4.1 Patients and controls recruited.

The study was approved by the institutional ethics committee, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh and an informed consent was taken before the enrollment of each subject into the study group.

In order to fulfill the objectives, the human subjects were enrolled (Figure 21) in the following study groups:

Group 1 (HIV only)  HIV-1 infected individuals
   Sub-group (1a): CD4 count >500 cells/ul (Asymptomatic phase)
   Sub-group (1b): CD4 count between 250 – 500 cells/ul (Progressive phase)
   Sub-group (1c): CD4 count <250 cells/ul (Advanced disease phase)

Group 2: (TB only)  Individuals infected with M. tuberculosis

Group 3: (HIV/TB)  Individuals co-infected with HIV-1 and M. tuberculosis

Group 4: (HC)  Healthy control subjects

Figure 21: Study participant recruited in different groups.
4.2 Sample collection

HIV-1 infected patients were enrolled from the Integrated Counselling and Testing Centre (ICTC) in the Department of Immunopathology, P.G.I.M.E.R, Chandigarh, coming for routine CD4 cell count test. At the time of recruitment, the patients were counselled and only those who had never been exposed to any antiretroviral (ART) or anti-tubercular treatment (ATT) were enrolled in the study. The patient history and previous records were properly scrutinized to ensure the treatment naive status of each patient.

Pulmonary tuberculosis infected individuals, and HIV-PTB co-infected individuals who were ART and ATT naïve were recruited from DOTS (Directly Observed Treatment, Short-course) centre, Department of Pulmonary Medicine, P.G.I.M.E.R, Chandigarh.

After a written consent, 3.0 ml of venous blood was drawn in a K$_3$EDTA vacutainer (Becton Dickinson, USA) vial for RNA isolation, total and differential lymphocyte count and CD4 cell count and another 10.0 ml blood was drawn in Heparin vacutainer vial (Becton-Dickinson, USA) for immunophenotyping and lymphocyte proliferation experiments. The samples were transported to ‘Molecular Immunology laboratory’ in the Department of Immunopathology, PGIMER, Chandigarh, for further processing and analysis (Figure 22).

Patients were confirmed positive for HIV-1 infection as per NACO guidelines i.e. the blood sample of the patient was positive by three rapid tests, each having different principle/antigen. Patients were confirmed positive for \textit{M.tuberculosis} by chest x-ray and sputum smear positivity.
4.3 Study plan

Figure 22: Schematic representation of the experimental design used in the present study.

4.4 HIV Disease status

4.4.1 CD4⁺ T Lymphocyte Count

Helper/inducer T-lymphocytes are a subset of T lymphocytes (CD3⁺) that carry CD4 molecule on their surface. The dual positive cell (CD3⁺CD4⁺) counts are used to characterize and monitor the progression of disease in HIV-1 infected individuals, who typically exhibit a steady decrease of helper/inducer T-lymphocyte counts as the infection progresses (Giorgia and Hultina, 1990).

Absolute CD4 lymphocyte counts were determined by flow-cytometry using BD
Tritest™ CD3 FITC/CD4 PE/CD45 PerCP with BD Trucount™ tubes (BD Biosciences, USA). BD Tritest™ CD3 FITC/CD4 PE/CD45 PerCP is a three colour direct immunofluorescence reagent for use with a suitably equipped flow cytometer to identify and determine the percentages and absolute counts of mature human T lymphocytes (CD3⁺) and helper/inducer (CD3⁺CD4⁺) T-lymphocyte subsets in erythrocyte-lysed whole blood. When used with BD Trucount™ tubes, absolute counts of these populations can be enumerated from a single tube. When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leucocyte surface antigens. CD3 identifies T lymphocytes, CD4 identifies helper/inducer T lymphocytes and CD45 identifies leucocytes. The BD Trucount™ tubes contain a freeze-dried pellet of fluorescent beads, and when a precise volume of EDTA blood sample is stained directly in a BD Trucount™ tube, the lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/μL) of positive cells in the sample is determined by comparing cellular events to bead events using BD Multiset™ software (BD Biosciences, USA).

