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Depletion of Regulatory T Cells in HIV Infection Is Associated with Immune Activation

Mark P. Eggena,‡ Banson Barugahare,† Norman Jones,‡ Martin Okello,‡ Steven Mutalya,‡ Cissy Kityo,‡ Peter Mugyenyi,‡ and Huyen Cao‡

Immune activation during chronic HIV infection is a strong clinical predictor of death and may mediate CD4+ T cell depletion. Regulatory T cells (Tregs) are CD4+CD25brightCD62Lhigh cells that actively down-regulate immune responses. We asked whether loss of Tregs during HIV infection mediates immune activation in a cross-sectional study of 81 HIV-positive Ugandan volunteers. We found that Treg number is strongly correlated with both CD4+ and CD8+ T cell activation. In multivariate modeling, this relationship between Treg depletion and CD4+ T cell activation was stronger than any other clinical factor examined, including viral load and absolute CD4 count. Tregs appear to decline at different rates compared with other CD4+ T cells, resulting in an increased regulator to helper ratio in many patients with advanced disease. We hypothesize that this skewing may contribute to T cell effector dysfunction. Our findings suggest Tregs are a major contributor to the immune activation observed during chronic HIV infection. The Journal of Immunology, 2005, 174: 4407–4414.

C hronic HIV infection is associated with a generalized state of immune dysregulation characterized by simultaneous hyperimmune activation and paradoxical anergy in both the CD4+ and CD8+ T cell compartments. The mechanism responsible for this immune dysregulation is unknown, but immune activation is positively associated with viral load and negatively associated with CD4 count (1–3). In addition, T cell activation has been suggested to be at least as powerful a predictor for disease progression and death as either CD4 count or viral load (4). Regulatory T cells (Tregs) are known to profoundly inhibit both CD4+ and CD8+ T cell responses (22). As disease progresses, effector function is lost before depletion of HIV-specific CD8+ T cells. Many mechanisms have been postulated to explain this anergy, including lack of CD4+ T cell help, interference with proper Ag presentation, CTL escape, and impaired memory formation (reviewed in Ref. 10).

CD4+CD25+CD62L+ regulatory T cells have gained prominence recently for their ability to down-regulate self-reactive T cell responses (11). Depletion of these cells in animal models has led to induction or exacerbation of an array of autoimmune diseases (12–14). Regulatory T cells (Tregs) have also been implicated in controlling responses to chronic pathogens (15–17). Tregs are known to profoundly inhibit both CD4+ and CD8+ T cell activation, proliferation, and effector function, although the mechanism of this inhibition remains unclear. Thus, Tregs may play a critical role in limiting immunopathology that results from persistent high level immune stimulation from chronic viral infections (18).

Multiple subtypes of Tregs share phenotypic markers, but inhibit via different mechanisms, adding to the complexity of this suppressor population. Some subtypes mediate inhibition by contact-dependent mechanisms, whereas others use cytokines such as IL-10 and TGF-β (reviewed in Refs. 19 and 20). Naturally occurring Tregs are derived in the thymus and are primarily directed toward self Ag, while adaptive Tregs are thought to be derived in the periphery from mature T cells under specific conditions of persistent antigenic stimulation (reviewed in Refs. 20 and 21). Regardless of their origin or mechanism of inhibition, Tregs need to be stimulated by their cognate Ag to suppress, but this suppression appears to act on all T cells (22).

In this study, we hypothesize that the immunodysregulation associated with chronic HIV infection is mediated in part by changes in Tregs. Tregs share many markers of recently activated cells, including CD38, HLA-DR, CD45RO, and CD25 (23). In fact, the only known surface markers that may distinguish them from activated cells are the level of CD25 (IL-2R) expression and CD62L (L-selectin) (23–25). Using this strict definition of Tregs, we evaluated this population in chronic HIV infection. We demonstrate that Tregs are depleted during the course of HIV infection and that their loss may facilitate the immune hyperactivation associated with HIV. Through in vitro depletion studies, we further confirm the results of recent studies suggesting that Tregs may also suppress HIV-specific T cell responses (26–28). However, our current data do not confirm this effect ex vivo.

