T-reg are the Predominant CD4+ T-Cell Subset Productively Infected with HIV-1 at Sites of Dual HIV/TB Infection

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Abstract

Background: Regulatory T-cells (T-reg) are expanded during active tuberculosis (TB) regardless of HIV-1-infection, particularly at sites of M. tuberculosis infection. In HIV-1 disease, T-reg are targeted by HIV-1 infection. However, whether they contribute to promotion of HIV-1 infection at sites of HIV/TB is unknown. Methods: Pleural fluid mononuclear cells (PFMC) from HIV/TB patients with pleural TB were characterized by immunostaining and FACS analysis for surface markers CD4, CD127, CCR5, CXCR4 and intracellular expression of Foxp3, HIVp24, IFN-g and Bcl-2. T-reg purified by immunomagnetic bead separation were assessed for HIV-1 strong stop (SS) DNA expression by real-time PCR. Results: High numbers of T-reg (defined as CD4+Foxp3+ or CD4+Foxp3+CD127−), that were HIV-1-infected were found in PFMC. T-reg displayed higher expression of the activation marker, HLA-DR (p<0.001), and HIV-1 co-receptors (CCR5 and CXCR4) (p<0.05 for both) as compared to non-T-reg. Purified T-reg exhibited higher HIV-1 infection, as measured by HIV-1 SS DNA, when compared to whole PFMC, and as compared to PFMC T-reg from an HIV-infected subject with mesothelioma. HIV-infected CD4+Foxp3+T-reg were significantly higher in Bcl-2 expression as compared to CD4+Foxp3+cells (p<0.001). Further, HIV-1-infected T-reg showed significantly higher Bcl-2 reactivity than un-infected T-reg (p<0.001). A small fraction of HIV-1-infected T-reg were also IFN-g-.

Conclusion: At sites of pleural HIV/TB infection higher frequencies of PFMC T-reg with survival advantage are predisposed to productive HIV-1 infection as compared to non-T-reg.

Introduction

Expansion of CD4 T-cells that express Foxp3 is a feature of active tuberculosis (TB). High numbers of Foxp3+ CD4 T-cells with a T-regulatory functional profile in blood [1,2] and at sites of Mycobacterium tuberculosis (MTB) infection [3,4] have been documented. The basis of expansion of T-reg at sites of dual HIV-1 and TB (HIV/TB) co-infection has been studied more recently. Expression of Foxp3, a molecular signature marker of T-reg [5], among pleural fluid mononuclear cells (PFMC) at sites of HIV/TB co-infection was significantly higher than that in autologous PBMC [6]. Other has reported expression of CD39 in addition to Foxp3 as a marker of T-reg in TB [7]. Pleural sites of active HIV/TB infection are prominent in HIV-1 activity as compared to that found systemically [8], and currently activated CD4 T-cells are believed to be the predominant source of HIV-1 in situ [9]. Whether T-reg contribute to productive HIV-1 infection at sites of HIV/TB is not known.

Foxp3 expression is necessary in establishing the regulatory T-cell lineage both for thymus-derived natural (n) T-reg, and induced (i) T-reg derived from naive CD4 T-cells outside of the thymus (Reviewed in [10]). Expression of Foxp3 in iT-reg is under control by epigenetic regulatory elements [11]. In peripheral lymphoid tissues, transforming growth factor beta (TGF-β) is critical to induction of expression of Foxp3 in T-cell receptor (TCR) activated naive CD4 T-cells [12,13]. Whereas induction of iT-reg requires TGF-β alone, expansion of TGF-β induced iT-reg is dependent on the NFκb/c-Rel [14] and/or IL-2/Stat5 signaling [15] pathways. At sites of pleural TB, TGF-β and pro-inflammatory cytokines (IL-8, IL-6, and IFN-g), but not IL-2, are abundant [6]. Further, Foxp3 mRNA expression in PFMC correlated with levels of IL-6, IL-8 and TGF-β in pleural fluid [6], suggesting that continuous TCR activation through cytokines in the cellular micro milieu at sites of pleural HIV/TB infection may be conducive to stabilization of Foxp3 expression in PFMC T-reg.

