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Expansion and productive HIV-1 infection of Foxp3 positive CD4 T cells at pleural sites of HIV/TB co-infection

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Abstract

Background—CD4 T-cells expressing Foxp3 are expanded systemically during active tuberculosis (TB) regardless of HIV-1 co-infection. Foxp3$^+$ CD4 T cells are targets of HIV-1 infection. However, expansion of HIV-1 infected Foxp3$^+$ CD4 T cells at sites of HIV/TB co-infection, and whether they contribute to promotion of HIV-1 viral activity is not known.

Methods—Pleural fluid mononuclear cells (PFMC) from HIV/TB co-infected patients with pleural TB were characterized by immune-staining and FACS analysis for surface markers CD4, CD127, CCR5, CXCR4, HLA-DR and intracellular expression of Foxp3, HIVp24, IFN-γ and Bcl-2. Whole PFMC and bead separated CD4$^+$CD25$^+$CD127$^-$ T cells were assessed for HIV-1 LTR strong stop (SS) DNA by real-time PCR, which represents viral DNA post cell entry and initiation of reverse transcription.

Results—High numbers of HIV-1 p24 positive Foxp3$^+$ and Foxp3$^+$CD127$^-$ CD4 T cells were identified in PFMC from HIV/TB co-infected subjects. CD4$^+$Foxp3$^+$CD127$^-$ T cells displayed high expression of the cellular activation marker, HLA-DR. Further, expression of the HIV-1 co-receptors, CCR5 and CXCR4, were higher on CD4$^+$Foxp3$^+$T cells compared to CD4$^+$Foxp3$^-$ T cells. Purified CD4$^+$CD25$^+$CD127$^-$ T cells isolated from PFMC of HIV/TB co-infected patients, were over 90% CD4$^+$Foxp3$^+$T cells, and exhibited higher HIV-1 SS DNA as compared to whole PFMC, and as compared to CD4$^+$CD25$^+$CD127$^-$ T cells from an HIV-infected subject with pleural mesothelioma. HIV-1 p24$^+$ Foxp3$^+$ CD4$^+$T cells from HIV/TB patients higher in Bcl-2 expression as compared to both HIV-1 p24$^+$ Foxp3$^-$ CD4 T cells, and Foxp3$^+$ CD4$^+$T cells without HIV-p24 expression.

Conclusion—Foxp3$^+$ CD4 T cells in PFMC from HIV/TB co-infected subjects are predisposed to productive HIV-1 infection and have survival advantage as compared to Foxp3 negative CD4 T cells.

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Keywords

Regulatory T-cell; Pleural TB; HIV/TB co-infection; HIV-1 p24; HIV-1 LTR; Apoptosis; Bcl-2; Foxp3

Introduction

During Human Immunodeficiency virus-1 and Tuberculosis (HIV/TB) co-infection, sites of active HIV/TB disease are the main source of viral activity as compared to virus generated systemically (1). High numbers of $Foxp3^+$ CD4 T-cells characterize sites of *Mycobacterium tuberculosis* (MTB) infection (2–4). $Foxp3^+$ CD4 T-cells have been found to be targets of HIV-1 infection among blood mononuclear cells (5, 6). Whether $Foxp3^+$ CD4 T-cells are expanded and productively HIV-1 infected at sites of HIV/TB co-infection is not known.

Foxp3 gene expression is key in establishing the regulatory T-cell lineage both for thymus-derived natural (n) T-reg, and induced (i) T-reg derived from naïve CD4 T-cells outside of the thymus (Reviewed in (7)). Transforming growth factor beta (TGF-β) is critical to induction of expression of Foxp3 in T-cell receptor (TCR) activated naïve CD4 T-cells (8, 9). Whereas induction of iT-reg requires TGF- β, expansion of TGF- β induced iT-reg is dependent on the NFκb/c-Rel (10) and/or IL-2/Stat5 signaling (11) pathways. However, stability of Foxp3 gene expression in iT-reg has been found to be dependent on presence of TGF-β alone (12, 13). At sites of pleural HIV/TB co-infection, bio-active TGF-β and the pro-inflammatory cytokines (IL-8, IL-6), but not IL-2, are abundant (14). In this latter study, Foxp3 mRNA expression in PFMC correlated with pleural fluid levels of IL-6 and IL-8 and to a lesser extent with TGF-β, but not with levels of the T-cell cytokine, IFN-γ (14). These data suggest that intense TCR activation in the context of the inflammatory micro milieu that includes excess TGF-β at sites of pleural HIV/TB co-infection may be conducive to expansion of PFMC CD4 T-cells with stable Foxp3 expression.

Under Th1 polarizing conditions, a fraction of $CD4^+Foxp3^+$ T cells have been shown to express IFN-γ (15, 16). However, IFN regulatory factor-1 has been shown to promote inhibition of Foxp3 expression (17), and ultimately may lead to conversion of T-reg to Th1-like cells (18). Whether the Th1 cytokine milieu at pleural sites of HIV/TB co-infection (14, 19) also promotes IFN-γ reactive $Foxp3^+$ CD4 T cells is not known.

Naïve T-cell precursors of Foxp3 positive T-reg are susceptible to HIV infection *in vitro* (20). Both heightened status of cellular activation (21) and increased expression of HIV-1 co-receptors (6) may underlie predisposition of T-reg to successful viral infection. Further, Foxp3 enhances NFκb occupancy at HIV-1 LTR in Jurkat T cell line (22), indicating the likely predisposition of $Foxp3^+$ CD4 T-cells to productive HIV-1 infection. On the other hand, others have shown that transfection of Foxp3 gene in primary CD4 T cells represses HIV-1 transcription under conditions of immune activation (23, 24). Whether the intense immune activation at pleural sites of HIV/TB co-infection (Meng submitted) leads to repression or promotion of HIV-1 infection in $Foxp3^+$ CD4 T cells is not known.

