Differential Expression of Rac-1, CXCR4 and CCR5 on CD4 T-Cells at Different Stages of HIV-1 Disease Relate To Its Progression in Therapy Naïve Individuals

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Abstract

Background: HIV-1 uses different co-receptors (CCR5/CXCR4) at different stages of disease to enter target cells. Any information in understanding this mechanism has important implication on both, the rate of disease progression and our understanding of the immunopathogenesis of this disease.

Methods: This cross-sectional study involved 67 treatment-naïve HIV-1 infected individuals. The expression of CCR5 and CXCR4 on T-cell subsets was evaluated in the peripheral blood of patients at different stages of HIV disease, active pulmonary tuberculosis (PTB), HIV/PTB co-infection and healthy controls, to assess their possible role or association with change in Treg frequency during the disease progression. Furthermore, we investigated the impact of Rac1 expression in relation to functionally active CXCR4 and NF-kB expression.

Results: Significantly higher CCR5 expression on CD4+CD25high Treg cells in HIV-1 subjects in early stage of the disease correlated well with initial decrease in Treg frequency. On the contrary, higher CXCR4 expression on CD4+CD25high T-cells (non-Treg cells) in advanced stage of disease explained the shift to X4 type with faster progression to AIDS. This shift is further supported by initial significant decrease in Rac1 expression in early disease followed by returning to normal expression with disease progression. Interestingly, PTB co-infection correlated significantly with increase in CCR5 expression on Treg population only, at the same time favoring increase in CXCR4 expression on CD4+CD25low T-cells subset.

Conclusion: The study indicates novel inter-relationships of co-receptor expression and their regulatory genes in therapy naïve individuals and how the virus manipulates the host machinery to its advantage. The Rac1 seems to regulate not only the functional conformation of CXCR4, but also the expression of both FoxP3 and NF-κB transcription factor to regulate not only the functional conformation of CXCR4, but also the expression of both FoxP3 and NF-κB transcription factor followed by returning to normal expression with disease progression. Interestingly, PTB co-infection correlated significantly with increase in CCR5 expression on Treg population only, at the same time favoring increase in CXCR4 expression on CD4+CD25low T-cells subset.

Keywords: HIV-TB co-infection; CCR5/CXCR4 co-receptors; HIV disease progression; Co-receptor utilization; T-regulatory cells

Introduction

Human Immunodeficiency virus, HIV-1 initiates infection via viral envelope (Env) glycoprotein gp120 interacting with cell surface CD4, followed by its association with a co-receptor that triggers the fusion of viral and host-cell membranes. Two chemokine receptors, CCR5 and CXCR4 are the predominantly known co-receptors for HIV-1 in vivo. All HIV-1 strains are classified phenotypically as R5 (Macrophage tropic), X4 (T-cell tropic), or R5X4 (dual tropic i.e. both M-tropic and T-tropic) depending on whether they preferentially utilize cysteine-cysteine receptor 5 (CCR5) or cysteine-X-cysteine receptor 4 (CXCR4) or mixed [1]. This inter-converted co-receptor expression may result from the number of changes in the Env V3 region. In early stage of HIV infection, only about 15% are dual/mixed tropic and is dominated by CCR5 tropism [2,3]. While in later stages of the disease, dual/mixed or pure CXCR4 tropism reaches up to 60% [4], and is highest in patients with CD4 ≤ 200 cells/μl [5,6]. This may vary with the prevalence of HIV-1 subtype in a region. Why there is selective advantage of CCR5 tropic viruses in establishing HIV-1 infection, switching over to CXCR4 utilization in the advanced stage of disease, is not clearly understood yet. This tropism switch has been associated with rapid disease progression and poor clinical prognosis [7-9]. Besides preferential CXCR4-utilizing virus, there may be other factors also playing crucial role in faster disease progression and needs a closer look. It has earlier been indicated that Rac1, a small (~21 kDa) signaling G protein a member of the Rac subfamily of the Rho family of GTPases, is important to maintain the functional conformation of CXCR4 receptor [10], and could be one of the candidate molecules that could possibly play an important role in preferential co-receptor utilization by the virus.

