-infected with HIV. It is well known that *M. tuberculosis* co-infection in HIV may create a microenvironment enhancing the productive infection of T-cells by HIV (Bentwich *et al.*, 1995; Fraziano *et al.*, 1999; Bentwich *et al.*, 2000; Djoba Siawaya *et al.*, 2007). We observed that HIV-1 individuals with active pulmonary tuberculosis manifested increased CCR5 (p=0.0364) and not CxCR4 (p=0.1914) expression per cell (MFI) on CD4 T-cells when compared with HIV-1 subjects with similar CD4 count (<250 cells/μl) suggesting preferential utilization of CCR5 rather than CXCR4 by virus in *M. tuberculosis* co-infected individuals. This falls in line with reports which demonstrated that R5 viral variants were preferentially recovered from patients with active TB (Morris *et al.*, 2003). Furthermore, increased frequency of CD4 cells expressing CCR5 and its up-regulated expression limited to CD25\textsuperscript{high} and CD25\textsuperscript{intermediate} populations only in co-infected individuals with no significant change in CD25\textsuperscript{low/negative} subpopulation indicated the influence of *M. tuberculosis* on these two subpopulations only making them more vulnerable to HIV-1 infection. Whereas, a significant decrease in frequency of CxCR4\textsuperscript{+} T-cells in co-infected individuals could mainly be attributed to a significant decrease in CD25\textsuperscript{low/negative} CD4 T-cell subpopulation. On the other hand, significantly increased CxCR4 expression on CD25\textsuperscript{low/negative} cells during *M. tuberculosis* infection makes it the main population affected in co-infected individuals as evidenced by their low frequency in these individuals. Thus, the data suggest that *M. tuberculosis* co-infection in HIV-1 individuals seems to be selectively modulating the differential expression of these chemokine receptors on different subpopulations of cells thereby leading to their selective depletion during different stages of disease.

In order to delineate the molecular mechanisms behind HIV and *M. tuberculosis* synergism, (Barnes *et al.*, 1991; Daley *et al.*, 1992; Whalen *et al.*, 1995) we evaluated the possible role of immune-regulators like FoxP3 splice variant A and variant B and HO-1 in HIV-1 individuals. HO-1 is a cytoprotective enzyme that breaks down heme to produce carbon monoxide, iron and biliverdin (Fredenburgh *et al.*, 2007). HO-1 induction provides important protection from oxidative stress and cellular damage (Abraham *et al.*, 1995; Lee *et al.*, 1996; Guo *et al.*, 2001). Over the
last two decades it has become apparent that the precursors and catabolic products of heme oxygenase system are capable of antimicrobial and antiviral activities. It therefore becomes important to examine, if HO-1 induction or over expression promotes antiviral activities in HIV-1 infection. The linear tetrapyrrole oxidation of heme, biliverdin, and its reduced derivative, bilirubin have also been reported to directly inhibit the HIV aspartic acid activated protease (DeCamp et al., 1992; McPhee et al., 1996). We found a significant increase in the expression of HO-1 in HIV patients as compared to controls. These results go with the previous findings where HO-1 has been shown to be induced by oxidative stress, and pro-inflammatory cytokines. As HO-1 derived CO can induce the DosR dormancy regulation in mycobacteria leading to latency and survival of the organism inside the host granuloma (Kumar et al., 2008; Regev et al., 2012), this finding supports and goes well with earlier reports and could be one of the reasons for people living with HIV to have a 20-30 times higher lifetime risk of developing active tuberculosis, compared with people without HIV (WHO, 2011c). On the other hand, HIV-\textit{M. tuberculosis} co-infected individuals had significantly lower HO-1 expression when compared to subjects with HIV-1 with similar CD4 count (<250 cells/\( \mu l \)). Decrease in HO-1 expression results in the increase in redox stress which have been shown to cause the reactivation of the latent HIV-1 provirus and apoptosis/depletion of CD4 cells (Pace and Leaf, 1995). The activation of the host cells is accompanied by the activation of the redox-sensitive transcription factor NF-\( \kappa B \) (Lander et al., 1993; Pantano et al., 2006) and its translocation to the nucleus (Greene, 1991), where it binds to the Long Terminal Repeats (Lopes et al.) of the integrated HIV-1 provirus and induces its replication (Nabel and Baltimore, 1987; Williams et al., 2007; Pyo et al., 2008). The redox state of the cell thus simultaneously affects both activation of NF-\( \kappa B \) and reactivation of the latent provirus. This hypothesis was further corroborated in our study, as we observed a significant increase in NF-\( \kappa B \) expression in correspondence to decrease in HO-1 in HIV-\textit{M. tuberculosis} co-infected individuals, which suggests that by \textit{M. tuberculosis} co-infection in HIV-1 individuals causes decrease in the HO-1 expression which may subsequently reactivate the latent HIV-1 leading to faster progression towards AIDS.
Interestingly, there was a very significant positive correlation of NF-κB with both FoxP3 splice variants. Though FoxP3 has been reported to inhibit NF-κB activity in transfected 293 cells or T-cells in some of the studies (Bettelli et al., 2005; Grant et al., 2006), yet others have failed to show such inhibitory effect in human T-cells (Wu et al., 2006). We observed initially low frequency of FoxP3+CD4+ Treg cells in early stages of HIV disease in patients with CD4 count >500 cells/µl, indicating preferential infection (Higher CCR5 HIV co-receptor expression), enhanced viral replication (high viral load in the acute phase of viral replication) and killing (significantly low Treg cell frequency) of these cells. Interestingly, the transcription factor FoxP3, critical for Treg cell development and function, has been shown to modulate the HIV-1 promoter’s transcription activity via enhancing NF-κB binding to HIV-1 LTR in Jurkat cells, thereby enhancing the viral replication (Holmes et al., 2007), although, HIV-1 transcription was shown to be inhibited in FoxP3 transfected primary human CD4 T cells (Grant et al., 2006; Selliah et al., 2008).

