FOXP3 Expression Is Upregulated in CD4⁺T Cells in Progressive HIV-1 Infection and Is a Marker of Disease Severity

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Abstract

Background: Understanding the role of different classes of T cells during HIV infection is critical to determining which responses correlate with protective immunity. To date, it is unclear whether alterations in regulatory T cell (Treg) function are contributory to progression of HIV infection.

Methodology: FOXP3 expression was measured by both qRT-PCR and by flow cytometry in HIV-infected individuals and uninfected controls together with expression of CD25, GITR and CTLA-4. Cultured peripheral blood mononuclear cells were stimulated with anti-CD3 and cell proliferation was assessed by CFSE dilution.

Principal Findings: HIV infected individuals had significantly higher frequencies of CD4⁺FOXP3⁺ T cells (median of 8.11%; range 1.33%–26.27%) than healthy controls (median 3.72%; range 1.3–7.5%; P = 0.002), despite having lower absolute counts of CD4⁺FOXP3⁺ T cells. There was a significant positive correlation between the frequency of CD4⁺FOXP3⁺ T cells and viral load (ρ = 0.593 P = 0.003) and a significant negative correlation with CD4 count (ρ = –0.423 P = 0.044). 48% of our patients had CD4 counts below 200 cells/μl and these patients showed a marked elevation of FOXP3 percentage (median 10% range 4.07%–26.27%). Assessing the mechanism of increased FOXP3 frequency, we found that the high FOXP3 levels noted in HIV infected individuals dropped rapidly in unstimulated culture conditions but could be restimulated by T cell receptor stimulation. This suggests that the high FOXP3 expression in HIV infected patients is likely due to FOXP3 upregulation by individual CD4⁺ T cells following antigenic or other stimulation.

Conclusions/Significance: FOXP3 expression in the CD4⁺ T cell population is a marker of severity of HIV infection and a potential prognostic marker of disease progression.

Introduction

Many aspects of HIV pathogenesis are still poorly understood. Despite the CD4 T cell depletion and resulting immunosuppression which are hallmarks of the disease, HIV infected individuals display increased levels of immune activation as evidenced by elevated expression of markers of cell activation such as HLA-DR, CD38 and CD69 [1,2]. The contributory role of preservation or destruction of regulatory T cells (Tregs), either in number or function, has not been established.

Naturally occurring Tregs are a subset of CD4⁺ T cells expressing the forkhead-winged-helix transcription factor, Forkhead box 3 or FOXP3 [3]. They are responsible for immunoregulation predominantly through cell-cell contact mediated suppression. It is plausible that preferential destruction or inactivation of Tregs by HIV could lead to excessive immune activation [4,5]. Treg-mediated suppression of HIV specific responses in vitro has been shown to be more effective with cells isolated from relatively healthy HIV infected patients compared with later stage AIDS patients, suggesting that Tregs (total or HIV-specific) were depleted or dysfunctional later in HIV disease [6].

On the other hand, preferential preservation of Tregs over other subsets of T cells could lead to suppression of immune responses to viral infections, leading to a high viral load [7,8]. Various authors have described increased Tregs as a proportion of CD4⁺ T cells in HIV positive patients, particularly in those with
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low CD4+ T cell counts (CD4 counts) [9,10,11]. In peripheral blood, Treg levels have been reported to remain elevated years after successful highly active antiretroviral therapy [9,12,13]. Certain authors [14] have suggested that with progression from HIV to AIDS the number of circulating CD4+CD25hi Treg as a proportion of CD4+ T cells increases but their function (which was measured by FOXP3 mRNA expression) decreases. Generation of T cells expressing FOXP3 or with suppressor activity has been reported to occur through incomplete activation of CD4+ T cells by immature, plasmacytoid or alternatively-activated dendritic cells [15,16,17,18,19,20,21] in HIV-infected individuals as well as in response to Vitamin D [22], all trans retinoic acid [23,24,25,26] or indoleamine deoxygenase (IDO) modulation in antigen presenting cells [20,27,28,29,30,31,32]. One or more of these mechanisms may be responsible for alternation of FOXP3 expression in HIV infected individuals. The end result may be a disproportionate increase in cells with a suppressive or tolerant rather than a proinflammatory phenotype, resulting in an inability of the host to combat pathogens.

There are a number of reasons why different studies may have had contrasting findings. Principally, there is still no validated marker with which to identify human Tregs. The best available to date is forkhead box transcription factor P3, written “Foxp3” in animals and “FOXP3” in humans. FOXP3 is a key control element in the development and function of CD4+ T cells with suppressor function [33,34,35,36].

Use of other markers to identify Tregs have varied from study to study and include CD25, Cytotoxic T lymphocyte associated protein 4 (CTLA-4 or CD152), glucocorticoid诱导 tumour necrosis factor receptor (GITR), CD27, OX40 ([37], CD44 [38], CD62L [39,40], CD39 [41] and decreased expression of CD127 [42,43,44]. Tregs are generally thought to be of memory phenotype expressing CD45RO [8,45,46], although there have been reports of naïve CD45RA Tregs [47,48,49]. None of these markers are exclusive to the Treg population and many are also expressed by activated CD4+ T cells.

While Tregs are easily defined in mice by concurrent expression of FOXP3 and high levels of CD25, in humans CD25 cannot be clearly delineated into low and high expressing subsets due to a continuum of expression. In addition, CD25 expression does not always correlate with FOXP3 expression in humans, particularly in HIV infected individuals [31]. FOXP3 expression can be triggered in FOXP3− CD4+ T cells during activation or division which may correlate with, perhaps transient, suppressive potential [50,51,52,53,54,55]. There is also however conflicting evidence suggesting that FOXP3 expression in humans may not be confined to cells with regulatory function [44,56,57,58,59].