Normal cell functioning of host is altered by invading viral proteins to the benefit of the virus [11]. There have been constant efforts to understand these interactions between viral and cellular gene products which together determine the host’s susceptibility to infection and disease progression in HIV-1 infection [12]. Working on few such molecules for their behavior in HIV-1 disease progression and M. tuberculosis co-infection, we have tried to analyze the role of HIV-1 co-receptors, CCR5 and CXCR4, variant 2 (shorter splice variant) of...
CXCR4, Rac1 (which is required to maintain CXCR4 in the responsive conformation) [10], NF-kB which has high affinity binding sight on long terminal repeat (LTR) enhancer region of HIV-1 and regulates HIV replication [13-16], and FoxP3 which enhances HIV-1 gene expression by modulating NF-kB occupancy at the LTR in human T-cells line [17].

Materials and Methods

Patients and controls

The study was conducted on different cohorts of individuals including 27 HIV-1 infected patients, 12 Mycobacterium tuberculosis (Mtib) infected patients (PTB), 8 HIV-1/Mtib co-infected patients (HIV-PTB), and 20 healthy controls (HC). HIV-1 infected patients confirmed by the blood sample being reactive by three rapid tests, as per National AIDS Control Organization (NACO) guidelines were enrolled from the Integrated Counselling and Testing Centre (ICTC) in the Department of Immunopathology, PGIMER, and Chandigarh, India. At the time of recruitment, the patients were interviewed by a counsellor to obtain informed consent and ascertain therapy naïve status. The status of disease was assessed by absolute CD4 cell count by flow cytometry using BD TriteSt™ containing antibody conjugates CD3-FITC/CD4-PE/CD45-PerCP with BD Trucount™ tubes (BD Biosciences, San Jose, USA). PTB and HIV-PTB patients confirmed positive for Mycobacterium tuberculosis by chest x-ray, sputum smear positivity and TB-PCR were recruited from DOTS centre, in our hospital. The study was approved by the Institutional Ethics Committee (IEC) of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India and peripheral blood was obtained from enrolled subjects after an informed consent. The peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by Ficoll-Hypaque density gradient centrifugation (HiMedia, Mumbai, India).

Treg Immunophenotyping

Freshly isolated PBMC were immunophenotyped for Treg number, FoxP3 expression per cell (MFI) and CCR5/CXCR4 expression using fluorophore-conjugated monoclonal antibodies (mAb): anti-CD4 PE/FITC, anti-CD25 PE-Cy7, anti-CCR5 PE, and anti-CXCR4 PE (all from BD Bioscience, San Jose, CA) for cell surface markers in combination with intracellular Fork-head box protein 3 (FoxP3) mAb conjugated with Alexa488 (eBioscience, San Diego, CA). Samples were acquired using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). The first strand cDNA was used in PCR reaction for the expression gene GAPDH. The primers for CXCR4 Total (CXCR4-T), CXCR4 splice variant 2 (NM_001008540.1), Rac-1 (NM_006908.4), NF-kB (NM_003998.3) were as follows: GAPDH forward, 5’-CAAGGTCATCTCATGACAACACCTTGTGCTGTA-3’ and reverse, 5’-GTCCACCACCCTGTTGC-3’; CXCR4-T forward, 5’-CTTCTACCCCAATGCTTGG-3’ and reverse, 5’-AATGTAGTAAGGCAGCCAACAG-3’; CXCR4 splice variant-2 forward, 5’-ACACCGCTTACCATGAGG-3’ and reverse, 5’-CCCATCACTGGCCAGTTAAGAGG-3’; Rac-1 forward, 5’-TTACGCCCTCCCATCCTCCAACAG-3’ and reverse, 5’-GCCGCCAGACCTCCAGGATTATT-3’; NF-kB forward, 5’-TTTACCAACAGCTGGCCCTTTGAC-3’ and reverse, 5’-CTGTCCTTGCGAACAGGCCTTGAGATTTTAGG-3’. The thermal cycle reprogramming was as follows: denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec and annealing at 58°C for GAPDH, 64°C for CXCR4-T, 56°C for CXCR4 splice variant-2, 66°C for Rac-1 and 62°C for NF-kB for 30 seconds, extension was carried at 72°C for 1 min.