Materials and Methods

Study population

A total of 81 HIV-1-infected Ugandan adults was enrolled at the Joint Clinical Research Centre (Table 1). Exclusion criteria included age <18, pregnancy, active tuberculosis (TB; defined as suspected TB or in the first 2 mo of anti-TB therapy), or moribund status. In addition, 25 healthy HIV-negative patients were enrolled as controls. All volunteers provided written informed consent. Institutional Review Board approval was obtained from the California Department of Health Services, University of California, and the Joint Clinical Research Centre.
CD4+ isolated PBMC were immunophenotyped for Treg quantitation as well as unstimulated (no peptide) PBMC as well as PHA-stimulated (10 μl ethylcarbazole peroxidase substrate coloring solution (100 μg/ml per peptide)). Tregs, but has no discernible impact on CD8+ T cell function. Beads were washed three times, and washes were pooled and assessed with intracellular staining using cytokerperm 2 reagents. Gating analysis was performed with FlowJo software (TreeStar) with the same gating applied to all samples. Absolute numbers of CD4+ or CD8+ T cells were determined by BD TruCount analysis using biotech (7-B6-1 biotin; Mabtech) at 0.5 μg/ml and pulsed with tritium-labeled thymidine for 12 h before harvest on day 7. Background was assessed in unstimulated wells at each dilution and subtracted from stimulated samples. Background proliferation was always <100 cpm. Suppression assays were repeated in three independent experiments with two different donors, all yielding comparable results.

Treg depletions

CD25 depletions were performed using anti-CD25 beads (Dynabeads). Briefly, 10^7 PBMCs were incubated with 80 μl of anti-CD25 beads for 45 min at 4°C. Beads were washed three times, and washes were pooled and centrifuged to obtain a CD25-depleted population. All depletions were verified by flow cytometry. Typically, this protocol depletes greater than 90% of Tregs, but has no discernible impact on CD8+ T cells. Tregs were eluted from beads and used for further immunophenotyping of surface markers.

Assessment of clade A and D Gag-specific CD4+ and CD8+ T cell responses by IFN-γ intracellular production and ELISPOT assays

Treg-depleted PBMCs were assayed in parallel with undepleted specimens for intracellular HIV-specific IFN-γ production, as previously described (29). Briefly, freshly isolated PBMCs (depleted and undepleted) were rested overnight in complete RPMI with 10% FCS and then stimulated for 6 h in the presence of brefeldin, anti-CD28, and anti-CD49d Abs. Stimulations were performed with pools of synthetic 15-mer peptides (11-aa overlap) spanning the entire consensus Gag protein for both clade A and D Gag proteins, using anti-IFN-γ FITC, anti-CD3 PerCP, and anti-CD8 allophycocyanin. Staphylococcus enterotoxin B (100 μg/ml; Sigma-Aldrich) was used as a positive control, and medium alone was used to establish background for every sample. Analysis was performed using FLOWJO software, and results were expressed as a percentage of responding CD8+ T cells after subtracting background. Responses were considered positive if more than twice background and at least 0.1% of CD8+ cells. Negative responses were assigned a value of zero.

For ELISPOT assay, 96-well nitrocellulose plates (Millititer; Millipore) were coated with mAb 1-D1K (Mabtech) overnight at 4°C using previously described methods (30–32). PBMC were added at 10^4 cells/well and stimulated with pools of overlapping Gag A and D peptides (40 peptides per pool at final 2 μg/ml per peptide). Negative and positive controls used were unstimulated (no peptide) PBMC as well as PHA-stimulated (10 μg/ml; Sigma-Aldrich) PBMC, respectively. Plates were incubated overnight at 37°C in 5% CO2 and washed with PBS before adding biotinylated anti-IFN-γ mAb (7-B6-1 biotin; Mabtech). Streptavidin-conjugated HRP (BD Biosciences) was subsequently added (1 μg/ml), followed by 3-amino-9-ethylcarbazole peroxidase substrate coloring solution (100 μl/well; Vector Laboratories). All plates were analyzed using an ELISPOT C.T.L. reader (C.T.L.). Results are expressed as the number of spot-forming cells (SFC) per 10^6 PBMC after subtraction of the background. Positive responses were defined as >100 SFC per million PBMC and more than twice the background values. Background values were <10 SFC per million PBMC.

Plasma HIV-1 RNA viral load

HIV-1 RNA level was determined from plasma using Roche Amplicor 1.5 assay (Roche). All undetectable values (below 400 copies) were assigned a value of 399. In addition, all samples with values greater than 750,000 were serially diluted and quantitated, as per the manufacturer’s recommendation.