Procedure: 20 μL of BD Tritest CD3/CD4/CD45 reagent was pipetted into the bottom of appropriately labelled BD Trucount™ tube. This was followed by the addition of 50 μL of well-mixed, anticoagulated K₃EDTA whole blood into the bottom of the tube without touching the sides of the tube. The mixture is gently mixed and incubated for 15 min in the dark at room temperature (20° to 25°C). 450 μL 1X BD FACS lysing solution was added to the tube, capped the tube and vortexed gently to mix. The tube was incubated for 15 minutes in the dark at room temperature (20° to 25°C). The sample was then acquired on the BD FACSCalibur™ (BD Bioscience, USA) flow cytometer and analyzed using the BD Multiset™ software.

4.5 Total and Differential Leukocyte Counts

Total and differential leukocyte counts from EDTA blood samples were obtained
using the Coulter® LH 750 Haematology Auto-analyzer (Beckman Coulter, USA).

4.6 **Enumeration of T-regulatory cells**

4.6.1 **Peripheral blood mononuclear cells (PBMCs) isolation**

PBMCs were isolated from heparinized venous blood by density gradient centrifugation using Ficoll Hipaque (HiMedia, Mumbai, India) in Leucosep-tubes (Greiner, Frickenhausen, Germany) using the following protocol.

- 8 ml anticoagulated peripheral blood was layered over 2.0 ml of Leucosep and centrifuged at 450 xg for 15 minutes at room temperature (with break option of centrifuge machine turned off using swing out rotor).
- Enriched PBMCs (buffy coat) ring at the interphase of leucosep and plasma was harvested and transferred into another centrifuge tube containing 6ml RPMI medium at room temperature.
- Cells were centrifuged at 450 xg followed by another washing with 6 ml RPMI medium at 250 xg.
- Cells were resuspended in 1 ml RPMI medium containing 10% heat inactivated Fetal calf serum (HI-FCS)
- Viability of cells was estimated by trypan blue exclusion test followed by cell count using the following formula:

\[
\text{Cell concentration (cell/ml) = } \frac{\text{Number of cells} \times 10^4 \times \text{dilution factor}}{4}
\]

Cells were immediately stained for immunophenotyping using flow cytometry and T-cell proliferation assay

4.6.2 **Immunophenotyping of T-regulatory cells**

Freshly isolated PBMCs were immunophenotyped for Treg quantification. Analysis was performed on a three-color flow cytometer (FACSCalibur; BD Biosciences). Treg quantification was performed by staining with anti-CD4 PE, anti-
CD25 PE-Cy7 for surface molecules and anti-foxp3 Alexa Fluor 488 antibody conjugates for intracellular FoxP3 (Pharmingen, USA) using the following protocol:

4.6.2.1 Preparation of buffers (Pharmingen, USA) before use.

- FoxP3 ‘Buffer A’ (10X concentrate) was diluted 1:10 (Buffer A: Water) with deionized water at room temperature (20°C to 25°C).
- Working solution of ‘Buffer C’ was made by diluting FoxP3 Buffer B into 1X FoxP3 Buffer A at a ratio of 1:50 (Buffer B:Buffer A).

The working solutions for Human FoxP3 Buffers A and C were made fresh for each experiment.

4.6.2.2 Cell Preparation and Staining Procedures for Conjugated Anti-Human FoxP3 Antibody

- The buffers were brought to room temperature before use. Working solutions of the BD Pharmingen Human FoxP3 Buffer Set Cat. No. 560098 were prepared (as described above).
- PBMCs were diluted with BD Pharmingen Stain Buffer (FBS) to 1x10^7 cells/ml.
- Previously titrated volumes of fluorochrome-conjugated monoclonal antibodies for surface antigen were pipetted to bottom of each 12 x 75 mm tube.
- 100 μl of cells per tube were added, vortexed and incubated for 20 minutes at room temperature protected from light.
- 2 ml of wash buffer was added and centrifuged at 250 xg for 10 minutes, and wash buffer was removed.
- Cells were fixed by gently re-suspending the cell pellet in residual volume of wash buffer and then adding 2ml of 1x Human FoxP3 Buffer A. Cells were gently mixed and incubated for 10 minutes at room temperature in the dark.
- The tube was centrifuged at 500 xg for 5 minutes, and Supernatant (fixative) was removed.
- To wash, the cells were re-suspended in 2ml of BD Pharmingen Stain Buffer (FBS), and centrifuged at 500 xg for 5 minutes. Wash buffer was removed.
To permeabilize, the cells were gently re-suspended in residual volume of wash buffer and then 0.5 ml of 1x working solution Human FoxP3 Buffer C was added to each tube. The cells were gently mixed and incubated for 30 minutes at room temperature in dark.