T-reg have been found to be targets of HIV-1 infection among...
blood mononuclear cells [16,17]. Also, naïve T-cell precursors of Foxp3 positive T-reg are susceptible to HIV infection in vitro [18]. Both heightened status of cellular activation [19] and increased expression of HIV-1 co-receptors [17] may underlie predisposition of T-reg to successful viral infection. Further, Foxp3 enhances NFκB occupancy at HIV-1 LTR [20], indicating that T-reg are predisposed to productive HIV-1 infection. However, others have shown that transfection of Foxp3 gene in CD4 T-cells in fact represses HIV-1 transcription [21,22]. Therefore, the transcriptional basis of HIV-1 infection in T-reg remains controversial.

Other studies have shown that CD4+Foxp3+ T-cells express IFN-γ, when activated under a Th1 cytokine polarizing environment [23,24], and a Th1 cytokine milieu characterizes sites of pleural TB [6,25]. Expression of IFN-γ among T-reg during HIV/TB co-infection is not known.

Based on our previous observations of both enhanced HIV activity and increased frequencies of T-reg at pleural sites of HIV/TB we hypothesized that T-reg expanded at pleural sites of dual HIV/TB infection may directly contribute to expansion of HIV-1 activity. We found that among CD4+ T-cells in PFMC, HIV-1 infection was enriched in the CD4+Foxp3+CD127+ T-cell population. Higher numbers of CD4+Foxp3+CD127+ T-cells co-expressed HIV-1 p24 and IFN-γ, as compared to non T-reg. Expression of both HIV-1 co-receptors (CXCR4 and CCR5) and HLADR was significantly higher and IFN-γ, as compared to non T-reg. Expression of both HIV-1 co-receptors (CXCR4 and CCR5) and HLADR was significantly higher and IFN-γ, as compared to non T-reg. Further, HIV infected T-reg were significantly more Bcl-2 positive than uninfected T-reg. Therefore, among CD4+ T-cells at sites of HIV/TB co-infection, the predominant population of productively HIV-1 infected T-cells are T-reg.

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 effusion 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 CW RU 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 [26]. Diagnosis of pleural TB was based on positive culture of sputum and/or pleural fluid, and/or biopsy as previously described [26]. Diagnosis of pleural TB was based on positive culture of sputum and/or pleural fluid, and/or positive histology of pleural tissue for Mtb. Only HIV+ patients were included in this study (n=16). All patients received standard short-course anti-TB therapy and were followed as before. Table 1 describes the characteristics of enrolled patients.

Preparation of cells and separation of T-reg from PFMC using magnetic beads

PFMC were prepared by ficollHypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation as described [26]. Viability was >98% as assessed by trypan blue exclusion. By immunostaining and FACS analysis PFMC were 60-60% CD4+ T-cells and contained only 1-5% CD14+ macrophages.

In a few experiments, T-reg were prepared by magnetic bead separation of PFMC using the human regulatory T-cell isolation kit II from Miltenyi Biotech (Auburn, CA) as instructed by the manufacturer. Purity of CD4+Foxp3+ T-cells obtained exceeded 90%. Aliquots of whole PFMC and purified T-reg were, dissolved in Tri-reagent (Molecular Research Center, Cincinnati, OH) and stored at -70°C until use.

Analysis of cell phenotype by flow cytometry

At the onset of study 4 color flow cytometry was used, as this was the limit of the flow cytometer available on site. Antibody and isotype control combination used to characterize T-reg included: 1. CD4 PerCpCy5.5, HLA-DR APC, CD127 Pac Blue and rat Foxp3 PE; 2. CD4 PerCpCy5.5, IgG2a APC, IgG1 Pac Blue and rat IgG2a PE (all from eBioscience, San Diego, CA); 3. CD4 PerCpCy5, CCR5 FITC, CXCR4 APC (both from Biologend, San Diego, CA) and Foxp3 PE; 4. CD4 PerCpCy5.5, IgG2a FITC, rat IgG2a APC and rat IgG2a PE; 5. CD4 PerCpCy5, p24 FITC (Beckman Coulter, Brea, CA), BcI-2 PE (BD Biosciences, San Jose, CA) and Foxp3 e-fluor660; 6. CD4 PerCpCy5, IgG1 FITC, IgG1 PE and rat IgG2a e-fluor660 (eBioscience).