Statistical analysis

Linear least-square regression model was used for statistical analysis. With nonlinear distribution, values were log transformed to permit the use of the linear model. All models were verified by viewing residual plots to ensure that the basic assumptions of linear regression were not violated and to assure the logarithmic transformation was appropriate. Statistical significance was defined as p < 0.05. Standardized coefficients were used to provide meaningful comparisons among the independent variables in a multivariate regression analysis. Standardized coefficients are measured by SD, and thus, the magnitudes of these coefficients are independent measures and provide a meaningful comparison among the regression coefficients. This approach allows the evaluation of the contribution of independent variables with differing units in a multivariate analysis. All dependent variables in our multivariate analysis were analyzed in separate models to evaluate correlation with independent variables in Table I. None of the interactions between different Treg representations was statistically significant.

Results

Demographic and clinical characteristics of patients

 Patients attending the outpatient HIV clinic at the Joint Clinical Research Centre were recruited to a cross-sectional study to assess T cell function, activation, and Tregs. Clinical characteristics for the HIV-positive study participants are summarized in Table I. Median CD4 count was 265 with a range of 56–1050 (interquartile range 190–429). Thirty-four percent of the participants were World Health Organization stage 3 or 4. All participants were anti-retroviral drug (ARV) naive. In addition, 25 healthy HIV-negative Ugandans were enrolled as controls. Median CD4 count in the HIV-negative volunteers assayed (n = 11) was 704 (range 547–1618), similar to previous reports from Uganda (33).

Development and validation of a Treg immunophenotyping strategy

Human Treg suppressor activity is primarily confined to CD4+CD25bright cells (23, 25), and almost every Treg surface marker described, including CD25, is also expressed on activated CD4+ cells. Therefore, the large number of activated CD4+ T
CD25bright CD62Lhigh population is in agreement with reports in Tregs (reviewed in Ref. 19). The high suppressor capacity of this population is demonstrated by sorting the CD25bright CD62Lhigh population and demonstrating that depletion of cells with suppressive activity might contribute to the immune hyperactivation associated with advanced HIV disease. We defined immune activation for both CD4+ and CD8+ T cells, respectively. Tregs are not associated with sex or viral load, but were inversely associated with age in multivariate models. In univariate and multivariate models, both CD4+ and CD8+ T cell activation are associated with Treg depletion. Tregs were represented as different ratios to T cell subsets to investigate potential mechanisms of inhibition. Statistically significant relationships are in bold.

Table II.  *Treg depletion is associated with increased immune activation*  

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Univariate ( r^2 ) (coefficient, ( p ) value)</th>
<th>Multivariate ( r^2 ) (adjusted) (coefficient, ( p ) value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 count</td>
<td>0.39 (0.0196, &lt; 0.0001)</td>
<td>0.43 (0.02, &lt; 0.0001)</td>
</tr>
<tr>
<td>HIV RNA</td>
<td>0.11 (−1.17, 0.0025)</td>
<td>0.43 (0.21, 0.5614)</td>
</tr>
<tr>
<td>Gender</td>
<td>0.0001 (−0.12, 0.9410)</td>
<td>NA</td>
</tr>
<tr>
<td>Age</td>
<td>0.13 (−0.24, 0.0017)</td>
<td>0.47 (−0.13, 0.0207)</td>
</tr>
<tr>
<td>Absolute Tregs</td>
<td>0.28 (−1.21, &lt; 0.0001)</td>
<td>0.36 (−0.81, 0.0217)</td>
</tr>
<tr>
<td>Tregs (% CD3)</td>
<td>0.24 (−12.25, &lt; 0.0001)</td>
<td>0.46 (−10.06, &lt; 0.0001)</td>
</tr>
<tr>
<td>Tregs (% CD4)</td>
<td>0.02 (−1.21, 0.2369)</td>
<td>0.37 (−2.19, 0.0135)</td>
</tr>
<tr>
<td>Treg:CD8</td>
<td>0.27 (−784.12, &lt; 0.0001)</td>
<td>0.43 (−560.82, 0.0006)</td>
</tr>
<tr>
<td>Absolute Tregs</td>
<td>0.20 (−1.07, 0.0002)</td>
<td>0.35 (−0.63, 0.0893)</td>
</tr>
<tr>
<td>Tregs (% CD3)</td>
<td>0.14 (−10.00, 0.0018)</td>
<td>0.40 (−7.73, 0.003)</td>
</tr>
<tr>
<td>Tregs (% CD4)</td>
<td>0.0001 (−0.09, 0.9307)</td>
<td>0.33 (−1.10, 2.245)</td>
</tr>
<tr>
<td>Treg:CD8</td>
<td>0.21 (−736.18, 0.0001)</td>
<td>0.41 (−507.37, .0031)</td>
</tr>
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</table>