To wash, the cells were re-suspended in 2ml of BD Pharmingen Stain Buffer (FBS), and centrifuged at 500 xg for 5 minutes. Wash buffer was removed and repeated the wash step.

10 μl of Alexa Fluor 488 conjugated FoxP3 antibody/isotype control was added at appropriate concentrations to re-suspend the pellet. Cell suspension was gently shaken and incubated for 30 minutes in the dark at room temperature.

Cells were washed with 2ml of BD Pharmingen Stain Buffer (FBS), and centrifuged at 500 xg for 5 minutes. Wash buffer was removed and the cells were finally resuspended in wash buffer and analyzed immediately.

4.7 Functional Assay

4.7.1 T-cell proliferation assay

To examine the functional defects of T-cells in patients with HIV-1, further experiment was designed for assessment of proliferative capability of T cells on stimulation with mitogen, PHA (phytohemagglutinin-P, Sigma) by determining tritiated (³H)-thymidine incorporation. PBMCs were isolated from heparinized peripheral blood by Ficoll/Hypaque-density gradient centrifugation (Leucosep tubes as described in previous section). The cells were cultured in the proliferation experiments in the presence or absence of mitogen as stimulant. Briefly, 1 × 10⁵ PBMCs/well were plated into 96-well plates in triplicates in complete RPMI 1640 medium (Sigma) supplemented with 10% HI-FCS, 20 IU/ml penicillin, 20 μg/ml streptomycin, 25 mM HEPES, and 2 mM L-glutamine for 96 hours at 37°C in 5% CO₂ (Thermo Scientific Forma Steri-Cycle CO₂ Incubator) in air. The cells were stimulated with PHA at a final concentration of 10 μg/ml of cell suspension. Eighteen
hours prior to termination of the culture, 1µCi of $^{3}$H-thymidine (Bhabha Atomic Research Centre (BARC), India) was added to each well.

The cells were then harvested on glass fibre mats (Skatron, Belgium) and counted in liquid scintillation counter (Beckman, USA). All measurements were conducted in triplicates and the results were expressed as the stimulation index (SI), which represented the mean cpm (counts per minute) in the presence of PHA divided by mean cpm in the absence of the PHA.

### 4.8 Gene Expression Studies

#### 4.8.1 mRNA isolation

Total RNA was extracted from EDTA treated blood using QIAmp RNA Blood Mini Kit (Qiagen, Germany). The protocol was followed as per manufacturer’s instructions (Figure 23).

A volume of 1ml of whole blood was mixed with 5ml of Buffer EL and incubated for 10–15 min on ice. It was thoroughly mixed by vortexing briefly, 2 times during incubation. Then it was centrifuged at 400 x g for 10 min at 4°C, and supernatant was discarded. 2ml of Buffer EL was added to the cell pellet. Cells were resuspended by vortexing briefly. The tube was centrifuged at 400 xg for 10 minutes at 4°C, and supernatant