Subsequently, a MiltenyMACSQuant Flow cytometer (allowing for the assessment of samples containing a maximum of 8 fluorochromes) became available. To assure that staining with FoxP3 antibody alone accurately identifies T-reg, subsequently duplicate samples for each patient were stained with CD127 Pac Blue (or IgG1 isotype antibody in combination with FoxP3 PE or FoxP3 alone as above). We chose CD127 as an additional marker for T-reg as studies by Fazekas et al. and Seddiqi et al. [27,28] indicate that addition of CD127 to Foxp3 and/or CD25 allows for the identification and isolation of a pure population of T-reg and allows for a better discrimination between T-reg and activated (CD25+) T-cells. We established that a strong correlation between frequencies of T-reg characterized as CD4+Foxp3+CD127+ and CD4+Foxp3- (R=0.87) existed. Antibody combinations for 5 color experiments were 1. CD4 PerCpCy5.5, CD127 Pac Blue, Foxp3 PE, p24 FITC and IFN-γ Alexa Fluor 647 (Biologend); 2. CD4 PerCpCy5.5, IgG1 Pac Blue, Rat IgG2a PE, IgG1 FITC and IgG1 Alexa Fluor 647 (Biologend).

The protocol and buffer set from eBioscience were used for all experiments involving intracellular staining. Samples were fixed and acquired within 1h of completion of staining. Data were analyzed in bulk using FlowJo software (TreestarInc, Ashland, OR) at the completion of study.

Measurement HIV-1 DNA 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 by ABI StepOnePlussothermal cycler (Applied Biosystems, Foster City, CA) was performed for HIV-1 minus-strand strong stop (SS) DNA (which represents HIV-1 transcripts post-cell entry that have initiated reverse transcription) [29], and human beta globin DNA. In each sample DNA copies were normalized to the copy number of beta globin (0.5x10⁶ copies in 1 million cells).

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 and correlation or spearman rank order correlation as appropriate. P ≤ 0.05 was considered significant.

Results

Preferential HIV-1 infection of CD4+Foxp3+IFNg+ PFMC

In combination immunostaining studies we assessed HIV-1 p24 and IFN-g reactivity of CD4+ Foxp3+ T-cells that were negative or positive for IL7 receptor (CD127) expression. This approach

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<th>Table 1: The characteristics of enrolled patients</th>
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<td><strong>Age</strong></td>
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fully differentiates “True T-reg” (CD4+Foxp3+CD127-) from non-T-reg (CD4+Foxp3+CD127+) and the “transitional” population (CD4+Foxp3+CD127+) [27,28]. An algorithm of analysis of HIV-1 p24 and IFN-g reactivity in CD4+Foxp3+CD127- and CD4+Foxp3+CD127+ PFMC co-expressing HIV-p24 and IFN-g alone or in combination is shown in Figure 1A. Cumulative data for 6 experiments is shown in Figure 1B. Higher frequencies of CD4+Foxp3+CD127-T-reg were dually HIV-1 p24+ and IFN-g+ as compared to CD4+Foxp3+CD127-T-cells (8.2-fold; P < 0.05) (Figure 1B right panel).

Productive HIV-1 infection of CD4 T-cells is dependent on cellular activation status. Therefore we also assessed the percentage of CD4+Foxp3+CD127+PFMC T-cells that were HLA-DR+. HLA-DR positivity among CD4+Foxp3+CD127-T-reg was 2.2-fold higher when compared to CD4+Foxp3+CD127+ non-T-reg (P<0.01) (Figure 1B mid panel). Further, greater frequencies of CD4+Foxp3+CD127-T-reg were dually HIV-1 p24+ and IFN-g+ as compared to CD4+Foxp3+CD127+ (8.2-fold; P < 0.05) (Figure 1B right panel).

We also found that IFN-g positivity among CD4+Foxp3+CD127-T-reg was 2.2-fold higher when compared to CD4+Foxp3+CD127+ non-T-reg (P<0.01) (Figure 1B mid panel). Further, greater frequencies of CD4+Foxp3+CD127-T-reg were dually HIV-1 p24+ and IFN-g+ as compared to CD4+Foxp3+CD127+ (8.2-fold; P < 0.05) (Figure 1B right panel).

Figure 1A:

Figure 1B:

Figure 1: Frequencies of CD4+Foxp3+CD127- and CD4+Foxp3+CD127+ PFMC co-expressing HIV-1 p24 alone or in combination with IFN-g.