We analysed FOXP3 expression, as well as other Treg markers, in South African patients with and without HIV infection. We found a significantly elevated percentage of FOXP3 expressing CD4+ T cells in HIV infected patients, particularly in those with lower CD4 counts. We explored the mechanism of FOXP3 upregulation by assessing the ability of cultured cells from HIV positive and negative patients to maintain FOXP3 expression in unstimulated conditions and following T cell receptor stimulation. While many previous studies have isolated CD4+CD25hi cells and mixed them with responder cells in a predetermined ratio such as 1:1 or 1:10, we avoided this approach as it does not reflect the situation in vivo at a physiological ratio of Tregs to responder cells. Additionally the isolation of Tregs by CD25hi sorting potentially leaves FOXP3-expressing Tregs behind in the responder population. We sought to investigate FOXP3 expression after T cell receptor stimulus while maintaining a physiological ratio of Tregs to responder cells. FOXP3 expression dropped rapidly in unstimulated cell culture but was restored by T cell receptor stimulation. This suggests that the high FOXP3 expression in HIV infected patients is likely due to FOXP3 upregulation by individual CD4+ T cells following antigenic or other stimulation.

Materials and Methods

Clinical Samples

In this cross-sectional study, twenty-seven HIV infected patients were recruited from the antiretroviral clinics of the Charlotte Maxeke Johannesburg Academic hospital and health care centres in Alexandra township, Johannesburg. Ten of the patients had active Tuberculosis as diagnosed on the basis of symptoms and sputum microscopy. As the HIV-infected patients with and without active Tuberculosis did not differ from each other with regards to CD4 count or FOXP3 expression (Figure 1A), they were grouped together for further analysis. Patients had access to the national antiretroviral programme but had not yet commenced antiretroviral therapy or received more than four days of TB treatment at study enrolment.

Twenty-two healthy controls were recruited from amongst health-care workers of the institutions named above as well as blood donors from the South African National Blood Transfusion Service. Controls and HIV infected subjects were similar with respect to gender and age. All subjects gave written informed consent for the study and ethics approval was granted by the University of the Witwatersrand Human Medical Ethics Committee (R14/49). The day of sample collection was regarded as Day 0.

HIV tests and haematological parameters

The HIV status of participants was confirmed using HIV rapid testing (Determine HIV-1/2, Abbott Laboratories, Abbot Park, IL 60064, USA). CD4 counting was performed using the PanLeuco-gating method [60]. Viral loads were performed using the COBAS AmpliPrep/COBAS Taqman HIV-1 Test (Roche Diagnostics Division, Basel, Switzerland).

Isolation of Peripheral Blood Mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated within 3–5 hours of sample collection using Ficoll Hypaque in LeucoSep tubes. Cells were washed twice in Hanks Buffered Salt Solution with 0.1% gentamycin and counted using a capillary cytometer (Guava technologies, Hayward, CA). Cells (2×10^6 cells/ml) were then rested overnight at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium with GlutaMAX and 25mM HEPES (Gibco, Scotland) supplemented with 20% fetal bovine serum (Gemini Bio-Products, USA) and 0.1% gentamycin (R20).

mRNA extraction and qRT-PCR

mRNA was extracted from one million PBMCs per sample using QiAamp RNA mini kit (QIAGEN, Germany). The mRNA was immediately converted to cDNA using the Applied Biosystems High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). cDNA was frozen at −20°C until needed. Multiplexed real-time reverse transcriptase PCR was then performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). FOXP3 expression was assessed using a fluorescently labelled probe (TaqMan Gene expression assays, probe Hs00203958_m1, Applied Biosystems, Foster City, CA) relative to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Applied Biosystems Foster City, CA) for normalization.
City, CA). All PCR reactions were run in triplicate in the presence of a blank control tube.

**Cell stimulations**

PBMC were rested overnight in R20 medium at 2x10^6 cells/ml with analysis of unstimulated samples on day 1. For assessment of cell proliferation, an equal volume of 10μM CFSE (Molecular Probes, Netherlands) was added to the unstimulated PBMC suspension. Samples were vortexed and incubated at room temperature in the dark for 7 minutes. To quench the CFSE reaction, a double volume of ice cold FBS was added. Samples were again vortexed and incubated at room temperature in the dark for 1 minute. PBMC were washed twice with warm RPMI 1640 medium with GlutaMAX and 25mM HEPES (Gibco, Scotland) supplemented with 10% human serum AB (Gemini Bio-Products, USA) and 0.1% gentamycin (R10) to remove excess CFSE. 2x10^6 CFSE stained cells were seeded in a 24-well culture plate (Nunc, Denmark) in R10 medium to a final volume of 2ml. Stimulated samples were treated with 0.1 μg/ml stimulatory anti-CD3 mAb (12F6). Unstimulated samples were used as negative controls. Plates were incubated at 37°C, 5% CO₂ for 4 days.

**Intracellular cytokine staining**

Harvested PBMC (both those rested overnight and those cultured for 4 days) were stained for intracellular FOXP3 and CTLA-4 expression and surface expression of CD25, GITR, CD3 and CD4 using the FOXP3 staining set (eBiosciences, UK) according to manufacturer’s instructions. Mean FOXP3% is reported from triplicate measurements. Preliminary experiments with propidium iodide showed efficacy of cell permeabilisation to be above 98%.