Statistical analysis

Data are expressed as mean ± SE and ranges. Difference between groups was tested by one-way student’s t-test. Correlation analysis was done using the non-parametric Spearman’s Rank Correlation Coefficient. Linear Regression Analysis was carried out and the residuals were calculated. The level of significance was set at p<0.05.

Results

Demographic and Immunological characteristics

The cross-sectional study included a total of 67 subjects: 27 HIV-1 infected patients, 12 Mycobacterium tuberculosis infected patients (PTB), 8 HIV-1/Mtib co-infected patients (HIV-PTB), and 20 healthy controls (HC). PTB and HIV-PTB patients confirmed positive for Mycobacterium tuberculosis by chest x-ray, sputum smear positivity and TB-PCR were recruited from DOTS centre, in our hospital. The study was approved by the Institutional Ethics Committee (IEC) of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India and peripheral blood was obtained from enrolled subjects after an informed consent. The peripheral blood mononuclear cells (PBMCs) were isolated from heparinised blood by Ficoll-Hypaque density gradient centrifugation (HiMedia, Mumbai, India).

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patients (age range between 21-53 years and CD4+ T-cell count range: 32-854 cells/μl). Table 1 summarizes their clinical characteristics.

High CD4+FoxP3 frequency correlates with poor T-cell stimulation in HIV-PTB co-infected and HIV advanced disease individuals

Within the HIV-1 infected group, there was significantly low frequency of Treg in individuals with CD4 count >500 cells/μl (mean ± SEM: 3.49 ± 0.31, p=0.0003) as compared to other groups and HC (Figure 2a) hence, indicating early selective depletion of Treg cells. This is followed by increase in the mean percent frequency of Treg as the disease progresses. Though statistically non-significant, we did see an increase in the Treg frequency in HIV-1 patients with Mtb co-infection when compared with only HIV-infected individuals with similar CD4 count (<250 cells/μl). Overall, there was a significant inverse relationship (p<0.0001) between percent Treg as well as FoxP3 expression with the disease progression in terms of CD4 count (Figure 2b). Following PHA stimulation, mean proliferative index of T-cells from HIV-PTB co-infected individuals was significantly lower (mean ± SEM: 39.90 ± 10.00, p=0.001) as compared to HC (mean ± SEM: 204.7 ± 30.76). Individuals with only PTB or HIV infected with CD4 count >500 cells/μl had higher proliferative index (mean ± SEM: 434.0 ± 138.8, p=0.0390 and 332.9 ± 97.74, p=0.1596 respectively). Overall, there was a significant negative correlation between T-cell proliferation and HIV-1 disease progression (Figures 2c and 2d). These results suggest that reduced T-cell proliferative potential in HIV-PTB co-infected as well as only HIV infected in advanced stage of disease is due to increased frequency of FoxP3+ Treg cells in these individuals.

HIV-1 subjects in early stage of disease had significantly higher number of CD4+CD25<sup>high</sup>Treg cells expressing CCR5

CD4 T-cell sub-populations were examined for surface expression of CCR5, which were calculated as percentage of total analyzed CD4 T-cells in peripheral blood. All HIV-1 infected individuals, irrespective of Mtb co-infection, had significantly higher percentage of CD4 T-cells expressing CCR5 as compared to HC. This difference was not seen in individuals with PTB infection (Table 2). However, looking at the CD4+CD25<sup>high</sup>Treg population within the CD4 T-cells, there was significantly higher percentage of cells with CCR5 expression in PTB individuals (mean ± SEM: 19.83 ± 2.52, p=0.0226) in comparison to HC (mean ± SEM: 12.72 ± 1.29) (Figure 3a). Similar results were seen in early stage of disease in only HIV-1 infected individuals having normal CD4 count (mean ± SEM: 32.17 ± 7.15, p=0.0021). Interestingly, there was a drop in the CCR5+ Treg cell numbers with decrease in CD4 count. There was significantly lower frequency of these cells in HIV patients.
when compared with healthy controls. T-cells positive for CCR5 and P value less than 0.05 indicates significant difference expressed as Means with SEM [standard error of the mean] of percentage CD4 individuals between healthy controls and other diseased patient groups. Data are