Though not consistently found, most studies including ours show an inverse relationship between Treg frequency and CD4 cell count. Treg cell expansion seems to be directly related to the degree of CD4 cell depletion. Contradictory to these studies showing expansion of Treg cells in infected patients, other reports suggested decreased level of Treg cells (Andersson et al., 2005; Apoil et al., 2005; Chase et al., 2008). Interestingly, in the latter studies, Treg levels were measured by quantitative RT-PCR of FoxP3 mRNA in PBMCs (Apoil et al., 2005), purified T cells (Andersson et al., 2005), or CD4 T cells (Chase et al., 2008). Taking a note of these studies, we carried out relative mRNA expression of FoxP3 gene in PBMCs of HIV-1 patients at different stages of the disease. As humans express two main isoforms of FoxP3, variant 1 (NM_104004) and variant 2 (NM_001114377), either of which can confer regulatory function when strongly over expressed (Yagi et al., 2004; Allan et al., 2005; Aarts-Riemens et al., 2008). The main deletional isoform of FoxP3, variant 2 lacks the proline-rich exon 2, which encodes the Leu-X-X-Leu-Leu motif that is required for the binding to the transcription factor retinoic acid receptor-related orphan receptor-α (RORα) (Du et al., 2008), and lacks amino-terminal residues that may mediate the interaction with nuclear factor of activated T cells (NFAT) resulting in transcriptional repression (Bettelli et al., 2005; Lopes et al., 2006). The role of
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FoxP3 variant 2 in human Treg cell biology remains unclear. In HIV-PTB co-infected and PTB infected groups, we found a significant increase in the mRNA expression of FoxP3 variant 1 in comparison to healthy controls, which corresponds well with FoxP3 protein expression per cell (MFI) in these groups. Though when looking at the total FoxP3 mRNA gene expression or FoxP3 variant 2, there was no significant difference in its expression within the HIV-1 groups with disease progression. On the other hand, we found a decrease in FoxP3 expression in PTB subjects, though statistically non-significant, in contrary to increased FoxP3 protein expression in these subjects in comparison to healthy controls.

Comparing the expression level of two Foxp3 splice variants, *M. tuberculosis* seem to be positively regulating Foxp3 variant 1 expression in HIV-1 patients and pulmonary tuberculosis patients. This pattern was not seen in Foxp3 variant 2 indicating a possible role of splice variants in infection. Both the variants showed similar expression profile in PBMC with significant positive correlation with HO-1 in HIV-1 patients indicating possible link between FoxP3 and HO-1 gene. HO-1 has been implicated in the activation as well as induction and/or expansion of Treg cells with constitutive HO-1 expression in human peripheral blood Treg cells but not in resting CD4+CD25- non-Treg cells (Lee *et al*., 2007; Xia *et al*., 2007; George *et al*., 2008; English *et al*., 2009; Burt *et al*., 2010; Carrion *et al*., 2010; Mougiakakos *et al*., 2011). This study lends supports to the later hypothesis indicating significant correlation between HO-1 and FoxP3 expression.