* Associations between Tregs and clinical/demographic characteristics were investigated using univariate and multivariate least square regression models controlling for viral load \( \text{log transformed, copies/ml}, \) CD4 count \( \text{cells/mm}^3 \), or viral load and CD4 count \( r^2 \). Coefficient represents the increase in the dependent variable per unit increase in the independent variable. CD4+ and CD8+ T cell activation represent HLA-DR CD38+ population expressed as percentage of CD4+ and CD8+ T cells, respectively. Traditionally, quantitation of Tregs has been expressed as a percentage of CD4+ T cells. However, CD4 counts decline in advanced HIV infection, and expressing Tregs as a percentage of CD4+ T cells will not adequately reflect the loss of Tregs or their stoichiometry with CD8+ cells. Therefore, we adopted the unique approach of expressing Tregs as an absolute number and as ratios of CD4+, CD8-, or CD3-positive cells. For this analysis, Tregs were measured independently as both percentage of CD4+ and percentage of CD3-positive cells by flow cytometry, while the absolute Treg count and Treg:CD8 ratios were calculated using the absolute CD4 and CD8 counts. This approach allows the comprehensive representation of the interaction of Tregs with their target cells yielding insight into their mechanism of action.

To examine the relationship between overall CD4 cell count and Tregs, we first expressed Tregs as either an absolute number or as a percentage of CD4+ T cells and performed linear regression against absolute CD4 cell count. We found a significant correlation between the decline in absolute CD4+ T cells and the decline in absolute Tregs (Fig. 2C; \( r^2 = 0.39, p < 0.0001 \)), thereby demonstrating that Tregs are depleted in the course of HIV disease. However, the strength of the correlation coefficient suggests that the decline of these two populations of CD4+ T cells may have distinct kinetics. Thus, CD4 count alone is not necessarily predictive of Treg count, and these populations can be analyzed independently. Furthermore, we observed increased numbers of Tregs (expressed as percentage of CD4+ T cells) in many HIV-positive volunteers with low CD4 cell count, suggesting a slower decline of this population; however, this trend did not reach statistical significance (\( r^2 = 0.04 \) and \( p = 0.068 \) (Fig. 2B).

*Treg depletion is associated with immune activation*

Because Tregs decrease as HIV disease progresses, we hypothesized that the depletion of cells with suppressive activity might contribute to the immune hyperactivation associated with advanced HIV disease. We defined immune activation for both CD4+ and CD8+ T cells as the simultaneous expression of both...
HLA-DR and CD38. This immunophenotyping approach has been extensively validated in prior studies and demonstrates T cell activation to be a strong prognostic indicator for progression to AIDS (1, 2, 4, 5, 36–42). In our study, HIV-negative Ugandans had levels of CD4 and CD8 T cell activation comparable to that reported for HIV-negative Ethiopians (5 vs 7–8% for CD4, and 13 vs 12–15% for CD8 T cells, respectively) (41). In contrast, antiretroviral naive HIV-positive individuals showed a striking increase in both mean CD4 and CD8 T cell activation (31% for CD4 and 63% for CD8 T cells, respectively), exceeding levels previously reported for HIV infection in developed countries (41) (Fig. 3A). CD4 and CD8 T cell activation were negatively associated with CD4 count (r² = 0.34, p < 0.001 and r² = 0.09, p = 0.0011, respectively) and positively associated with viral load (r² = 0.146, p < 0.0001 and r² = 0.193, p < 0.0001, respectively) in this population (Fig. 3, B and C, and data not shown). No significant association between CD4 count and CD4⁺ or CD8⁺ T cell activation was observed in HIV-negative Ugandans (p = 0.335 and p = 0.861, respectively; data not shown).