Table 2: Comparison of frequency of CCR5 expression on CD4+ T-cells in individuals between healthy controls and other diseased patient groups. Data are expressed as Means with SEM [standard error of the mean] of percentage CD4 T-cells positive for CCR5 and P value less than 0.05 indicates significant difference when compared with healthy controls.

| Groups          | Mean    | Std. Error | P value |
|-----------------|---------|------------|---------|
| HC              | 2.731   | 0.2586     |         |
| PTB             | 3.295   | 0.8588     | 0.3215  |
| HIV-1 [CD4 >500 cells/μl] | 3.876     | 0.4110     | 0.0243  |
| HIV-1 [CD4 500>x<250 cells/μl] | 5.254    | 1.208      | 0.0194  |
| HIV-1 [CD4 <250] | 5.835     | 1.569      | 0.0127  |
| HIV-PTB         | 9.424   | 0.0243     |         |

Table 3: Comparison of frequency of CXCR4 expression on CD4+ T-cells in individuals between healthy controls and other diseased patient groups. Data are expressed as Means with SEM [standard error of the mean] of percentage cells positive for CXCR4.

| Groups          | Mean    | Std. Error | P value |
|-----------------|---------|------------|---------|
| HC              | 2.731   | 0.2586     |         |
| PTB             | 3.295   | 0.8588     | 0.3215  |
| HIV-1 [CD4 >500 cells/μl] | 3.876     | 0.4110     | 0.0243  |
| HIV-1 [CD4 500>x<250 cells/μl] | 5.254    | 1.208      | 0.0194  |
| HIV-1 [CD4 <250] | 5.835     | 1.569      | 0.0127  |
| HIV-PTB         | 9.424   | 0.0243     |         |

with CD4 count <250 cells/μl (p=0.002) in comparison to those with CD4 count >500 cells/μl. In individuals with PTB co-infection, there was a significant increase in CCR5+ Treg number (mean ± SE: 28.15 ± 9.41) when compared with only HIV-1 infected individuals with similar CD4 count (CD4 count <250 (p=0.0260)). There was however no significant change in CCR5 expression seen in CD4+CD25low/negative subpopulation among these groups (Figure 3a). This indicates that in the early stage of HIV infection there is higher number of Treg cells having very high CD25 expression co-expressing CCR5 also which may be the reason for their early depletion in these individuals.

M. tuberculosis co-infection in HIV-1 induces CCR5 expression in CD25high and CD25intermediate/Treg subpopulations but not in CD25low/negative cells

Looking at the quantitive (MFI) expression of CCR5 on CD4 T-cell subsets, we observed significantly lower expression in HIV infected individuals in advanced stage of disease (CD4 count <250 cells/μl (mean ± SEM: 13.63 ± 1.79, p=0.0496)) as compared to healthy controls (mean ± SEM: 17.84 ± 1.68), while it was significantly in PTB subjects (mean ± SEM: 26.52±3.93, p=0.0214) (Figure 3b). Presence of PTB co-infection had similar effect in HIV-1 individuals with significantly higher expression of CCR5 (mean ± SEM: 21.48±3.54, p=0.0364) as compared to only HIV infected patients with similar CD4 count (i.e.<250 cells/μl: mean ± SEM: 13.63±1.79).This quantitative increase in the CCR5 expression on CD4 T-cell in PTB individuals was seen on CD4+CD25high and CD4+CD25intermediate cell populations but not on CD4+CD25low/negative (mean ± SEM: 24.44±4.28, p=0.0549) subpopulation indicating the presence of Mtb influences the expression of CCR5 on CD25+ Treg cells only and not the CD25low/negative effector cells.