True T-reg (CD4+Foxp3+CD127-) and non-T-reg (CD4+Foxp3+CD127+) among PFMC from HIV/TB patients were characterized for their expression of HIV-1 p24 and IFN-g.

A. Algorithm for assessment of frequencies of CD4+Foxp3+CD127- and CD4+Foxp3+CD127+ PFMC co-expressing HIV-p24 and IFN-g alone or in combination. B. Among CD4+Foxp3+CD127+ PFMC frequencies of HIV-1 p24+ (p<0.01), IFN-g+ (p<0.01) and dual HIV-p24+IFNg+ (P<0.05) T-cells were higher as compared to CD4+Foxp3+CD127- T-cells (n=6 for all comparisons).
Expression of HIV co-receptors by CD4 Foxp3+PFMC

HIV-1 infection is dependent on expression of HIV-1 co-receptors (CCR5 and CXCR4) by CD4+ T-cells. In prior observations we had found that PFMC T-cells characterized by high CD25 expression had significantly higher expression of CCR5 as compared to CD25 low or negative CD4 T-cells (unpublished data).

In experiments outlined above (n=6) a strong correlation between frequencies of T-reg characterized as CD4+Foxp3+CD127- and CD4+Foxp3+(R=0.87) was found. Here we examined both CCR5 and CXCR4 expression by CD4+Foxp3+PFMC as compared to CD4+Foxp3- PFMC. Figure 2A shows these results: expression of both CCR5 and CXCR4 was increased on CD4+Foxp3+ T-reg as compared to CD4+Foxp3- PFMC (P<0.01 for both, n=5) (A). HIV-1 entry was assessed by PCR following immunomagnetic separation of PFMC to obtain T-reg (CD4+Foxp3+ CD127- ) and compared to that in whole PFMC. Results shown are for 2 HIV/TB patients and one HIV infected subject with pleural mesothelioma (B).

To confirm enrichment of HIV infection among PFMC T-reg, whole PFMC and T-reg (CD4+Foxp3+ CD127-) purified by immunomagnetic separation were compared. Cellular DNA was assessed by HIV-1 strong stop (SS) DNA (HIV-1 transcripts that have initiated reverse transcription). Results from two HIV/TB subjects were compared to PFMC and T-reg obtained from an HIV-infected subject with pleural mesothelioma. HIV-1 SS DNA was enriched among PFMC T-reg as compared to whole PFMC in HIV/TB subjects (Figure 2B). HIV-1SSDNA in PFMC from the subject with mesothelioma was low and not enriched in their T-reg.

HIV infected Foxp3+ T reg cells have survival advantage among PFMC

Previously we have observed a survival advantage (by Tunel assay) of T-reg over non-T-reg among PFMC in HIV/TB co-infected subjects (unpublished data). Here, the intracellular co-expression of Bcl-2 and HIV-1 p24 among CD4+Foxp3+ and CD4+Foxp3+ T-cells was assessed (n=16). A 5 fold higher Bcl-2 expression among T-reg as compared to non-T-reg was found (P<0.001; Figure 3A). Interestingly, there was a correlation of Bcl-2 reactivity and Fox p3+ HIV-1 p24+dual reactivity (R=0.551, p=0.02), but not with HIV-1 infected non-T-reg (Fox- p24-). Interestingly, further analysis showed that expression of Bcl-2 reactivity was significantly enriched among HIV-1 infected as compared to HIV uninfected Foxp3+ T-reg (p<0.001) (Figure 3B).

Discussion

Recent literature indicates that CD4+Foxp3+ T-reg are predisposed to HIV infection [16,18] and thus may contribute to HIV-1 disease.
progression through viral production. This is in addition to the role of T-reg in suppression of antigen-specific CD4 and CD8 anti-HIV-1 T-cell responses [38]. Here we found significantly higher numbers of T-reg defined as CD4+Foxp3+ CD127− that were HIV-1 infected in PFMC at sites of pleural HIV/TB. T-reg were characterized by both significantly higher cellular activation (HLA-DR) and expression of HIV-1 co-receptors (CCR5 and CXCR4) as compared to non-T-reg, underscoring their susceptibility to productive HIV-1 infection. Interestingly, HIV-1 infected T-reg were Bcl-2 reactive, implicating their survival advantage not only over non-T-reg, but also over HIV-uninfected T-reg. Also, a significantly greater fraction of T-reg were both HIV-1 infected and IFN-γ+, when compared to non-T-reg. Cumulatively, these data pose T-reg as a dominant HIV-infected CD4 T-cell population with survival advantage at sites of TB. Whether increased frequencies of HIV-infected T-reg directly contribute to HIV production at sites of HIV/TB through HIV release or infection of other mononuclear cells needs to be established in future studies.