The relationships between Tregs and immune activation in HIV-positive ARV naïve volunteers were next examined using all four representations of Tregs (Table II). Univariate analysis revealed a negative correlation between Tregs and both CD4⁺ and CD8⁺ T cell activation. In multivariate regression analysis controlling for the effect of overall CD4⁺ T cell number and viral load on immune activation, this relationship remained highly significant for all four representations of Treg number, except between CD8⁺ T cell activation and Tregs expressed as absolute number or as a percentage of CD4⁺ T cells. Interestingly, Tregs expressed as a percentage of CD3⁺ T cells appeared to correlate best with overall higher r² values. All associations remained significant whether CD4 count was represented as an absolute number, CD4:CD8 ratio, or as a percentage of T cells (data not shown). Finally, we found no correlation between Tregs and sex or viral load in multivariate analysis; however, a negative association was apparent with age (Table II).

FIGURE 1. Validation of a Treg immunophenotyping strategy. Freshly isolated PBMCs from HIV-negative and HIV-positive Ugandan volunteers were stained with anti-CD62L FITC, anti-CD25 PE, anti-CD3 PerCP Cy5.5, and anti-CD4 allophycocyanin. After gating on CD3⁺CD4⁺ cells, Tregs were identified as CD25bright and CD62Lhigh (a). Treg function was validated by sorting this population and performing suppressor assays on responder cells (CD4⁺CD25negCD62Lhigh) (Tresp) to show dose-dependent inhibition of anti-CD3-induced proliferation (b). Tregs were added at varying ratios to responder cells (Treg:Tresp) ranging from 1:1 to 1:64. Cells were stimulated with soluble anti-CD3, and proliferation was measured by tritium incorporation. Background proliferation was always <100 cpm and subtracted from net values.

FIGURE 2. Tregs as compared with CD4 count in HIV-positive and HIV-negative volunteers. Greater variability in Tregs (represented as a percentage of CD4⁺ cells) is seen in HIV-positive (b) compared with HIV-negative volunteers (a). This variability is especially apparent in individuals at late stages of disease. Absolute Treg number is correlated with CD4 count in HIV-positive volunteers (c).
In contrast, similar associations between Tregs and CD4+ and CD8+ T cells expressing activation markers were not observed for any of the four representations of Treg number (Fig. 3D and data not shown) in HIV-negative Ugandan volunteers.

Because Tregs are a subset of CD4+ T cells, we next asked whether the previously described associations between CD4 count and T cell activation were actually attributable specifically to Tregs rather than to CD4+ T cells in general. We focused our analysis on Tregs as expressed as a percentage of CD3+ T cells, as this variable had the strongest correlation with immune activation (shown above) and also most accurately represents Treg interactions with both CD4+ and CD8+ T cells. Using standardized coefficients in multivariate analysis to compare the strength of the associations between immune activation and Tregs, CD4 count, and viral load, we found that both CD4+ and CD8+ T cell activation correlate more strongly with Tregs than with CD4 count (standardized coefficient 0.40 vs 0.29 and 0.29 vs 0.19 for CD4+ and CD8+ T cell activation, respectively) (Table III). The increased relative strength of this relationship between immune activation and Tregs held true for all representations of Tregs, except for Tregs represented as a percentage of CD4+ T cells (data not shown). This suggests that Treg depletion in HIV infection plays a critical role in immune activation that has previously been attributed only to CD4+ T cell depletion. Viral load continued to have a significant, positive association with CD4+ and CD8+ T cell activation in our analysis. Compared with viral load, Tregs appear to have a stronger association with CD4+ immune activation (standardized coefficient 0.40 vs 0.28), but a weaker association with CD8+ T cell activation (standardized coefficient 0.29 vs 0.42) (Table III).

Tregs and CTL dysfunction

Next, we investigated whether Tregs contribute to T cell effector dysfunction as measured by IFN-γ assays. Impaired CD8+ T cell function in HIV infection has previously been attributed to loss of CD4+ T cell help (8). We hypothesized that CD8+ T cell dysfunction may alternatively be caused by the observed increasing ratio of Tregs to T helpers in advanced diseases. To test this principle, we first depleted Tregs using anti-CD25 magnetic beads and examined the effect of in vitro depletion on Gag-specific CD8+ T cell responses. On average, our depletions achieved greater than 10-fold reduction in the CD25brightCD62Lhigh Treg population. We found that Treg depletion in our study population resulted in an increase in CD8+ HIV-specific responses in six of six randomly selected ARV naive individuals assayed (Fig. 4A), thereby confirming that Tregs appear to have the ability to suppress HIV-specific responses at least in vitro, similar to previous reports (26, 27).