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Based on our previous observations of enhanced HIV activity, increased TGF-β bioactivity, and high Foxp3 expression of PFMC at pleural sites of HIV/TB co-infection, we hypothesized that $CD4^+Foxp3^+$ T cells in PFMC may be HIV-1 infected and contribute to expansion of HIV-1 activity *in situ*. We found that among PFMC CD4+ T-cells, HIV-1 infection was enriched in the $Foxp3^+CD127^-$ CD4 T-cell population. Co-expression of HIV-1 p24 and IFN-γ among $Foxp3^+$ $CD127^-$ CD4 T-cells was higher than $Foxp3^-CD127^+$ CD4 T cells. Expression of both HIV-1 co-receptors (CXCR4 and CCR5) and HLA-DR was significantly higher among $Foxp3^+$than $Foxp3^-$ CD4 T cells in PFMC. Also, HIV-1 $p24^+$ $Foxp3^+$ T cells were higher in Bcl2 expression as compared to both HIV-1 $p24^+Foxp3^-$ and HIV-1 $p24^-$ $Foxp3^+$ CD4 T cells.

Methods

Study Subjects

Patients hospitalized at Mulago Hospital at Makerere University in Kampala Uganda with symptoms of fever, cough, night sweats, dyspnea for at least 2 weeks who had moderate to large pleural effusions were identified and referred to the TB clinic of the TB Research Unit for evaluation of pleural TB. Informed consent (approved by the Institutional Review Board at CWRU and the Ugandan National AIDS Research Subcommittee) was obtained from all subjects. All patients underwent HIV-1 testing, and thoracocentesis and pleural biopsy as previously described (25). Diagnosis of pleural TB was based on positive culture of sputum and/or pleural fluid, and/or positive histology of pleural tissue for MTB infection. Only HIV^+ patients were included in this study. HIV-1 viral load in plasma and pleural fluid was determined using the Roche Amplicor (1.2) System. All patients received standard short-course anti-TB chemotherapy and were referred to Makerere Joint AIDS Programme for anti-retroviral therapy.

A total of 16 HIV/TB co-infected patients were studied. Table 1 describes the characteristics of enrolled subjects.

Preparation of PBMC and PFMC

PFMC and PBMC were prepared by Ficoll Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation as described (25). Viability was > 98% as assessed by trypan blue exclusion. By immune staining and FACS analysis PFMC were 40–60% CD4 T-cells and contained only 1–5% $CD14^+$ macrophages, while autologous PBMC were 10–15% $CD14^+$ monocytes.

Analysis of cell phenotype by flow cytometry

Antibody combination panels for four or five color immune-staining were as follows:

1. CD3PerCP, CD4 FITC, CD25APC, and rat Foxp3PE (all from eBioscience, San Diego, CA).
2. CD4 PercpCy5.5, HLA-DR APC, CD127 Pac Blue and rat Foxp3 PE (eBioscience, San Diego, CA).

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3. CD4 PercpCy5.5, CCR5 FITC, CXCR4 APC (Biolegend, San Diego, CA) and Foxp3 PE.
4. CD4 PercpCy5.5, HIV-1 p24 FITC (Beckman Coulter, Brea, CA), Bcl-2 PE (BD Biosciences, San Jose, CA) and Foxp3 e-fluor660 (eBioscience).
5. CD4 PercpCy5.5, CD127 Pac Blue, FoxP3 PE, HIV-1 p24 FITC and IFN-γ Alexa Fluor 647 (Biolegend).

Isotype antibodies used included: IgG2a APC, IgG1 Pac Blue, rat IgG2a PE, IgG2a FITC, rat IgG2a AP, IgG1 FITC, IgG1 PE, IgG2a e-fluor660, and IgG1 Alexa Fluor 647.

The protocol and buffer set from eBioscience were used for all experiments involving intracellular staining. All samples were fixed and acquired within 1h of completion of staining, and analysis was by Miltenyi MACSQuant Flow cytometer.

Data were analyzed by FlowJo software (Treestar Inc, Ashland, OR) at the completion of study.

TUNEL assay of apoptosis

Terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) was used to detect apoptotic mononuclear cells as before (25). Briefly, mononuclear cells were surface stained with CD4 Percp and CD25 APC antibodies. Cells were fixed, permeabilized and incubated with Brd UTP (Sigma) in the presence or absence of TdT (Boehringer Indianapolis, IN). DNA breaks were identified by incubation with FITC antibody to Brd UTP (Becton Dickinson). Then, three color analysis was performed by FACScan flowcytometer (Becton Dickinson) and analyzed using WINDMD 2.8 software (Scripps Institute, La Jolla, CA).

Separation of PFMC using magnetic beads

The Human regulatory T-cell isolation kit II from Miltenyi Biotech (Auburn, CA) was used to isolate $CD4^+CD25^+CD127^-$ T cells as instructed by the manufacturer. Purity of isolated CD4 T cells for $Foxp3^+$ expression exceeded 90%. Aliquots of whole PFMC and purified T cells were dissolved in Tri-reagent (Molecular Research Center, Cincinnati, OH) and stored at −70°C until use.

Measurement HIV-1 DNA and Foxp3 mRNA by real-time PCR

Cellular DNA was extracted from the lower phase of cell lysates in Tri-reagent according to the manufacturer's instructions. Real time PCR using the Taqman methodology was by StepONEPlus thermo cycler (Applied Biosystems, Foster City, CA) for HIV-1 minus-strand strong stop (SS) DNA (26), and human beta globin DNA. In each sample DNA copies were normalized to the copy number of beta globin ($0{\cdot}5 \times 10^{10}$ copies in $1{\times}10^6$ cells).