Significant decrease in the frequency of CXCR4+ cells in CD4+CD25low/negative sub-population of HIV-PTB co-infected individuals

There was no significant difference seen in frequency of CXCR4+ cells within HIV-1 sub-groups with the disease progression. However, we found a decrease in the percentage of CXCR4+ cells in HIV-PTB co-infected individuals (mean ± SE: 86.43 ± 4.95) when compared with only PTB infected (mean ± SE: 94.99 ± 1.76, p=0.0232) and HC (mean ± SE: 95.28 ± 1.13, p=0.048) (Figure 4a). Interestingly, this difference was not seen either in Treg population or in CD25low/negative sub-populations but only in the CD25low/negative populations. Further, CD25high Treg cells showed lower frequency of CXCR4+ cells in comparison to CD25low/negative in all studied subjects. These results indicate that significantly lower number of CD25low/negative effector cells expressing CXCR4 receptor were seen in PTB co-infected individuals as compared to only HIV-1 infected individuals having similar CD4 count. On the other hand, quantitatively the CXCR4 expression (MFI) was significantly increased on CD4 cells in subjects with only PTB infection (mean ± SEM: 148.4...
patients with the disease progression (Figure 5A, 5B, 5F). Further with the onset of AIDS the HIV-PTB co-infected individuals had higher CXCR4 expression (mean ± SEM: 126.0 ± 6.2, p=0.1201) in comparison to HIV-1 subjects with similar CD4 count (CD4 count <250 cells/µl) though statistically non-significant. Similar results were observed in CXCR4 variant-2 expression with significantly high expression in PTB and HIV-1 patients (Figure 5B). Interestingly, there was significant decrease in the expression of variant-2 with PTB co-infection (mean ± SEM: 124.6 ± 7.2) when compared to only HIV-1 infected subjects with CD4 count <250 cells/µl (mean ± SEM: 103.6 ± 5.0, p<0.0175). This suggests that with the progression of HIV disease CXCR4 expression is increased both in only HIV infected as well as PTB co-infected individuals. Further, this increase in CXCR4 expression positively correlated with NF-kB expression in HIV-1 patients (Figure 5E and 5F). This significant positive correlation of CXCR4 was extended with hemeoxygenase 1 (HO-1) but only with variant 2 (Figure 5G and 5H).

**Mycobacterium tuberculosis infection results in down regulation of Rac-1 expression in HIV-1 patients**

Rac1 expression was significantly lower in PTB patients (mean ± SEM: 112.4 ± 4.7, p=0.0063), HIV-1 subjects with CD4 count >500 cells/µl (mean ± SEM: 109.7 ± 9.5, p=0.0283) and HIV-PTB co-infected individuals (mean ± SEM: 110.1 ± 2.3, p=0.0062) when compared to healthy controls (mean ± SEM: 131.7 ± 4.5). We saw a trend of initial decrease in Rac1 expression in early stage of HIV-1 infection followed by returning to normal expression as the disease progresses (Figure 6A). Further, there was a significant decrease in the Rac1 expression in HIV individuals with onset of Mtb co-infection when compared with individuals infected with HIV-1 only with CD4 count between 250 and 500 cells/µl (p=0.0128) and <250 cells/µl (p=0.0325). This indicates that onset of Mtb infection down regulates the expression of Rac-1 gene making it unavailable to maintain the CXCR4 conformation suitable for HIV utilization. In addition, Rac1 significantly, positively correlates with immunosuppressive genes FoxP3 (Figure 6F and 6G) and HO-1 (Figure 6H and 6I) in HIV-1 individuals only (unpublished personal data).

**Discussion**

The results in the present study elucidate some of the cellular factors manipulated by HIV-1 at different stages of disease progression and in the event of *M.tuberculosis* co-infection. It has earlier been revealed that CCR5 and CXCR4, the major co-receptors critical for viral entry into the host cells, are differentially expressed on CD4+ T-cells, and their relative expression is closely associated with disease pathogenesis [18-24]. We studied their expression on different subsets of T-cells including T-regulatory cells and evaluated their role in changing the frequency of these cells with disease progression. We also evaluated the change in Rac1 expression, critical for functional CXCR4 receptor [10], for its possible role in M5 to T4 HIV-1 viral shift based on availability of co-receptor.