We next examined whether we could detect an ex vivo influence of Tregs on HIV-specific responsiveness. ELISPOT assays were used instead of intracellular flow cytometry to accommodate the large number of samples. For this analysis, Tregs were represented as a percentage of CD3+ cells and as a percentage of CD4+ cells to assess their effects on all responding cells, and the impact of

Table III. Strength of variables on T cell activation using standardized coefficients

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Standardized Coefficient</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dependent variable = CD4+ T cell activation (r² = 0.46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs (% CD3)</td>
<td>-0.40</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4 count</td>
<td>-0.29</td>
<td>0.0126</td>
</tr>
<tr>
<td>HIV RNA</td>
<td>0.28</td>
<td>0.0147</td>
</tr>
<tr>
<td>Dependent variable = CD8+ T cell activation (r² = 0.40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs (% CD3)</td>
<td>-0.29</td>
<td>0.0043</td>
</tr>
<tr>
<td>CD4 count</td>
<td>-0.19</td>
<td>0.1154</td>
</tr>
<tr>
<td>HIV RNA</td>
<td>0.42</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

The relative strengths of the association between Tregs, CD4 count, and viral load with CD4+ or CD8+ T cell activation were assessed in a multivariate regression analysis (adjusted r²) using standardized coefficients. The magnitude of standardized coefficients allows meaningful comparisons among the regression coefficients within a multivariate regression analysis. The higher the absolute value of the coefficient, the stronger the contribution of this variable within an analysis. Tregs show the strongest association with CD4+ activation, while viral load has the strongest association with CD8+ T cell activation. CD4 count does not have a statistically significant direct effect on CD8+ T cell activation after controlling for Tregs.
Depletion of Tregs in vitro increases HIV-specific CD8⁺ T cell responses. In vitro depletion of Tregs in all six HIV⁺, ARV naive individuals results in an increase in the percentage of Gag-responding CD8⁺ T cells (as measured by intracellular IFN-γ) (a). Correlation of Gag-specific responses (as measured by IFN-γ ELISPOT), with CD4 count (b) and Tregs expressed as a percentage of CD3⁺ T cells (c).

Discussion

Our study is the first to comprehensively quantitate Tregs in ARV naive HIV-infected individuals at all stages of disease, and suggests that Treg depletion may play a critical role in the pathogenesis of AIDS. We report in this work an intimate association between Treg number, CD4 count, and T cell activation. Because HIV-associated immune activation is a strong predictor of disease progression, these findings may have far-reaching significance.

Immune activation has been associated with viral load and CD4⁺ T cell depletion, although the specific mechanism responsible for this activation has remained unknown. It has been proposed that immune activation is driven by HIV viremia through bystander activation. This hypothesis, however, does not explain what mediates bystander activation or the inverse relationship between activation and CD4 count. The depletion of CD4⁺ T cells may lead to activation through nonspecific homeostatic proliferation, or conversely, activation may be driving depletion by activation-induced cell death (2, 44–48). In fact, CD4⁺ T cell activation has been postulated as the primary mechanism of CD4⁺ T cell depletion in the pathogenesis of AIDS (45). We propose that Tregs are depleted in HIV infection, and that this depletion, in conjunction with viremia, drives CD4⁺ and CD8⁺ T cell activation. The resultant immune activation may, in turn, lead to increased death of Th cells by activation-induced cell death or increased viral replication and infection. The observed strong association between immune activation and Treg number supports our hypothesis and is in agreement with a recent report showing increased immune activation in HIV-positive patients with low Foxp3 expression (49). The fact that this relationship between immune activation and Tregs (absolute or percentage of CD3⁺ T cells) is not seen in HIV-negative patients suggests that this effect may be HIV specific and requires the presence of persistent Ag to induce and activate Tregs. Alternatively, absolute Tregs may have to be decreased to a lower threshold that was not reached in our HIV-negative volunteers, before an effect on activation is apparent.