Total RNA was obtained from PFMC cell lysate. RT-PCR for Foxp3 mRNA was as before (14). Foxp3 mRNA levels in each sample were corrected to the copy numbers of ribosomal 18S (R18) ($1{\times}10^{10}$ copies of R18 in $1{\times}10^6$ cells).

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Statistics

Normally distributed data sets were analyzed by student t-test. Wilcoxon or Kruskall Wallis tests were used for data sets that were not normally distributed. Correlation between variables was assessed using linear regression or spearman rank order correlation as appropriate. $P \leq 0.05$ was considered significant.

Results

Increased frequencies of Foxp3^{+} CD25^{hi+} CD4 T cells at pleural sites of HIV/TB co-infection

First, PFMC and autologous PBMC from HIV-1/TB co-infected patients were examined for frequencies of CD4^{+}CD25^{hi+} T-cells and co-expression of Foxp3. CD4^{+}CD25^{hi+} T-cells were increased by 2.7-fold in PFMC as compared to autologous PBMC ($p < 0.01$) (Figure 1). Foxp3^{+} reactivity was enriched among CD4^{+}CD25^{hi+} T-cells in PFMC and PBMC by 85% and 70% respectively. Foxp3^{+}CD4^{+}CD25^{hi+} T-cells were higher in PFMC as compared to PBMC ($P < 0.05$) (Figure 1).

Next, Foxp3 mRNA expression in PFMC from HIV/TB co-infected subjects (Methods) was correlated to their pleural fluid viral loads. Foxp3 mRNA expression and HIV-1 viral load in pleural fluid were found to correlate to a moderate degree ($r=0.780$, $p<0.002$) ($n=16$).

Cumulatively, the data above indicate expansion of PFMC Foxp3^{+}CD4^{+}CD25^{hi+}T-reg at sites of pleural HIV/TB co-infection and a positive association of Foxp3 expression with HIV-1 viral load at pleural sites of HIV/TB co-infection.

HIV-1 infection of Foxp3+ CD4 T cells in PFMC from HIV/TB co-infected patients

Differentiation of CD4^{+}Foxp3^{+} CD127^{-} from transitional CD4^{+}Foxp3^{+} CD127^{+} T cell (27, 28), and from CD4^{+}Foxp3^{-}CD127^{+} was performed on PFMC from 6 HIV/TB co-infected subjects. For this purpose, protocols employing five color immunostaining and FACS analysis were employed (Methods). HIV-1 p24 and IFN-γ reactivity of CD4^{+} Foxp3positive or negative PFMC T-cells that were positive or negative for CD127 (IL7 receptor) expression were assessed. An algorithm for analysis of HIV-1 p24 and IFN-γ reactivity in CD4^{+}Foxp3^{+} CD127^{-}and CD4^{+}Foxp3^{-}CD127^{+} T-cell subsets is shown in Figure 2A. As noted, a relatively small % of transitional CD4^{+}Foxp3^{+}CD127 $^{+}$T-cells were identified in PFMC (Figure 2A).

Cumulative data is shown in Figure 2B. Frequencies of CD4^{+}FoxP3^{+}CD127^{-} T-cells positive for HIV-1 p24 were 7.8 fold higher when compared to CD4^{+}FoxP3^{-}CD127^{+} T cells ($p < 0.01$) (Figure 2B left panel). Also, IFN-γ positivity in CD4^{+}FoxP3^{+}CD127^{-} T cells was 2.2-fold higher when compared to CD4^{+}FoxP3^{-}CD127^{+} T cells ($p < 0.01$) (Figure 2B mid panel). Interestingly, higher (8.2 fold) frequencies of CD4^{+}FoxP3^{+}CD127^{-} T cells that were both HIV-1 p24^{+} and IFN-γ^{+} as compared to CD4^{+}FoxP3^{-} CD127^{+} T cells ($p < 0.05$) were found (Figure 2B right panel).

Thus, CD127^{-} Foxp3^{+} CD4 T cells that express HIV-1 p24^{+} are enriched in PFMC from HIV/TB co-infected patient. Further, almost 25% of HIV-1 p24^{+} CD127^{-} Foxp3^{+} CD4 T cells are IFN-γ reactive.

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Activation status of CD127^{-} Foxp3^{+} CD4 T cells in PFMC from HIV/TB patients

Productive HIV-1 infection of CD4 T-cells is dependent on their status of cellular activation (29). At sites of HIV/TB co-infection HLA-DR^{+} CD4 T cells are expanded and HIV-1 originating from HLA-DR mononuclear cells in pleural fluid is higher than that in the blood (30). Here, we assessed the percentage of CD4^{+}Foxp3^{+} CD127^{-} T cells and CD4^{+}Foxp3^{-}CD127^{+} T cells in PFMC that were HLA-DR positive or negative. HLA-DR positivity among CD4^{+}Foxp3^{+} CD127^{-} T cells was 3 fold higher as compared to CD4^{+}Foxp3^{-}CD127^{+} T cells ($p<0.001$) (Figure 3).

These data identify Foxp3^{+} CD127^{-} CD4 T cells in PFMC from HIV/TB co-infected patients with high levels of immune activation.