Antibody-fluorochrome conjugates used included CD3 APC, CD3 PerCP, CD4 FITC, CD4 PerCP, CD8 FITC,CD25 APC (BD Biosciences, San Jose, CA), GITR APC, CTLA-4 FITC (R&D Systems, Minneapolis, USA) and FOXP3 PE (clone PCH101, eBiosciences, UK).

**Flow cytometry**

Flow cytometry was performed using FACSCalibur (BD Biosciences) and LSRII (BD Biosciences) flow cytometers with acquisition enabled by CellQuest Pro or FACS Diva software (BD Biosciences) respectively. Colour compensation was achieved using an appropriate single fluorochrome-labelled sample. Data was analysed using FlowJo 6.4.2 (TreeStar, USA). For CD25 quantitation, expression of CD25 on CD4+ T cells was used as a reproducible reference point.
50 000 to 1 million events were collected per sample. There was no use of biexponential axes. Not all samples were available for analysis of all parameters, depending on CD4 count and numbers of PBMCs available for culture.

Statistical Analysis
Statistical analyses were performed using SPSS version 15 and GraphPad Prism version 4.0. Groups were compared by Mann Whitney analysis and correlations performed using the Spearman correlation coefficient. Significance was chosen at the 5% level. Group medians are reported.

Results
CD4 counts of HIV-infected subjects
HIV infected subjects had CD4 counts ranging from 0 to 712 cells/ul (median CD4 count 216 cells/ul) with 48% of patients <200 cells/ul group, 19% 200–350 cells/ul and 33% >350 cells/ul.

HIV infected subjects display higher percentages of CD4+ T cells that express FOXP3 despite lower absolute numbers
FOXP3 expression in CD4+ T cells has been reported to confer a regulatory phenotype and may be dysregulated in HIV infection. To explore whether FOXP3 expression by CD4+ T cells differs in HIV infected individuals compared with controls, we isolated PBMCs and performed intracellular cytokine staining after overnight rest. In the control group, 3.72% of CD3+CD4+ T cells were positive for FOXP3 (range from 1.3–7.5%), in keeping with that described in the literature [10,56]. The HIV infected group showed significantly elevated FOXP3 expression when expressed as a percentage of total CD3+CD4+ cells (median of 8.11%, range 1.33–26.27%, P = 0.002, Figure 1B,C). Absolute numbers of FOXP3+CD4+ T cells were lower in HIV positive patients than controls (12.6 cells/µl versus 30.47 cells/µl, P = 0.005, Figure 1C) because of the lower CD4+ T cell count. Similarly, there was lower FOXP3 mRNA levels in HIV positive patients versus controls (0.92 vs 1.55, P = 0.03, data not shown).

FOXP3 expression in CD8+ T cells, while found at a much lower proportion than CD4+ T cells, has also been reported [9,52,56,57,61,62,63,64] and may confer regulatory functions. It is unknown if there is an alteration in CD8+ T cell FOXP3 expression during HIV infection. We found FOXP3 expression at low levels in the CD8+ T cell population but no significant difference between the control and HIV infected groups (0.20% of CD3+CD8+ versus 0.39% respectively). FOXP3 expression on the CD3+CD4+ population could be upregulated by anti-CD3 stimulation (data not shown). There was however no significant difference in stimulated FOXP3 expression of CD4+ T cells or CD8+ (CD3+CD4−) T cells between the HIV and control groups.

FOXP3 expression does not correlate with the activation marker CD25
CD25 is often used as a marker of regulatory T cells but can also act as an activation marker. CD25 expression in humans appears as a continuum of expression rather than a discrete positive and negative population, therefore to ensure reproducible gating, CD25 was gated by selecting the total CD3+ population and plotting CD25 against CD4. FOXP3 expression on the CD4+ population was used to set the positive gate. The resulting percentage obtained was used to calculate the CD25 expression as a percentage of the total CD3+CD4+ population (Figure 2).

CD25 expression gated in this manner did not differ significantly between the HIV infected and control group. While FOXP3∗T helper cells were visually CD25+ as expected, FOXP3% showed no significant correlation with CD25 expression in either the control or HIV infected group. CD25 expression did not show a statistical correlation with either viral load or CD4 count.

Examining other postulated markers of regulatory T cells, CTLA4∗FOXP3+ coexpression (as a percentage of CD3+CD4+ cells) was higher in the HIV group than the controls (0.78% versus 0.39%, P = 0.009, Figure 2A). There was no significant difference between the two groups in CD25+, CTLA4+, GITR+ or GITR∗FOXP3+ coexpression.

CD4+ T cell FOXP3 expression is negatively correlated with CD4+ T cell count and positively correlated with viral load
As FOXP3 expression may be affected by the degree of immunodeficiency, we analysed the relationship between the CD4+ T cell count and the percentage of CD4+ T cells expressing FOXP3. There was a negative correlation demonstrable between CD4 count and FOXP3 percentage in the HIV infected group (rho = −0.423 P = 0.044, Figure 3A) but no significant correlation in the control group (Figure 3B). Stratification based on CD4 count revealed that it was only the samples with CD4 count <200 cells/µl that showed a marked elevation in FOXP3 percentage (median 10%), while there was no elevation in FOXP3 percentage in samples with higher CD4 counts (5.09% in samples with CD4 counts of 200–350 cells/µl and 5.36% in samples >350 cells/µl) (Figure 3C).