During in vitro infection of T-cells by either HIV-1 [17,18] or FIV [31], T-reg support higher viral replication than non-T-reg. Both degree of HIV-1 co-receptor expression and activation status determines successful productive HIV-1 infection of mononuclear cells. Previously, we had found higher expression of both CCR5 [29] and CXCR4 (unpublished) by CD4 T-cells in PFMC from dually infected HIV/TB subjects. Here we identify CD4+Foxp3+T-cells as the subset with significantly higher expression of CCR5 and CXCR4 as compared to CD4+Foxp3-non-T-reg in PFMC. Consistent with increased HIV-1 co-receptor expression by T-reg, we found higher levels of initiated viral transcripts (HIV-1 SS DNA) in immunomagnetically isolated PFMC T-reg as compared to un-separated PFMC. Further, HIV-1 SS DNA was notable in T-reg from two HIV/TB patients as compared to a HIV-1 infected subject with mesothelioma.

In the current study, we found more PFMC T-reg that were dually positive for HIV-1 p24 and Bcl-2 than non-T-reg. Even among T-reg, HIV-1 infection was associated with significantly higher expression of Bcl-2. Therefore, HIV-1 infected T-reg at sites of HIV-1/TB are presumably characterized by lesser predisposition to apoptosis as compared to other T-cells. However, others have shown that Foxp3 targeted genes in iT-reg, expression of Bcl-2 is actually down-regulated [32], and inhibition of Bcl-2 expression appears to be based on interaction of Foxp3 with the transcriptional repressor, Eos [33]. While it is possible that at sites of HIV/TB, interaction of Foxp3 and Eos is defective in PFMC T-reg, this needs to be investigated. On the other hand, TCR engagement leads to expression of anti-apoptotic molecules Bcl-2, B6-1 and Bcl-x in T-cells [34,35]. Strong Th1 signaling provided at sites of HIV/TB infection [6,25], in the presence of continuous TGF-β signaling necessary in maintaining Foxp3 expression [36], likely underlies the basis of expanded Bcl-2+T-reg among PFMC found here. The higher Bcl-2 expression of HIV-1 infected Foxp3+ T-cells and the correlation of Bcl-2 with Foxp3+ p24- (and not Foxp3+ p24+) reactivity underscores survival advantage of HIV-1 infected T-reg. Thus, infected T-reg at sites of HIV/TB may be the predominant CD4+ T-cell source of HIV-1 due to their survival advantage. This may explain the correlation of Foxp3 mRNA to viral load in the pleural fluid of HIV/TB patients found here (data not shown, n=16, r=0.780, p<0.002). Of note, however, was there only a modest and insignificant correlation of Foxp3 mRNA expression in PFMC with pleural fluid viral load in our previous study (n=10, r=0.3) [6].

Recently, it has been shown that CD4+Foxp3+ T-cells can produce IFN-γ when activated under a Th1 cytokine polarizing environment [23,24]. In fact, it has been suggested that IFN-γ production identifies pathogen epitope-specific T-reg during viral infections [37]. Here, up to 6.1% of T-reg (CD4+Foxp3+CD127+T-cells) were IFN-γ+. IFN-γ production by T-reg may be important to their immunoregulatory function as shown in the model of Graft-versus-Host Disease [38]. However, the true role of IFN-γ producing T-reg at sites of dual HIV/TB remains to be deciphered.

In summary, higher frequencies of T-reg as compared to non-T-reg are HIV-infected and display survival advantage among PFMC at sites of pleural HIV/TB. Whether expanded T-reg at sites of dual HIV/TB infection contribute to HIV-1 viral activity also by undermining anti-HIV-1 immune responses of CD4 and CD8 effector T-cells needs to be further studied.

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References


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