Expression of HIV co-receptors by CD4^{+}Foxp3^{+}PFMC

HIV-1 infection is dependent on expression of HIV-1 co-receptors (CCR5 and CXCR4) on CD4^{+} T-cells. In prior observations of PFMC from HIV/TB co-infected subjects we had found that T-cells characterized by high CD25 expression, had significantly higher expression of CCR5 as compared to CD4 T-cells that were low or negative in CD25 expression (unpublished data). Here we examined both CCR5 and CXCR4 expression by CD4^{+}Foxp3^{+} PFMC as compared to CD4^{+}Foxp3^{-} PFMC (n=5). Fig 4A shows these results: expression of both CCR5 and CXCR4 was higher on CD4^{+}Foxp3^{+} as compared to CD4^{+}Foxp3^{-} T-cells ($P < 0.01$, for either comparisons).

Next, we assessed successful HIV-1 entry in PFMC from HIV/TB co-infected patients (n=2) and one HIV-infected subject who proved later to have pleural mesothelioma. CD4^{+} CD25+ CD127^{-} were purified from PFMC by immune-magnetic separation (Methods). Cellular DNA was extracted from each population and assessed for HIV-1 strong stop (SS) DNA, which represents all HIV-1 that has initiated reverse transcription post HIV-1 cell entry. HIV-1 SS DNA was enriched in the isolated CD4^{+} CD25+ CD127^{-} T cells as compared to whole PFMC in the HIV/TB co-infected subjects, but not in the subject with pleural mesothelioma (Fig 4B).

Therefore, higher HIV-1 co-receptor expression and predisposition to successful HIV-1 infection are features of CD25+ CD127^{-} CD4 T cells that are 90% Foxp3^{+} in PFMC from HIV/TB co-infected subjects.

Survival of HIV infected Foxp3^{+} T cells in PFMC

First, we assessed the apoptotic profile of CD4 T cells in PFMC from HIV/TB co-infected subjects. For these experiments TUNEL positivity of PFMC identified as CD4^{+}CD25^{hi+} was compared to CD4^{+}CD25$^{lo+/-}$ T-cells. Frequencies of TUNEL positive CD4^{+}CD25$^{lo+/-}$ T-cells exceeded those of CD4^{+}CD25^{hi+} T cells by 3.7 fold ($P < 0.001$) (Figure 5A). As noted above (Fig 1), 85% of CD4^{+}CD25^{hi+} PFMC were Foxp3 positive.

Next, the intracellular co-expression of the anti-apoptotic molecule, Bcl-2, and HIV-1 p24 in Foxp3^{+} and Foxp3^{-} CD4T cells in PFMC were assessed. CD4^{+}Foxp3^{+} T cells were 5 fold higher in co-expression of Bcl-2 and HIV-1 p24 as compared to CD4^{+}Foxp3^{-} T cells ($P < 0.001$) (Fig 5B). A correlation of Bcl-2 expression and Foxp3^{+} HIV-1 p24^{+} dual reactivity

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was found (R=0.551, p=0.02). Interestingly among $CD4^+Foxp3^+$ T cells, Bcl-2 reactivity was significantly higher among HIV-1 $p24^+$ as compared to HIV-1 $p24^-$ cells ($p<0.001$) (Fig 5C).

Therefore, in PFMC from HIV/TB co-infected subjects HIV-1 p24 positive $CD4^+Foxp3^+$ T cells have a survival advantage over both HIV-1 $p24^+$ $Foxp3^-$ CD4 T cells, and HIV-1 p24 negative $CD4^+Foxp3^+$ T cells.

Discussion

Current literature indicates that $Foxp3^+CD4$ T cells are predisposed to HIV infection (5, 20), and therefore may contribute to HIV-1 disease progression through viral production. This is in addition to the role of $CD4^+Foxp3^+$T cells in suppression of antigen-specific HIV-1 T-cell responses (31). Here we found significantly higher numbers of $CD4^+Foxp3^+$ in PFMC as compared to autologous PBMC from HIV/TB co-infected patients with pleural disease. Higher HIV-1 p24 expression was found in $Foxp3^+$ $CD127^-$ as compared to $Foxp3^-$ $CD127^+$ CD4 T cells in PFMC. $CD4^+Foxp3^+$ T cells in PFMC were also characterized by both significantly higher cellular activation (HLA-DR reactivity) and expression of HIV-1 co-receptors (CCR5 and CXCR4) as compared to $Foxp3^-$ CD4 T cells, underscoring their susceptibility to productive HIV-1 infection. Interestingly, HIV-1 $p24^+$ $Foxp3^+$ CD4 T cells were highly Bcl-2 reactive as compared to both HIV-1 $p24^+$ $Foxp3^-$ CD4 T cells and $Foxp3^+$ CD4 T cells without HIV-1 expression. A significantly greater fraction of $CD4^+Foxp3^+$ $CD127^-$ T cells were both HIV-1 $p24^+$ and IFN-γ^+, when compared to $Foxp3^-$ CD4 T cells. Cumulatively, these data indicate $CD4^+Foxp3^+$ as a source of HIV-1 among CD4 T cell population at pleural sites of HIV/TB co-infection, with survival advantage over other CD4 T cells.