As anticipated, there was a significant negative correlation between viral load and CD4 count (rho = −0.536 P = 0.005, data not shown). We further determined that FOXP3 percentage correlated positively with viral load (rho = 0.593 P = 0.003, Figure 3D).

High FOXP3 frequencies in HIV infected individuals are not due to increased FOXP3+ T cell lifespan nor increased T cell proliferation
In order to assess the mechanism by which the FOXP3 frequency of total CD4 T cells is elevated in HIV infected patients, we analysed FOXP3 expression in four-day cultures with and without stimulation through the T cell receptor with anti-CD3.

Unexpectedly, unstimulated FOXP3 expression, expressed as a percentage of CD3+CD4+ cells, was significantly lower on day 4 than on day 1 in the HIV infected group (1.95% versus 9.75%, P<0.001) whilst there was no significant drop in expression in the control group (2.56% versus 3.36%) (Figure 4A). This suggests that FOXP3+ cells were not maintaining high levels due to a longer lifespan than FOXP3− cells. After stimulation with anti-CD3, however, day 4 FOXP3 expression in the HIV infected group was comparable with baseline and with day 4 control group levels (Figure 4A), suggesting that mechanisms of FOXP3 upregulation after T cell receptor triggering are intact in HIV infected individuals.

To assess whether FOXP3 upregulation was due to increased CD4+ T cell proliferation or increased expression in proliferated CD4+ T cells, we assessed FOXP3 expression following anti-CD3 stimulation of CFSE stained cells. Total CD4+ T cells proliferated less in the HIV infected group than in the control group (13.09% of CD3+CD4+ cells versus 60.08%, P = 0.02, Fig 4B). FOXP3 was upregulated in daughter cells (Figure 4C) but the percentage of proliferating cells expressing FOXP3 did not differ in the HIV infected and control groups (11.31% versus 11.78% Figure 4B).

Together, these findings suggest that high FOXP3 percentages in HIV infected patients is not due to longer lifespan, increased cell
proliferation nor increased expression in proliferated cells. We conclude that the increased FOXP3 frequencies in HIV infected patients is likely due to upregulation of FOXP3 expression by individual CD4\(^+\) T cells, possibly following antigenic or other stimulation.

**Discussion**

We used intracellular cytokine staining to analyse FOXP3 expression and other postulated markers of regulatory T cells (CD25, CTLA4 and GITR) in HIV infected individuals. We
found that HIV infected individuals showed a significantly higher percentage of CD4⁺ T cells that expressed FOXP3 compared with control individuals. The percentage of FOXP3 expressing CD4⁺ T cells correlated negatively with CD4 count and positively with viral load. Due to their lower absolute CD4 count, the absolute number of FOXP3 expressing cells, as well as FOXP3 mRNA expression, was found to be lower in HIV infected individuals than in controls.

The finding of higher FOXP3 levels in patients with lower CD4 counts and higher viral loads is in keeping with the findings of others [11,37,65]. The inverse relationship between CD4 count and FOXP3 expression has not been consistently described and is well illustrated in our sample group possibly due to the inclusion of HIV infected patients with very low CD4 counts. This relationship also explains why studies with patients with relatively high CD4 counts at enrolment may have failed to demonstrate elevations of FOXP3 expression in peripheral blood. If the data is stratified into patients according to CD4 count, it is only the patients with CD4 counts below 200 cells/µl who show a marked elevation in FOXP3 percentage of CD3⁺CD4⁺ T cells (median 10%) while there is no significant difference in FOXP3 percentage between the 200–350 cells/µl or >350 cells/µl group and the control group (Figure 3C).

We noted that FOXP3 positivity was not limited to the CD4⁺ T cell subset but was also observed on other CD4⁻ T cells, albeit at much lower levels. Interestingly, FOXP3 upregulation after anti-CD3 stimulation, was noted on CD4⁻ T cells as well as on CD4⁺ T cells (data not shown). FOXP3 expression in CD8⁺ T cells has been described [9,52,56,57,61,62,63,64], as have other subsets of FOXP3⁻CD8⁺ cells with suppressor function [64,66,67,68,69]. In contrast to the CD4⁺FOXP3⁺ subset, we saw no significant difference in unstimulated or anti-CD3 stimulated percentages of CD4⁻FOXP3⁺ cells between the control and HIV infected groups.

HIV infected individuals are known to exhibit high levels of T cell activation and debate reigns as to whether FOXP3 is expressed by activated T cells that do not possess suppressive functions [52,56,58]. In this study, stimulated FOXP3 expression did correlate with in vitro proliferation of both CD4⁺ and CD8⁺ T cells to anti-CD3 stimulation but there was no correlation between expression of FOXP3 and the activation marker CD25. The correlation of FOXP3 with cell proliferation may suggest that FOXP3 acts as a marker of cell activation, however its discordance with CD25 expression indicates otherwise. The lack of statistical correlation between FOXP3 and CD25 expression, gated in a reproducible manner, lends weight to the argument that the elevated FOXP3 levels are not merely a marker of activated cells in HIV infection, in keeping with findings [70] describing dissociation between FOXP3 mRNA and CD25 expression in a SIV model. We took great care to gate CD25 using a reproducible gating strategy, namely using CD25

![Figure 3. Correlation of FOXP3 with CD4 count and viral load. Panel A: CD4 count was negatively correlated with day 1 FOXP3 percentage in the HIV infected group. Panel B: The control group showed no correlation between CD4 count and FOXP3 percentage. Panel C: FOXP3 percentage stratified by CD4 count. Panel D: FOXP3 percentage was positively correlated with viral load. doi:10.1371/journal.pone.0011762.g003](image-url)
expression on CD4− T cells as the cutoff for CD25 positivity. This proved a more reliable strategy than setting an arbitrary threshold for CD25+ and CD25++. Future studies should, however, include reference beads to allow more accurate CD25 determination. In previous studies, FOXP3 has also been shown not to correlate with the activation markers CD69 [31] or CD38 [9] although there was a correlation with HLA-DR expression in CD4+ T cells in the latter study.