In this study, we adopted the unique approach whereby Tregs were expressed as an absolute number or as ratios to CD3-, CD4-, and CD8-positive cells. This analytical method is critical when the T cell number and ratios change dramatically over time as in HIV disease. Additionally, this approach sheds light on potential mechanisms of Treg action. Although the mechanism of Treg inhibition is unknown, Tregs are believed to exert suppressive effects on both CD4⁺ and CD8⁺ T cells through either direct cell contact or cytokines. Expressing Tregs as a percentage of all target cells should best approximate their regulatory role. Our finding that Tregs expressed as a percentage of CD3⁺ T cells have the stronger associations with CD4⁺ and CD8⁺ T cell activation supports this argument and lends biologic credence to this methodology. The stronger correlations seen with Tregs expressed as a percentage of CD3⁺ cells may be reflective of the widely divergent total T cell number seen in HIV-positive individuals. In addition, when we analyzed the effect of Tregs expressed as a percentage of CD4⁺ cells on CD8⁺ T cell activation, the lack of statistical significance suggests that this representation of Tregs, which is the most often used in Treg studies, may be biologically inappropriate in HIV disease. Persistent, high viremia and perhaps homeostatic proliferation may also contribute substantially to immune activation. Although our analysis suggests Treg number has a stronger effect than viral load on CD4⁺ T cell activation, the relative impact of these two factors needs further evaluation.

How could Treg depletion lead to immune activation, but at the same time not result in autoimmunity or exaggerated pathogen-specific responses? If Tregs suppress HIV-specific immune responses in vitro as demonstrated by us and others (26–28), shouldn’t we expect to see greater HIV-specific CD8 responses at late stages of disease when Tregs are depleted? A possible explanation may be apparent in the finding that Tregs appear to decline more slowly than other CD4⁺ T cells in some patients, thereby establishing an increased suppressor to helper ratio in advanced disease. However, we failed to find a significant association between Treg number, CD4 cell count, and Gag-specific responses ex vivo. A possible explanation for this lack of correlation might be found in the great complexity of factors that are required to generate an effective T cell response (reviewed in Refs. 9, 50, and 51). Perhaps this complexity masks any impact that Tregs may have on this process ex vivo. Alternatively, IFN-γ production in
response to HIV peptides may not be the best measure to assess Treg-mediated suppression of effector T cells.

Our immunophenotyping strategy for Tregs has several limitations. Because regulatory activity may not be strictly confined to the CD25highCD62Llow population, our study may have underestimated the true number of Tregs (52–54). Despite this limitation, the observed strong correlation between activation and Treg number strengthens our hypothesis. Literature suggests that the best marker for Treg activity may be the transcription factor Foxp3 (55). However, recently, Foxp3 has been shown to be up-regulated in activated human CD4+ cells, thus potentially complicating the use of this marker in HIV-infected individuals (56, 57). Until distinct markers are identified to clearly distinguish Tregs from activated CD4+ T cells, the full effect of this population cannot be evaluated.

Many questions remain unanswered concerning the origin and identification of Tregs and their mechanism of action. This level of complexity is further augmented in defining their role in HIV infection. One intriguing speculation is that the Tregs in advanced HIV disease are of the adaptive phenotype and, as recently suggested, have HIV specificity (27, 28). If substantiated, this hypothesis could explain why viremia (necessary to activate Treg-suppressive function) is associated with T cell anergy in many studies (58, 59). In addition, because antiretroviral drugs rapidly decrease immune activation, it is important to evaluate the relative contribution of Treg reconstitution vs control of viremia on activation during initiation of therapy. The fact that Tregs are a subset of CD4+ T cells should also prompt a closer re-examination of previously observed associations with CD4 count that may actually be attributable to Tregs.

It remains to be determined whether Tregs play a protective or detrimental role in HIV disease. Expansion of adaptive HIV-specific Tregs hypothetically could decrease the magnitude of T cell responses in viremic patients and make them more susceptible to other pathogens. Alternatively, Tregs may have a protective effect and limit the massive immunopathology that could be caused by high level viremia. Because of the negative association between Tregs and immune activation, we postulate that Treg depletion may hasten progression to AIDS and death. A longitudinal study examining Treg depletion and clinical progression will most likely address this issue.

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Disclosures

The authors have no financial conflict of interest.

References


CD4 cell count increases in HIV-infected adults experiencing 4 years of viral  

munodeficiency virus type 1.  


terin requirements of CD4+ T cells that prevent spontaneous autoimmune en-  


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