During *in vitro* infection of human T-cells by HIV-1 (6, 20), high viral replication is supported by Foxp3 expressing CD4 T cells. Here we identify $Foxp3^+$ CD4 T cells as the subset with significantly higher expression of CCR5 and CXCR4 as compared to $Foxp3^-$ CD4 T cells in PFMC. Consistent with increased HIV-1 co-receptor expression, we found higher levels of HIV-1 SS DNA in immune-magnetically isolated $CD4^+CD25^+CD127^-$T cells as compared to un-separated PFMC. HIV-1 SS DNA was notably higher in $CD4^+CD25^+CD127^-$PFMC T cells from the two HIV-1 infected patients who were MTB culture positive as compared to the subject who proved to have pleural mesothelioma and not TB. All together, these data indicate that $Foxp3^+$ CD4 T cells at sites of pleural HIV/TB co-infection are predisposed to successful and productive HIV-1 infection. Strong correlation of Foxp3 mRNA in PFMC with viral load in the pleural fluid of HIV/TB patients corroborates this contention. Thus, HIV-1 $p24^+$ $Foxp3^+$ CD4 T cells at sites of HIV/TB may be a significant source of HIV-1. However, only a modest and insignificant correlation of Foxp3 mRNA expression in PFMC with pleural fluid viral load was found in our own previous study (14). Whereas, the number of patients in the current study were higher (16 vs 10) than previously (14), differential cell handling may have contributed to this discrepancy also.

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Here, HIV-1 $p24^+$ $Foxp3^+$ CD4 T cells in PFMC were higher in expression of the anti-apoptotic molecule, Bcl-2 as compared to HIV-1 $p24^+$ $Foxp3^-$ CD4 T cells. Strong Th1 signaling provided at sites of HIV/TB co-infection (14, 19), in the presence of continuous TGF-β signaling necessary in maintaining expression of Foxp3 (32), likely underlies the expanded $Bcl\text{-}2^+$ $Foxp3^+$ CD4 T cells in PFMC. However, others have shown that among Foxp3 targeted genes, expression of Bcl-2 is actually down-regulated (33), possibly based on interaction of the transcriptional factor Eos with Foxp3 gene (34). While it is possible that at sites of HIV/TB co-infection, the interaction of Foxp3 and Eos is altered, this needs to be investigated. The higher Bcl-2 expression of HIV-1 $p24^+$ $Foxp3^+$ T-cells as compared to HIV-1 $p24^-$ $Foxp3^+$ T-cells, and the correlation of Bcl-2 positivity with $Foxp3^+p24^+$ reactivity of PFMC T cells, implies a survival advantage of HIV-1 infected Foxp3+ CD4 T cells.

The stability of Foxp3 expression in natural T-reg has been ascribed to the highly de-methylated conserved non-coding DNA sequence 2 (CNS2) in the Foxp3 promoter, which is not present in induced T-reg. However, TGF-β has been shown to decrease methylation of CNS2 thus stabilizing Foxp3 expression in iT-reg (12, 13). It is possible that at sites of HIV/TB co-infection, high levels of bioactive TGF-β induce and maintain Fox p3 expression. Further, recent studies have shown that bystander inflammation, conditions naïve T cells to Foxp3 expression and reduced effector cell differentiation (35). The correlation of Foxp3 mRNA expression in PFMC from HIV/TB co-infected patients with pleural fluid levels of IL-6 and IL-8 and to a lesser extent TGF-β, but not at all with levels of IFN-γ (14), support this latter scenario. Overall, these data implicate induction of Foxp3 mRNA expression by inflammatory cytokines and maintenance of its expression by TGF-β at sites of HIV/TB co-infection.

Recently, it has been shown that $CD4^+Foxp3^+$ T-cells can produce IFN-γ when activated under a Th1 cytokine polarizing environment (15, 16). In fact, it has been suggested that IFN-γ production identifies pathogen epitope-specific $CD4^+Foxp3^+$ T-cells during viral infections (36). Here, over 25% of $CD4^+$PFMC T cells that were $Foxp3^+CD127^-$ T-cells were also IFN-γ^+. Similar to the model of Graft-versus-Host Disease (37) IFN-γ expression by $Foxp3^+$ CD4 T cells may be important to their immune-regulatory function. However, IFN-γ positivity may actually identify $Foxp3^+$ CD4 T cells that are predisposed to loss of Foxp3 expression and conversion to a Th1 T cell phenotype (38, 39). Also, at sites of inflammation, certain $Foxp3^+$ CD4 T cells may undergo rapid reprogramming into Th1 like T-cells, without loss of expression of Foxp3 (40). The true fate of IFN-γ producing $Foxp3^+$ CD4 T cells at sites of dual HIV/TB co-infection remains to be explored.

In summary, high frequencies of HIV-1 infected $Foxp3^+$ CD4 T cells that display features of survival at pleural sites of HIV/TB co-infection were found. Whether HIV-1 24 expressing $Foxp3^+$ CD4 T cells convert to CD4 T cells with ‘silent’ HIV-1 infection upon resolution of TB needs to be further studied.