We found a significantly low frequency of FoxP3+CD4 cells in early stage of 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 up-regulated 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 [25-27]. There was higher percentage of CD25hiTreg cells expressing CCR5 in comparison to

![Graph showing correlations and expression levels](image)

**Figure 5: CXCR4 expression**. A. & B. Total CXCR4 and CXCR4 splice variant 2 mRNA relative gene expression as percent of GAPDH in unstimulated PBMCs from HC, patients with PTB, HIV-1 infected with or without PTB co-infection. C. & D. CXCR4 mRNA relative gene expression is up-regulated in HIV-1 infected subjects with disease progression with significant negative correlation with absolute CD4 count. E. & F. NF-kB positively associated with CXCR4 variants expression in HIV-1 patients. The relative NF-kB mRNA expression in PBMC cells positively correlated with CXCR4 expression in HIV-1 subjects and not in healthy controls [HC]. G. & H. Relative HO-1 mRNA expression in PBMCs positively correlate with CXCR4 variant 2 in HIV-1 subjects and not in healthy controls. The solid line is the linear regression of the points and dotted lines are the 95% confidence band for the mean. Rho and p values are Spearman rank correlation test.

![Graph showing correlations and expression levels](image)

**Figure 6: FoxP3 and HO-1 expression**. A. & B. Total FoxP3 and FoxP3 splice variant 2 mRNA relative gene expression as percent of 18S in unstimulated PBMCs from HC, patients with PTB, HIV-1 infected with or without PTB co-infection. C. & D. FoxP3 mRNA relative gene expression is up-regulated in HIV-1 infected subjects with disease progression with significant negative correlation with absolute CD4 count. E. & F. NF-kB positively associated with CXCR4 variants expression in HIV-1 patients. The relative NF-kB mRNA expression in PBMC cells positively correlated with CXCR4 expression in HIV-1 subjects and not in healthy controls [HC]. G. & H. Relative HO-1 mRNA expression in PBMCs positively correlate with CXCR4 variant 2 in HIV-1 subjects and not in healthy controls. The solid line is the linear regression of the points and dotted lines are the 95% confidence band for the mean. Rho and p values are Spearman rank correlation test.

± 20.40) as compared to the HC (mean ± SEM: 73.1 ± 6.90, p=0.0011). The expression was also higher in PTB-HIV co-infected subjects (although statistically non-significant at mean ± SEM: 94.77 ± 16.05), when compared to only HIV-1 infected individuals with comparable CD4 count (count <250 cells/µl; mean ± SEM: 105.3 ± 16.16), but was significantly higher than HC (mean ± SEM: 73.10 ± 6.90, p=0.0342). Interestingly, this significant difference in CXCR4 expression in PTB subjects was mainly contributed by CD25hiFoxP3+ subpopulation showing nearly two fold increase in its expression in comparison to HC (p=0.0011). These results indicate that Mtb infection favors increase in CXCR4 as well as% expression.

**Upregulation of CXCR4 and its splice variant-2 expression is associated with HIV disease progression and presence of Mbtco-infection**

There was a trend of increase in the CXCR4 expression in HIV-1 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 up-regulated 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 [25-27]. There was higher percentage of CD25hiTreg cells expressing CCR5 in comparison to the healthy controls, irrespective of the stage of the disease, which is in concordance with the previous findings [25-27]. There was higher percentage of CD25hiTreg cells expressing CCR5 in comparison to
CD25<sup>low/negative</sup> effector cells (p<0.0001) and which could make them preferential targets for R5 viral attack in the initial stages of disease (Figure 3a). On the other hand, in advanced stage of the disease with CD4 count <250 cells/μl, the number of CCR5 positive Treg were significantly lower when compared to HIV-1 individuals in early stage of disease (p=0.0022). With previous reports in non-subtype C infections, the depletion of CCR5 expressing memory cells in acute infection may drive viral evolution towards CXCR4 [28,29], 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 [30-32]. This change in CCR5 expression might be responsible for increase in the frequency of Treg cells in the advanced stages of the disease.