![Figure 23: QIAamp RNA Blood Mini](image-url)
was completely removed. To the pelleted leukocytes 350 μl of Buffer RLT was added and cells were suspended by pipetting in and out repeatedly. The lysate was directly pipetted into a QIAshredder spin column in a 2 ml collection tube and centrifuged for 2 min at maximum speed to homogenize. QIAshredder spin column was discarded and 350 μl of 70% ethanol was added to the homogenized lysate and mixed by pipetting. Whole sample including precipitates were transferred into a new QIAamp spin column in a 2 ml collection tube and centrifuged for 15 seconds at 8000 xg (10,000 rpm). QIAamp spin column was transferred into a new 2 ml collection tube. 700 μl Buffer RW1 was added to the QIAamp spin column and centrifuged for 15 seconds at 8000 xg (10,000 rpm) to wash. QIAamp spin column was placed in a new 2 ml collection tube. 500 μl of Buffer RPE was added into the QIAamp spin column and centrifuged for 15 seconds at 8000 xg (10,000 rpm). Final washing was done by adding 500 μl of Buffer RPE and centrifuged at full speed (20,000 xg, 14,000 rpm) for 3 minutes. QIAamp spin column was transferred into a 1.5 ml microcentrifuge tube and 30-50 μl of RNase-free water was added directly onto the QIAamp membrane. The RNA was eluted by centrifuging at 8000 xg (10,000 rpm) for 1 minute. RNA samples were quantified by A$_{260}$ measurement and purity was checked by taking the A$_{260}$/A$_{280}$ ratio. Samples with A$_{260}$/A$_{280}$ ratio between 1.7 to 2.0 were processed further. The RNA was immediately reverse transcribed to cDNA as per the protocol given in the next section.

4.8.2 First strand cDNA synthesis

Total RNA was reverse-transcribed to make complimentary DNA (cDNA) copies using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). For the reaction mixture of 20 μl, the mixture containing 2 μg of total RNA, 0.2 μg of random hexamers was prepared and made to 12 μl with DEPC-treated water. The reaction mixture was gently mixed, incubated at 70°C for 5 minutes and quick chilled on ice water. This was followed by the addition of 4.0 μl of 5X reaction buffer, 20 units of RiboLock™ Ribonuclease Inhibitor, and 0.1mM dNTPs mix (mixture of dATP, dCTP, dGTP and dTTP). The contents were gently mixed. After incubating at 25°C for 5 minutes, 200 units of Revert Aid™ Moloney-murine leukemia virus (M-MuLV) reverse transcriptase enzyme was added. The reaction
mixture was incubated at 42°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes followed by chilling on ice. The first strand cDNA was used in PCR reaction for the following expression studies.

4.8.3 Reverse transcription - Polymerase chain reaction (RT-PCR).

4.8.3.1 FoxP3 splice variants expression

The protein encoded by this gene is a member of the forkhead/winged-helix family of the transcriptional regulators. There are two major splice variants reported (Figure 24), Variant 1 (gi |167466188| ref |NM_014009.3|) represents the longer transcript and Variant 2 (gi |167466189| ref |NM_001114377.1|) lacks an in-frame segment of the coding region, compared to Variant 1. The first strand cDNAs were used as template in the PCRs to amplify 172 base pair (bp) (conserved region in all splice variants) Foxp3 mRNA and its splice variants represented by 421 bp (Variant-1 representing full length Foxp3 gene expression), and 316 bp (Variant-2 representing 105 bp deleted variant) fragment in all the cases and controls.

**Figure 24:** Schematic diagram of FoxP3 alternately spliced transcript variants 1 (2397 bp) and variant 2 (2292 bp).

The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (New England Biolabs (NEB)), 0.8 picomole (pmol) of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 35
cycles with denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute (68°C for 1 minute for FoxP3 splice variants) and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minute at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers designed for the present study were as follows:

Primer pair for FoxP3 expression is as follows:
- FoxP3 F: 5'-CGGACCATGTTCCTGGATGAG-3'
- FoxP3 R: 5'-TTGTCGGATGATGCCACAG-3'

Primer pair for FoxP3 splice variant 1 and variant 2 mRNA expression:
- FoxP3 F1: 5'-TTCACCAAGCCTGCCCTTGGAC-3'
- Foxp3 R1: 5'-GCTGATCATGGCTGGGCTCTC-3'

4.8.3.2 IFN-gamma expression

This gene encodes a member of the type II interferon family. It is an important activator of macrophages and clearance of viral and intracellular bacterial infections. The first strand cDNAs were used as template in the PCR to amplify 283 bp fragment of IFN-γ gene from all the cases and controls. The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 35 cycles with denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.5% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers designed for the present study were as follows:

- IFN-gamma F1: 5'-CCTTAAGAAATATTTTAATGCA-3'
- IFN-gamma R1: 5'-ATTCAAGTCAGTTACCGAAT-3'
4.8.3.3 Heme Oxygenase (HO)-1 expression

HO-1, an essential enzyme in heme catabolism, cleaves heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide. Heme is known to have anti-HIV activity by inhibiting RT and bilirubin/biliverdin has anti-protease activity. On the other hand, carbon monoxide plays an importance role in latency and survival of mycobacteria. The first strand cDNAs were used as template in the PCR to amplify 139 bp fragment of HO-1 gene (gi|4504436| ref |NM_002133.1| HO-1) from all the cases and controls. The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95ºC for 5 minutes, for 34 cycles with denaturation at 95ºC for 1 minute, annealing at 58ºC for 1 minute and extension at 72ºC for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72ºC. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers designed for the present study were as follows:

HO-1F  5'-AGGCCAAGACTGCGTTCCT-3'
HO-1R  5'-GCAGAATCTTGCACTTTGTTG -3'

4.8.3.4 Nuclear factor (NF)-κB expression (p50 subunit)

NF-κB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, bacterial or viral products. It initiates and enhances HIV-1 replication by binding to the 5' LTR region of HIV-1. The first strand cDNAs were used as template in the PCR to amplify 406 bp fragment of nuclear factor of kappa light polypeptide gene enhancer in B-cell (NF-κB), transcript variant 1 (gi|259155300| ref |NM_003998.3|) gene from all the cases and controls. The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR
buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 35 cycles with denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequences of primers were taken from study by Mitamura et al., 2003 and were as follows:

NF-κB F 5'- CACTTATGGACAACTATGAGGTCTCTGG -3'  
NF-κB R 5'- CTGTCTTGTGGACAACGCAGTGGAA TTTTAGG -3'

4.8.3.5 Interleukin (IL)-10 expression

IL-10 has a uniquely broad immune-modulatory effect, especially in suppressing cell-mediated immunity through down-regulating pro-inflammatory cytokines, co-stimulatory molecules, as well as major histocompatibility complex (MHC) class II proteins. It has been implicated in HIV-1 infection and pathogenesis. The first strand cDNAs were used as template in the PCR to amplify 182 bp fragment of interleukin 10 gene (NM_000572.2, 1629 bp mRNA) from all the cases and controls. The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 34 cycles with denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers was taken from the study by Faal et al., 2006, is as follows:

IL-10-F 5'-TGAGAACCAAGACCCAGACA-3'  
IL-10-R 5'-TCATGGCTTTGTAGATGCCT-3'
4.8.3.6 Rac1 expression

The first strand cDNAs were used as template in the PCR to amplify 280 bp fragment of transcript variant Rac1 gene (Homo sapiens ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1), gi|156071503|ref|NM_006908.4|) from all the cases and controls. The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 34 cycles with denaturation at 95°C for 1 minute, annealing at 66°C for 1 minute and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers designed for the present study was as follows:

Rac1-F  5'-TTACGCCCCCTATCCTATCCGCA-3'
Rac1- R  5'- GCGCCGAGCACTCCAGGTATT-3'

4.8.3.7 GAPDH expression

GAPDH gene is a constitutively expressed housekeeping gene that takes care of any minor differences in the amount of RNA subjected to reverse transcription as well as any variability in reverse transcription efficiency. The quantitative estimation of a constitutively expressed GAPDH gene was taken as control and used to normalize the values for semiquantitative estimation study. The first strand cDNAs from all the samples were used as template in the PCR to amplify a 180 bp fragment of GAPDH gene. The reaction mixture (20 µl) for PCR comprised of 3 µl of cDNA, 2 µl of 10X PCR buffer supplied with the enzyme (consisting of 0.1 M Tris-HCl, pH 8.8, 15 mM MgCl₂, 0.5 M KCl and 1% Triton-100), 200 µM dNTPs (NEB), 3 µl of
25mM MgCl₂ (Sigma), 1.0 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). The thermocycling was carried after an initial denaturation at 94°C for 3 minutes, for 35 cycles with denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 45 seconds in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. The sequences (5'-3') of the primers (MBI Fermentas, Lithuania) used in the study were as follows:

GAPDH-F:  5’-CAAGGTCATCCATGACAACCTTTGG-3’
GAPDH-R:  5’-GTCCACCACCCTGTGCTGTA-3’

4.8.4 Agarose Gel Electrophoresis

Agarose extracted from seaweed is a linear polymer of D-galactose and 3, 6-anhydro-L-galactose units. It is soluble in hot water (or aqueous buffer) and, when cooled, forms a matrix that serves as a molecular sieve to separate DNA fragments on the basis of size, under the influence of an electric field.