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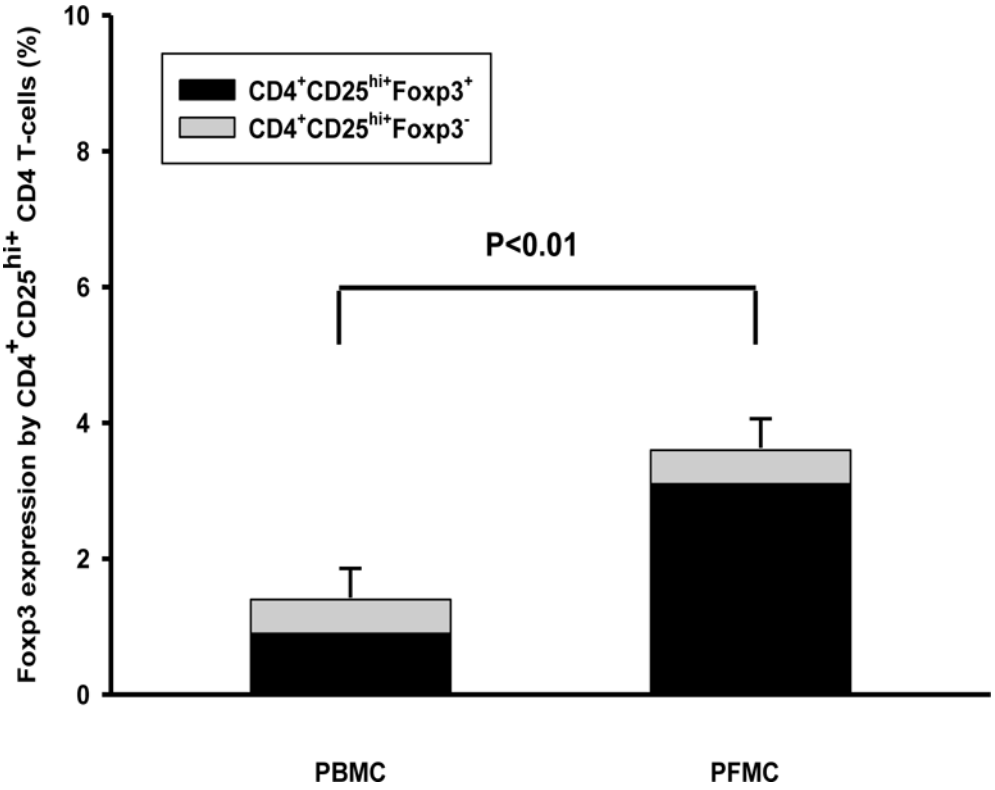

Figure 1. Frequencies of T-reg are increased at sites of HIV/TB co- infection

$CD4^+CD25^{hi+}$ in PFMC and autologous PBMC from HIV/TB co-infected patients with pleural TB were assessed for intra-cellular Foxp3 expression (n=16). As compared to autologous PBMC, PFMC contained higher $CD4^+CD25^{hi+}$ T cells ($p<0.01$) and higher proportion of $Foxp3^+CD25^{hi+}$ T cells ($p < 0.05$).

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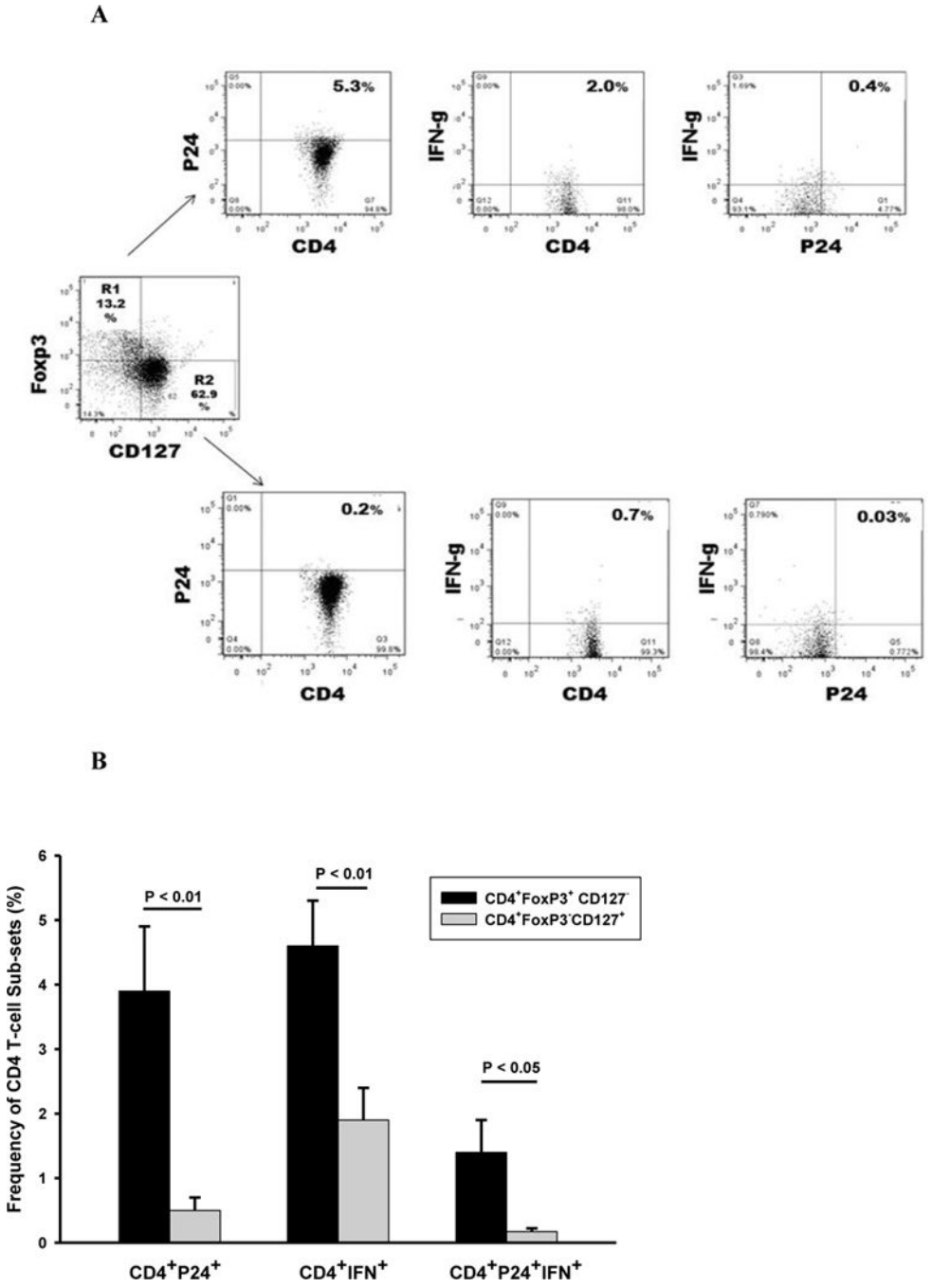