Over the course of infection, the co-receptor usage of HIV changes from CCR5 to CxCR4 in 50% of the infected individuals (Fenyo *et al*., 1988; Tersmette *et al*., 1989; Schuitemaker *et al*., 1992; Berger *et al*., 1999; Blaak *et al*., 2000; Karlsson *et al*., 2005). It is generally believed that relatively late appearance of X4 virus variants is driven by evolution of virals population during the course of disease (Holmes, 2001). This assumption is in conflict with viral property of rapid turnover of about 10^{10} to 10^{12} virions every two days combined with the high mutation rate of HIV (De Jong *et al*., 1992; Fouchier *et al*., 1992; Mansky and Temin, 1995; Holmes, 2001; Perelson, 2002; Jensen *et al*., 2003; Pastore *et al*., 2004; Bozek *et al*., 2009; Bunnik *et al*., 2011). This should result in emergence of X4 variants fairly early during infection, especially because, in some cases, only two mutations are thought to
be necessary for co-receptor switch. Hence factors that influence this evolution of the virus remain largely unknown. It has been demonstrated recently that Rac1 is specifically involved in regulating the conformation of CxCR4, thereby controlling signaling efficiency of the receptor. Furthermore, the conformation adopted by CxCR4 after Rac1 inhibition blocks HIV-1 infection, most probably by interfering with viral binding and subsequent entry into the host cells (Zoughlami et al., 2012). In this study, we observed that Rac1 expression was significantly lower in patients in initial stages of the HIV disease as compared to healthy controls. With the disease progression there was an increase in its expression in individuals infected with only HIV-1. These results indicate and support the previous findings that initially there is R5 virus predominance. As we found low Rac1 expression in these individuals, X4 viruses even if present would not be able to use CxCR4 co-receptor. Rac1 has no effect on CCR5 conformation or its utilization by the virus (Zoughlami et al., 2012).

However, in advanced stage of HIV-1 infection we found an up-regulated Rac1 expression which would make CxCR4 conformationally favorable for HIV-1 attachment and possibly is one of the reasons for R5 to X4 viral shift with HIV disease progression. We found a significant positive correlation of Rac1 with NF-κB and CxCR4 expression in healthy controls. Though this correlation of Rac1 and CxCR4 was not obvious in HIV-1 patients, NF-κB retained significant positive correlation in HIV-1 subjects indicating this pathway was not being affected by the disease condition. However, with *M. tuberculosis* co-infection, the Rac1 was significantly down-regulated making the CxCR4 receptors non-utilizable by the virus. This observation is in line with the finding that R5 viral variants were preferentially recovered from HIV infected patients with active *M. tuberculosis* (Morris et al., 2001).

In order to resolve the possible mechanism for the up-regulation of CxCR4 expression during Mtb co-infection in HIV infected individuals, we found a significantly higher expression of NF-κB in CD4 cells of HIV-PTB co-infected individuals with positive correlation between NF-κB and CxCR4 gene expression in these subjects. NF-κB has been shown to bind to the CxCR4 promoter, and treatment with NF-κB inhibitor parthenolide has been shown to reduce CxCR4 expression in MDA-MB-231 breast cancer cells (Helbig et al., 2003). With *M. tuberculosis* co-infection up-regulating NF-κB expression in HIV subjects, we observed a quantitative
increase in CxCR4 expression on CD4 cells from these subjects, when compared to their HIV counterparts with similar CD4 count. As CxCR4 receptor using X4 virus is linked with faster progression of the HIV disease, a decrease in CxCR4 receptor expression could lead to a slower progression. This association between CxCR4 and NF-κB could be exploited and could be of clinical importance.

In conclusion, our study delineates the novel interactions and interplay between HIV and *M.tuberculosis* assessing the impact of TB on HIV-1 disease progression. Our findings appear to indicate that CCR5 expression was selectively up-regulated in CD4+CD25high Treg and CD4+CD25intermediate T cells while CxCR4 expression was up-regulated on CD25low/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 was significantly down-regulated making CxCR4 non-utilizable. This observation corresponds well with the finding that R5 viral variants were preferentially recovered from HIV infected patients with active *M.tuberculosis* (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. This study, to the best of our knowledge, represents the first report reflecting the delicate link between NF-κB, FoxP3 splice variants and HO-1 in HIV with disease progression and how *M.tuberculosis* co-infection modulates these links to HIV-1’s advantage. Our findings support previous reports showing strong mutual interaction between HIV-1 and *M.tuberculosis* infection (Toossi, 2003) and found increased NF-κB and FoxP3 expression and decreased HO-1 expression in HIV-PTB co-infection, leading to increased HIV-1 replication and enhanced HIV disease progression. In addition, we addressed the controversy regarding Treg number in HIV, showing different Treg frequency at different stages of the disease. After initial drop in the CD4 count in asymptomatic HIV-1 subjects, there was a negative correlation of Treg frequency and FoxP3 expression per cell with absolute CD4 count correlating with increase in suppressive function. Further larger studies are required to validate the findings related to Treg/T-effector cell imbalances.