CTLA-4 and GITR did not coincide with FOXP3 expression i.e. cells did not co-express FOXP3 with CTLA4 or GITR (Figure 1c).

This is in keeping with findings by Weiss et al. [8] of intracellular CTLA-4 positivity in only 30% of Tregs (identified by CD4+CD25hi expression) and Lim et al. [9] who found CTLA-4 or GITR expression in less than 10% of Tregs (CD4+CD25+CD127−). A limitation of this study is that CD127 had not been included, which may have given additional discriminatory power of the Treg subset from activated cells. We therefore limited our functional analysis to FOXP3 expression.

The finding of increased percentages of CD4+ T cells expressing FOXP3 in HIV infected individuals suggests that an imbalance of

Figure 4. FOXP3 expression after T cell receptor stimulation. Panel A: FOXP3 expression as a percentage of T lymphocytes at baseline and after 4 days of cell culture, with and without T cell receptor stimulation with anti-CD3. Panel B: Proliferation of total CD4+ T cells and FOXP3+ expression in proliferated CD4+ T cells following T cell receptor stimulation with aCD3. Panel C: Representative plot of FOXP3+ expression in proliferated T cells (left-hand plot) following anti-CD3 stimulation compared with an unstimulated sample (right-hand plot). The small plots above show ancestry - lymphocytes were gated; followed by exclusion of events with high CFSE; followed by selection of CD3+CD4+ T cells. Proliferation is demonstrated by halving of CFSE fluorescence in cells that have divided (large plots below). In the anti-CD3 stimulated sample, FOXP3 expression is noted in cells which have proliferated (top-left quadrant) as well as those that have not proliferated (top-right quadrant).

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regulatory to effector T cells could be responsible for susceptibility to opportunistic infection. Higher FOXP3 expression could be the result of one of four mechanisms: upregulated expression of FOXP3 in individual CD4+ T cells compared with HIV negative individuals, increased proliferation of cells expressing FOXP3+ cells, increased lifespan of FOXP3+ cells or increased cell death of FOXP3+ CD4+ T cells.

To analyse the mechanism of increased expression of FOXP3 amongst CD4+ T cells from HIV infected individuals, we cultured PBMCs from HIV infected and uninfected individuals in the presence of a stimulating antibody against the T cell receptor (anti-CD3). We found FOXP3 to be markedly upregulated by antigenic stimulation with anti-CD3 stimulation.

We did not directly address the lifespan or susceptibility to cell death of FOXP3 expressing cells in this study, however we noted that during unstimulated culture conditions, the elevated FOXP3 levels in the HIV infected groups declined rapidly to control levels. This is in keeping with a previous study suggesting that Tregs (CD3+CD25hi) are highly susceptible to apoptosis in vivo due to low levels of the antiapoptotic molecule Bcl-2 [54] and suggests that they do not have a longer lifespan than FOXP3+ cells.

In addition, FOXP3 expression in the HIV infected group could be rescued by anti-CD3 to levels comparable with and the stimulated control group. We explored whether this was due to increased CD4+ T cell proliferation or increased FOXP3 expression in cells which had proliferated. Using dual labelling with CFSE and FOXP3 we found that total CD4+ T cell proliferation was lower in the HIV group than in the control group, and the percentage of proliferated cells that expressed FOXP3 was similar in both groups. Thus the mechanism by which FOXP3 expression was upregulated in vivo in response to anti-CD3 stimulation is not likely to be due to CD4+ T cell proliferation. Together, these findings suggest that elevated FOXP3 percentages in HIV infected individuals is due to upregulation of FOXP3 expression in individual CD4+ T cells in HIV, likely due to antigenic or other stimulation. The downstream effect of FOXP3 upregulation was not directly addressed in this study. Transient FOXP3 upregulation after T cell receptor engagement may indicate that FOXP3 is an activation marker, however does not exclude the possible acquisition of suppressive potential after activation.

The debate over FOXP3 as activation marker versus marker of suppressive activity may eventually prove both sides right. The mechanisms of activation-induced cell death in healthy individuals is poorly understood, but every activated cell must eventually die or be suppressed, otherwise result in leukaemia. FOXP3 may indeed be both an “activation marker” and a marker of cells that have become unable to carryout effector functions. We have shown that FOXP3 expression was transient and could be lost and restimulated in HIV infected patients. Thus there did not seem to be an increase in number of natural Tregs with lineage-dependent FOXP3 expression in these patients, rather an increase in FOXP3 expression induced peripherally. This may be a result of cell activation, but the question remains as to why the increased numbers of activated T cells in HIV infected individuals fail to clear pathogens and are ineffectual in their actions. While not directly addressed by this study, it is plausible that continuous stimulation through the T cell receptor in HIV infected individuals may induce chronic rather than transient FOXP3 upregulation, resulting in dysfunctional effector T cells. A difference in function between FOXP3 and other activation markers may be an explanation for the discordance found in this study between FOXP3 expression and expression of the activation marker CD25.