Figure 2. Frequencies of CD4$^+$Foxp3$^+$ CD127$^-$ and CD4$^+$Foxp3$^-$CD127$^+$ PFMC co-expressing HIV-1 p24 alone or in combination with IFN-γ

CD4$^+$Foxp3$^+$ CD127$^-$ and CD4$^+$Foxp3$^-$CD127$^+$ in PFMC from HIV/TB patients (n=6) were characterized for their expression of HIV-1 p24 and IFN-γ. **A.** Algorithm for assessment of frequencies of CD4$^+$Foxp3$^+$ CD127$^-$ and CD4$^+$Foxp3$^-$CD127$^+$ PFMC co-expressing HIV p24 and IFN-γ alone or in combination. **B**. Among CD4$^+$Foxp3$^+$ CD127$^-$ T cells, higher frequencies of HIV-1 p24$^+$ ($p<0.01$), IFN-γ$^+$ ($p<0.01$) and dual HIVp24$^+$IFNγ$^+$ ($P<0.05$) as compared to CD4$^+$Foxp3$^-$CD127$^+$ T cells were found.

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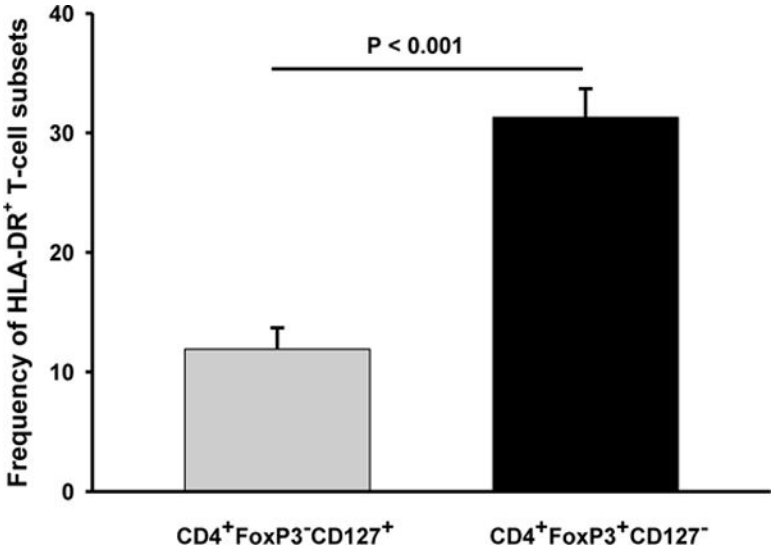

Fig 3. Frequencies of HLADR expression by $CD4^{+}Foxp3^{+}$ $CD127^{-}$ versus $CD4^{+}Foxp3^{-}CD127^{+}$ T cells

PFMC from HIV/TB co-infected patients were assessed for CD4, $Foxp3^{+}$, CD127 and HLA-DR reactivity (n=16). Frequency of HLADR positive $CD4^{+}CD127^{-}Foxp3^{+}$ T cells was significantly higher than $CD4^{+}$ CD127 $Foxp3^{-}$ T cells ($p<0.001$).

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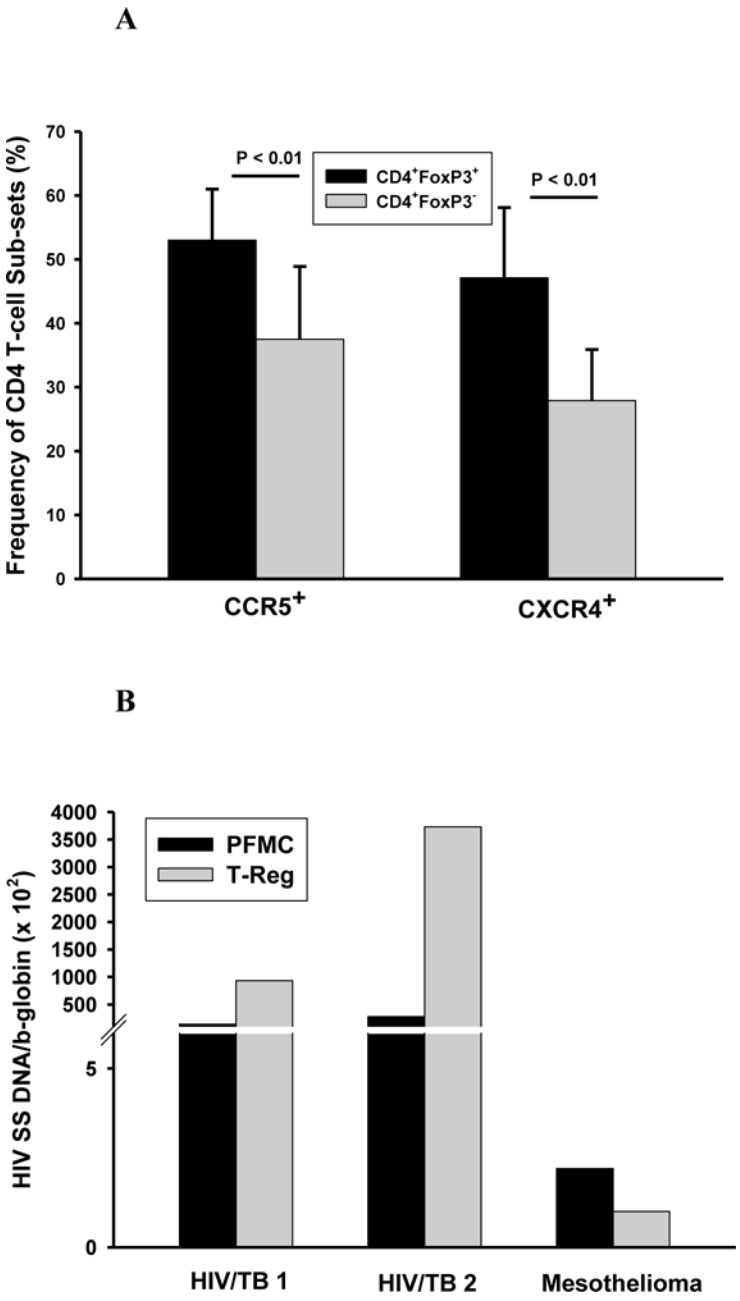