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 (Figure 4a). This is in concordance with previously reported observation in HIV subjects [25-27]. It is interesting to note that there is a reverse scenario when we considered the quantitative expression of CXCR4 per cell (MFI) on CD4 cells in these subjects (Figure 4b). There was an increase in the CXCR4 expression in advanced stages of HIV-1 when compared to asymptomatic early stage HIV-1 subjects. This could explain decrease in the frequency of CXCR4 positive CD4 T-cells in later stages of the disease due to X4 tropic viral shift and killing of these cells. Again, contrary to CCR5, there was significant decrease in percent CD4 T-cells expressing CXCR4 (p=0.0033) even quantitatively (MFI, p=0.0004) in CD25<sup>high</sup> Treg when compared with CD25<sup>low/negative</sup> cells in healthy controls. This could be a possible explanation for the decrease in percent of CXCR4 expressingCD4 T-cells in CD25<sup>low/negative</sup> subpopulation of cells within HIV-1 groups negatively correlating with disease progression but with no effect on Treg cell number with low expression of CXCR4.

However the situation was totally different if we look at the data from patients infected with only Mtb or co-infected with HIV. It is well known that Mtb co-infection in HIV may create a microenvironment enhancing the productive infection of T-cells by HIV [33-36]. We observed that HIV-1 individuals with active pulmonary tuberculosis displaying increased CCR5 (p=0.0364, Figure 3b) and not CXCR4 (p=0.1914, Figure 4b) 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 co-infected individuals. This falls in line with reports which demonstrated that R5 viral variants were preferentially recovered from patients with active TB [37]. Furthermore, increased frequency of CD4 cells expressing CCR5 and up-regulated expression limited to CD25<sup>high</sup> and CD25<sup>intermediate</sup> populations only in co-infected individuals with no significant change in CD25<sup>low/negative</sup> subpopulation indicated the influence of MTB on these two subpopulations only making them more vulnerable to HIV-1 infection. Whereas, a significant decrease in frequency of CXCR4<sup>+</sup> T-cells in co-infected individuals could mainly...
be attributed to a significant decrease in CD25<sup>hi</sup>/CD45RA<sup>−</sup> CD4 T-cell subpopulation. On the other hand, significantly increased CXCR4 expression on CD25<sup>hi</sup>/CD45RA<sup>−</sup> cells during Mtb infection makes it the main population affected in co-infected individuals as evidenced by their low frequency in these individuals. Thus, the data suggests that *M. tuberculosis* co-infection in HIV-1 individuals seems to be selectively affecting the differential expression of these chemokine receptors on different subpopulations of cells thereby leading to their selective depletion during different stages of disease.

Over the course of infection, the co-receptor usage of HIV changes from CCR5 to CXCR4 in 50% of the infected individuals [1,7,38-41]. It is generally believed that relatively late appearance of X4 virus variants is driven by evolution of virus population during the course of disease [42]. This assumption is in conflict with viral property of rapid turnover of about 10<sup>10</sup> to 10<sup>12</sup> virions every two days combined with the high mutation rate of HIV [42-50]. 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 virus binding and subsequent entry into the host cells [10]. It was interesting to note in our study that Rac1 expression was significantly lower in patients in early stage of disease when compared to healthy controls (Figure 6A). With the disease progression we observed an increase in its expression in individuals infected with only HIV-1. These results indicate and support the other previous findings that initially there is R5 virus predominance as there is low Rac1 expression and X4 viruses even present are unable to use CXCR4 co-receptor. Rac1 has no effect on CCR5 conformation or its utilization by the virus [10]. Though, in advanced stage of HIV-1 infection we found an up-regulated Rac1 expression which would make CXCR4 conformationally favorable to 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 (Figure 6D) and CXCR4 (Figure 6B) expression in healthy controls. Though this correlation of Rac1 and CXCR4 was not obvious in HIV-1 patients (Figure 6C), NF-κB retained significant positive correlation in HIV-1-infected patients with detectable viremia. J Infect Dis 194: 926-930.

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