A gel casting unit was set up with appropriate comb. Required amount of molecular grade Agarose (Sigma Aldrich, USA) and 1X TAE electrophoresis buffer were added to a clean flask (estimating the volume based on a 0.8 cm thick gel). Swirled gently to mix and heated in a microwave oven for 1 minute to melt the Agarose and boiled. The mixture was cooled to about 50°-60° C and 2 µl of 10 mg/ml ethidium bromide was added (Sigma Aldrich, USA) per 100 ml Agarose solution. The Agarose suspension was gently mixed and poured onto the gel casting unit. Air bubbles were removed and the gel was allowed to solidify for 30-40 minutes at room temperature. The comb was removed carefully and gel tray was placed in the electrophoresis tank containing 1X TAE buffer. The DNA samples and ladder were mixed with 6X Bromophenol blue (Sigma Aldrich, USA) gel loading dye and loaded into the slots of submerged gel very carefully. The gel tank lid was closed and the electrical leads were attached such that the DNA migrates towards the anode. A voltage of 5 volt/cm was applied till the dye migrated to the appropriate distance. The
gel was removed from the tank and DNA bands were visualized and photographed under UV light using the G-Box gel documentation (Syngene, USA) system (Sambrook and Russell, 2001).

4.5 Chemokine receptor expression.

4.5.1 Characterizing CxCR4 and CCR5 on T-cell regulatory cells by flowcytometry.

One million PBMCs suspended in 200 μl of complete RPMI medium were taken in a 12 × 75 mm tube (BD Biosciences, USA). To the cell suspension, 8-10 μl (previously titrated volumes) of each of fluorescent labelled antibodies were added with a combination of three of the following antibodies (BD Pharmingen):

- Tube 1. Unstained
- Tube 2. CD4 (FITC), CD25 (PE-Cy7)
- Tube 3. CD4 (FITC), CD25 (PE-Cy7), CxCR4 (PE)
- Tube 4. CD4 (FITC), CD25 (PE-Cy7), CCR5 (PE)

The tube was vortexed gently and incubated for 30 minutes in dark at room temperature. 2 mL of BD FACS™ Flow sheath fluid (BD Biosciences, USA) was added and centrifuged at 500g for 5 minutes. Supernatant was removed. Now 0.5 ml of 1% paraformaldehyde solution was added and mixed thoroughly. The samples were acquired on BD FACS Calibur flow cytometer and analyzed using BD Cell Quest™ Pro software (BD Biosciences, USA). Ten thousand gated events were analyzed by selecting the lymphocyte cluster on the Forward Scatter (FSC) vs Side Scatter (SSC) dot plot. The gated events were then differentiated on a second dot plot to identify the percent T-helper cells as CD4⁺ Vs Side Scatter. On CD4 T-cell, percent CD4 T-cells expressing CxCR4 and CCR5 were calculated. On the bases of CD25 expression, CD4 T-cells were divided into three groups: CD4⁺CD25^high (top 2% of the CD4⁺CD25⁺ cells with very high intensity of CD25 signal), CD4⁺CD25^intermediate (middle 15% of the CD4⁺CD25⁺ cells with intermediate intensity of CD25 signal) and
CD4CD25^{low/negative} (lower 83% of CD4^{+}CD25^{+} cells with very low or negative signal for CD25) cells.