Figure 4. Expression of CCR5 and CXCR4 by Foxp3$^+$ and Foxp3$^-$ CD4 T cells in PFMC and HIV-1 entry in PFMC

Foxp3$^+$ and Foxp3$^-$ CD4 T cells in PFMC from HIV/TB co-infected patients were assessed for expression of HIV-1 co-receptors CCR5 and CXCR4 and HIV-1 entry. **A**. Expression of CCR5 and CXCR4 were increased in CD4$^+$Foxp3$^+$ as compared to CD4$^+$Foxp3$^-$ T cells ($p < 0.01$ for both, n=5). **B**. HIV-1 entry was assessed by PCR for HIV-1 SS DNA following immune-magnetic separation of PFMC to obtain CD4$^+$Foxp3$^+$ CD127$^-$ T cells and compared to that in whole PFMC. Results are shown for 2 HIV/TB co-infected patients and one HIV-1 infected subject with pleural mesothelioma.

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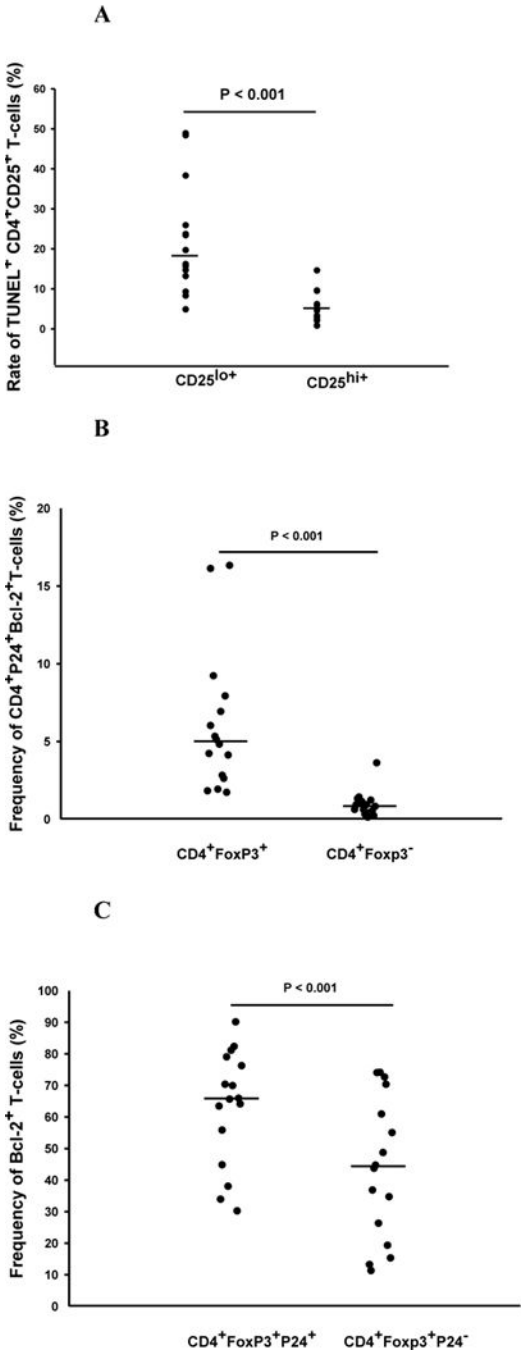

Figure 5. Resistance to apoptosis and differential expression of Bcl2 in PFMC subpopulations in HIV/TB co-infected patients

In **A**, apoptosis of $CD4^+CD25^{hi+}$T cells and $CD4^+CD25^{lo/-}$ T cells were assessed by TUNEL methodology (n=10). In **B** and **C** PFMC from HIV/TB patients were assessed for co-expression of intra-cellular Foxp3, HIV-1 p24 and Bcl-2 (n=16). **A.** Frequencies of TUNEL positive $CD4^+CD25^{lo/-}$ T cells exceeded those of $CD4^+CD25^{hi+}$ T cells by 3.7 fold ($P < 0.001$). **B.** Frequencies of HIV-1 $p24^+Foxp3^+$ CD4 T cells co-expressing Bcl-2 exceeded HIV-1 $p24^+Foxp3^-$ T cells by more than 5 fold ($p < 0.001$). **C.** Among Foxp3

positive CD4 T cells, intracellular expression of Bcl-2 was enriched among HIV-1 $p24^{+}$ as compared to HIV-1 $p24^{-}$ CD4 T-cell subsets ($p<0.001$) (n=16).

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Table 1

Age*	Female/male (%)	CD4 (/μl)*	Plasma VL (/ml)*	Pleural Fluid VL (/ml)*	TB diagnosis (%) Culture/Histology
34 (25–50 yrs)	18.7%/81.3%	169 (59–445)	2.8×10^5 ($0.2–9.8\times10^5$)	2.1×10^6($0.07–5.2\times10^6$)	93.7%/6.3%

* Median (range)