It is recognised that CD4 count is not a perfect prognostic tool for monitoring of HIV infected individuals, with some patients appearing well even at low CD4 counts and some patients doing poorly even with relatively high counts (in our study one patient with a CD4 count of 2 appeared healthy). We suggest that FOXP3 expression in the CD4+ T cell subset may prove a more accurate prognostic tool to monitor disease progression and response to antiretroviral therapy. Longitudinal studies should be conducted to assess the use of FOXP3 frequency as a prognostic monitoring tool.

The inclusion in our HIV patient cohort of some HIV infected individuals with active Tuberculosis can be criticised, as there is evidence suggesting changes in Treg number or function result from Tuberculosis alone [71,72,73,74,75,76,77,78,79,80,81,82,83] although these studies remain inconclusive. Roberts et al. [84] found no difference in the levels of CD4+CD25+ Tregs in active tuberculosis cases compared with latentely infected controls in unstimulated peripheral blood mononuclear cell cultures. Many of these studies, as with studies of Tregs in HIV infection, have assessed FOXP3 mRNA expression rather than FOXP3 protein expression at the single cell level. Further, most quantitated Tregs based on CD25 expression without the use of a reference marker by which to set the CD25 gate. We conducted a subgroup analysis which showed no significant difference in levels of FOXP3 expression in HIV infected individuals with and without Tuberculosis (Figure 1A). Additionally, it can be hypothesized that any defect in Treg number or function may be of a similar nature in both HIV and Tuberculosis, given their propensity to occur simultaneously. Thus the co-infected group may illustrate more extreme changes not demonstrable in a small group of HIV infected patients alone.

In conclusion, we have shown that HIV infected individuals had significantly higher percentages of CD4+ T cells positive for FOXP3 than HIV uninfected individuals and that this is likely due to upregulation of FOXP3 expression by CD4+ T cells. FOXP3 expression as a percentage of CD4+ T cells correlated positively with viral load and negatively with CD4 count, with a marked elevation in FOXP3 percentage in patients with CD4 counts below 200 cells/µL. While correlation does not imply causation, this data support the hypothesis that FOXP3 plays a functional role in disease progression and may suppress responses to pathogens. The basis of the increased FOXP3 expression appears to be upregulation of FOXP3 expression by individual CD4+ T cells following T cell receptor stimulation. FOXP3 expression as a percentage of the CD4+ T cell population is a potential prognostic marker of disease progression.

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Author Contributions
Conceived and designed the experiments: MSS EM VAG SS LDWS. Performed the experiments: MSS EM VAG. Analyzed the data: MSS. Contributed reagents/materials/analysis tools: MSS SS LDWS CMG CTT. Wrote the paper: MSS. Reviewed manuscript: EM VAG SS WS CMG CTT.
References

1. Savarino A, Bottarel F, Malavasi F, Dianzani U (2000) Role of CD38 in HIV-1 infection: an epiphenomenon of T-cell activation or an active player in virus-host interactions? AIDS 14: 1079–1089.

2. Simon AE, Carmignani L, Meier-Schellersheim M, Grossman Z, Victorino RM (2002) CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol 169: 3900–3906.

3. Sankar S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing the IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 153: 1151–1164.

4. Oswald-Richter K, Grill SM, Shariat N, Lekvassig M, Sundrud MS, et al. (2004) HIV infection of naturally occurring and genetically programmed human regulatory T-cells. PLoS Biol 2: E198.

5. Apoil PA, Puissant B, Roubinet F, Abbal M, Massip P, et al. (2005) FOXP3 is upregulated in HIV-1-infected individuals regulate CD4+ and CD8+ T-cell responses in vitro and are associated with favorable clinical markers of disease status. J Exp Med 200: 331–343.

6. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, et al. (2004) CD25+ regulatory T cells in the peripheral blood of asymptomatic HIV-infected individuals regulate CD4+ and CD8+ T-cell responses in HIV-positive patients. J Acquir Immun Deff Syndr 39: 381–385.

7. Kinter AL, Hennessy M, Bell A, Kern S, Lin Y, et al. (2004) CD25+ regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4+ and CD8+ T-cell responses in HIV-positive patients. J Acquir Immun Def Syndr 39: 381–385.

8. Weiss L, Donkova-Petrini V, Caccavelli L, Balbo M, Carbonelli C, et al. (2004) Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. Blood 104: 3249–3256.

9. Lim A, Tan D, Price P, Kamarulzaman A, Tan HY, et al. (2007) Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. AIDS 21: 1255–1264.

10. Montes M, Lewis DE, Sanchez C, de Castilla DL, Graviss EA, et al. (2006) Foxp3+ regulatory T cells in antiretroviral-naive HIV patients. AIDS 20: 61–67.

11. Kallon NJ, Lopez M, Soriano V, Garcia-Samaniego J, Romero M, et al. (2009) Level, phenotype and activation status of CD4+Foxp3+ regulatory T cells in patients chronically infected with human immunodeficiency virus and/or hepatitis C virus. Clin Exp Immunol 153: 34–42.

12. Gaardbo JC, Steenken SD, Veld S, Erbsbll AK, Harkinson L, et al. (2008) Regulatory T cells in human immunodeficiency virus-infected patients are elevated and independent of immunological and virological status, as well as initiation of highly active antiretroviral therapy. Clin Exp Immunol 154: 80–86.