### 4.5.2 CxCR4 splice variant expression

CxCR4 is the main HIV-1 coreceptor used for entry into the CD4 T-cells in the later stage of the disease. The first strand cDNAs were used as template in the PCRs to amplify total CxCR4 mRNA expression (gi|56790926|ref|NM_001008540.1|: Variant (1), also known as CxCR4-Lo, represents the longer transcript and encodes the longer isoform (a)) and CxCR4 transcript variant 2 (gi|56790926|ref|NM_001008540.1|: Variant (2) contains a distinct 5' UTR and lacks an in-frame portion of the 5' coding region, compared to variant 1. The resulting isoform (b) has a shorter N-terminus when compared to isoform (a).

The reaction mixture (25 µl) for PCR comprised of 3 µl of cDNA, 2.5 µl of 10X PCR buffer supplied with the enzyme, 200 µM dNTPs (NEB), 0.8 pmol of each primer and 1.5 units of Taq DNA polymerase (NEB). PCR was carried out after an initial denaturation at 95°C for 5 minutes, for 35 cycles with denaturation at 95°C for 1 minute, annealing at 64°C for 1 minute for total CxCR4 expression and 56°C for 1 minute for variant 2, and extension at 72°C for 1 minute in a thermocycler (Eppendorf, Germany). The last cycle was extended for 10 minutes at 72°C. After PCR, the amplified products were visualized following electrophoresis in 2.0% agarose gel containing 0.5 µg/ml ethidium bromide and documented in the Gel documentation System (Bio Rad). The sequence of primers designed for the present study was as follows:

- **Primer for Total CXCR4 Expression**: Product size- 208
  - CXCR4/Tot/F 5'-CTTCTACCCCAATGACTTGTGG-3'
  - CXCR4/Tot/R 5'-AATGTAGTAAGGCAGCCAACAG-3'

- **Primer for CxCR4 Splice Variant 2(b)**: Product size- 178bp
  - CXCR4/SP(b)/F 5'-AACCAGCGGTTACCATGGAG-3'
  - CXCR4/SP(b)/R 5'-CCCACAATGCCAGTAAAGAGA-3'
4.6 Statistical Analysis

Data are expressed as mean, standard error (SE) and ranges. P-values less than or equal to 0.05 with 95% confidence intervals were considered statistically significant. Differences between groups were tested by Student $t$ test.

**Mann Whitney U test**: This is a non-parametric test that compares the distribution of two unmatched groups. Non-parametric tests are preferred if the sample size is small and are based on the presumption that the samples do not follow a Gaussian distribution. Comparison between the different studied groups was done by Mann-Whitney U test.

**Table 3**: P value interpretation for Mann-Whitney U test.

<table>
<thead>
<tr>
<th>P value</th>
<th>Wording</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.001</td>
<td>Extremely significant</td>
<td>***</td>
</tr>
<tr>
<td>0.001 to 0.01</td>
<td>Very significant</td>
<td>**</td>
</tr>
<tr>
<td>0.01 to 0.05</td>
<td>Significant</td>
<td>*</td>
</tr>
<tr>
<td>&gt;0.05</td>
<td>Not significant</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Spearman Correlation**: Correlation quantifies the degree to which two variables are related. Correlation is used when both X and Y variables are measured, and is not appropriate if X is a variable that is experimentally manipulated. Spearman Correlation assumes that the sample population does not follow a Gaussian distribution. The correlation analysis reports the value of the correlation coefficient. The correlation coefficient, r, ranges from -1 to +1. The nonparametric Spearman correlation coefficient, abbreviated rs, has the same range. Linear Regression Analysis was carried out and the residuals were calculated. The level of significance was set at p<0.05.
Table 4: Interpretation of Spearman Correlation r-value.

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<thead>
<tr>
<th>Value of r (or rs)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Perfect correlation</td>
</tr>
<tr>
<td>0 to 1</td>
<td>The two variables tend to increase or decrease together.</td>
</tr>
<tr>
<td>0.0</td>
<td>The two variables do not vary together at all.</td>
</tr>
<tr>
<td>0 to -1</td>
<td>One variable increases as the other decreases.</td>
</tr>
<tr>
<td>-1.0</td>
<td>Perfect negative or inverse correlation.</td>
</tr>
</tbody>
</table>

Correlation between CD4$^+$ T cell counts, T-regulatory cell frequency in CD4 cells and FoxP3 expression was done by Spearman Correlation Co-efficient.