13. Kohler L, Gaardbo JC, Skoglund K, Ryder LP, Erbsbll AK, et al. (2006) Increased levels of regulatory T-cells (Tregs) in human immunodeficiency virus-infected patients after 5 years of highly active antiretroviral therapy may be due to increased thymic production of naive Tregs. Clin Exp Immunol 143: 44–52.

14. Tamamis S, Ishovak T, Imado T, Higasa S, Kakishita E, et al. (2009) Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients. AIDS 19: 879–886.

15. Kornbluth RS, Stone GW (2006) Immunostimulatory combinations: designing the next generation of vaccine adjuvants. Leuk Blood 80: 1048–1052.

16. Choungurt C, Gesani S (2006) Role of gp120 in dendritic cell dysfunction in HIV infection. J Leukoc Biol 80: 994–1000.

17. Granelli-Piperno A, Golebiowska A, Thump.Threadinger C, Siegel FP, Steinman RM (2004) HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. J Immunol 169: 3400–3406.

18. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR (2008) The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol 181: 5396–5404.

19. Modulation of tryptophan catabolism by regulatory T cells. J Exp Med 171: 38–50.

20. Goleva E, Cardona ID, Osu LS, Leung DY (2005) Factors that regulate naturally occurring T regulatory cell-mediated suppression. J Allergy Clin Immunol 116: 1094–1100.

21. Nelson J, Boasso A, Velilla PA, Zhang R, Vaccari M, et al. (2006) HIV-1-driven regulatory T cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. Blood 108: 3808–3817.

22. Sauson DM, Manzotti CN, Zhang Y (2003) What’s the difference between CD80 and CD86? Trends Immunol 24: 314–319.

23. Bennett CL, Christie J, Ramdani F, Brunhouk ME, Ferguson J, et al. (2001) The immunoregulatory properties of virus-specific T cells of the X-linked lymphoproliferative disorder (XLP) vary with age and disease manifestations. J Exp Med 197: 1023–1033.

24. Willers NN, Ramdani F, Peake J, Faravelli F, Casanova J, et al. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 27: 68–73.

25. Ziegler SF (2006) Foxp3, of mice and men. mRNA Rev 24: 209–226.

26. Faizel de S Groth B, Landay AL (2008) Regulatory T cells in HIV infection: pathogenic or protective participants in the immune response? AIDS 22: 671–683.

27. Fischer M, Dhqin S, Estes P, Siegelman MH (2006) Suppressor activity and potency regulatory T cells is discriminated by functionally activated CD4+ T cells. Blood 107: 619–627.

28. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, et al. (2005) Depletion of regulatory T cells in HIV infection is associated with immune activation. J Immunol 174: 4407–4414.

29. Hueln H, Hamann A (2005) Homing to suppress: address codes for Treg migration. Trends Immunol 26: 632–636.

30. Sereti I, Immich H, Natarajan V, Immich R, Tchaumandliadis M, et al. (2005) In vivo expansion of CD4+CD45RO+CD25+ T cells expressing FoxP3 in IL-2-treated HIV-infected patients. J Clin Invest 115: 1839–1847.

31. Frieschng B, Oberle K, Pfeffer F, Bauer J, et al. (2006) Naive regulatory T cells: a novel subpopulation defined by resistance toward CD95L-mediated cell death. Blood 108: 3371–3378.

32. Sereti I, Symerman-Nunn B, Martinsson J, Zanders J, Sauson S, et al. (2006) Expression of interleukin (IL)-7 receptor in HIV-1-infected and controls: HIV correlates. Blood 107: 619–627.

33. Liu W, Pumam AL, Xi-Yu Z, Santt GL, Lee MR, et al. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203: 1701–1711.

34. Sereti I, Symerman-Nunn B, Martinsson J, Zanders J, Sauson S, et al. (2006) Expression of interleukin (IL)-7 receptor in HIV-1-infected and controls: HIV correlates. Blood 107: 619–627.

35. Levy L (2006) Cycokine-based modulation of immune function in HIV infection. Curr Opin HIV AIDS 1: 69–73.

36. Sereti I, Immich H, Natarjan V, Immich R, Tchaumandliadis M, et al. (2005) In vivo expansion of CD4+CD45RO+CD25+ T cells expressing FoxP3 in IL-2-treated HIV-infected patients. J Clin Invest 115: 1839–1847.

37. Chen W, Jin W, Hardegen N, Lei KJ, Li L, et al. (2003) Correlation of peripheral CD4+CD25+ naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 167: 1875–1858.

38. Wu W, Zhang N, Yopp AC, Chen H, Mas M, et al. (2004) TGF-beta induces Foxp3+ Tregulatory cells from CD4+CD25− precursors. J Exp Med 197: 1614–1627.

39. Pillai V, Ortega SB, Wah CK, Karzandinar NJ (2007) Transient regulatory T-cell states attained by all activated human T-cells. Clin Immunol 123: 119–129.

40. Walker MR, Kasprzak DJ, Gersuk VH, Benard A, Van Laerdegem M, et al. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25− T cells. J Clin Invest 112: 1437–1443.

FOXP3 is Upregulated in HIV
54. Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, et al. (2006) Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 116: 2423–2433.

55. Lith S, Huber S, Schramm C, Bach T, Zander S, et al. (2006) Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuritis and induces antigen-specific Tregs. J Clin Invest 118: 3403–3410.

56. Gavin MA, Torgeron TR, Houston E, DeRoo P, Ho WY, et al. (2006) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci U S A 103: 6659–6664.

57. Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, et al. (2006) Human CD4+CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 116: 2423–2433.

58. Tran DQ, Ramsey H, Shevach EM (2007) Induction of FOXP3 expression in human activated non-regulatory CD4+ T cells. Eur J Immunol 37: 129–138.

59. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE (2008) Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 37: 129–138.

60. Glencross D, Scott LE, Janni IV, Barnett D, Janossy G (2002) CD45-assisted PanLeucocytogating for accurate, cost-effective dual-platform CD4+ T-cell enumeration. Cytometry 50: 69–77.

61. Bukińska B, Colgan J, Laban J, Bluestone JA, Herald KC (2005) TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. J Clin Invest 115: 2904–2913.

62. Hoji A, Coro A, Ng HL, Jamieson BD, Yang OO (2008) Proliferation and Foxp3 expression in virus-specific memory CD8+ T lymphocytes. AIDS Res Hum Retroviruses 24: 1087–1095.

63. Mahir M, Henjum K, Yaqub S, Bjornetha BA, Torgeresen KM, et al. (2008) Generation of highly suppressive adaptive CD8(+)CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. Eur J Immunol 38: 640–646.

64. Sharabi A, Mozes E (2008) The suppression of murine lupus by a tolerogenic retrovirus. Retroviruses 24: 1087–1095.

65. Zhang Z, Jiang Y, Zhang M, Shi W, Liu J, et al. (2008) Relationship of frequency of CD4+CD25hi Foxp3+ regulatory T cells with suppressor function in CD4+CD25+ T cell cultures upon polyclonal stimulation ex vivo. J Immunol 181: 8767–8775.

66. Billerbeck E, Thimme R (2008) CD8+ T lymphocytes in tuberculous pleural effusion. Chin Med J (Engl) 121: 581–586.

67. Jiang H, Wu Y, Liang B, Zheng Z, Tang G, et al. (2008) Foxp3-expressing CD8+ T cells in regulation of the immune response during human tuberculosis. Clin Exp Immunol 144: 25–34.

68. Vogtenhuber C, O'Shaughnessy MJ, Vignali DA, Blazar BR (2006) Outgrowth of CD4+Foxp3−CD25+ T cells with suppressor function in CD4+CD25+ T cell cultures upon polyclonal stimulation ex vivo. J Immunol 181: 8767–8775.

69. Koch SD, Vis E, van Ler A, ten Berge JJ (2008) Alloantigen-induced regulatory CD4+CD103+ T cells. Hum Immunol 69: 737–744.

70. Boasso A, Vaccari M, Hyniewicz A, Fuchs D, Nacsa J, et al. (2007) Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. J Virol 81: 11593–11603.

71. Liu L, Hill PC, Jeffries DJ, Holland MJ, Fox A, et al. (2007) FOXP3 gene expression in a tuberculous case contact study. Clin Exp Immunol 149: 117–122.

72. Chen W, Perruche S, Li J (2007) CD4+CD25+ T regulatory cells and TGF-beta in mucosal immune system: the good and the bad. Curr Med Chem 14: 2245–2249.

73. Gazzola L, Tiscati C, Gori A, Saresella M, Marventano I, et al. (2006) Foxp3 mRNA expression in regulatory T cells from patients with tuberculosis. Am J Respir Crit Care Med 174: 356; author reply 357.

74. Guyot-Revol V, Innes JA, Hackford S, Hinks T, Lahavani A (2006) Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 175: 893–810.

75. Hougardy JM, Place S, Hildebrand M, Dowsart A, Debrée AS, et al. (2007) Regulatory T cells depress immune responses to protective antigens in active tuberculosis. Am J Respir Crit Care Med 176: 499–416.

76. Hougardy JM, Verscheure V, Locht C, Mascart F (2007) In vitro expansion of CD4+CD25highFOXP3+CD127−/− regulatory T cells from peripheral blood lymphocytes of healthy Mycobacterium tuberculosis-infected humans. Microbes Infect 9: 1325–1332.

77. Baron J, Maranghi E, Leclerc C, Majlessi I (2008) Effect of attenuation of Treg during BCG immunization on anti-mycobacterial Th1 responses and protection against Mycobacterium tuberculosis. PLoS One 3: e2833.

78. Kursar M, Koch M, Mittrucker HW, Nouailles G, Bonhagen K, et al. (2007) Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol 178: 2661–2665.

79. Qin XJ, Shi HZ, Liang QL, Huang LY, Yang HB (2008) CD4+CD25+ regulatory T lymphocytes in tuberculosis pleural effusion. Chin Med J (Engl) 121: 581–586.

80. Ribeiro-Rodrigues R, Resende Co T, Rojas T, Troossi Z, Dietze R, et al. (2006) A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis. Clin Exp Immunol 144: 25–34.

81. Scott-Browne JP, Shafiani N, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, et al. (2007) Expansion and function of Fop3-expressing T regulatory cells during tuberculosis. J Exp Med 204: 2159–2169.

82. Wu B, Huang C, Kato-Osaeda M, Hopewell PC, Daley CL, et al. (2007) Messenger RNA expression of IL-10, FOXP3, and IL-12beta differentiates latent tuberculosis infection from disease. J Immunol 178: 3688–3694.

83. Roberts T, Beyers N, Aguirre A, Wald G (2007) Immunosuppression during active tuberculosis is characterized by decreased interferon-gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor-beta, and interleukin-4 mRNA levels. J Infect Dis 195: 870–878.