Normalization of FoxP3+ Regulatory T Cells in Response to Effective Antiretroviral Therapy

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Regulatory T cells (Tregs) blunt uncontrolled immune responses. In advanced human immunodeficiency virus (HIV) infection, the total number of Tregs is decreased, but the proportion of T cells with a regulatory phenotype is highly variable. We studied CD4+CD25+FoxP3+ T cells from patients successfully treated with combination antiretroviral therapy (ART). The proportion of CD4+CD25+FoxP3+ cells transiently increased and then decreased from a median of 13% at baseline to 5.1% at 48 weeks, similar to values in normal subjects. These data suggest that with effective therapy, the regulatory cell numbers normalize, and that the inflammatory signals driving their production may also abate.

HIV infection is associated with a progressive decrease in CD4+ T cell count [1]. In advanced AIDS, lower CD4+ cell counts are responsible for the increased incidence of opportunistic infections and death [2]. The interaction between HIV infection and the host is complex and involves multiple components of the immune system including proinflammatory effector T cells and regulatory immune responses. Chronic immune activation is associated with ongoing viral replication and gradual immune depletion [3].

Effective ART blunts viral replication and increases the CD4+ T cell count in peripheral blood, a phenomenon known as immune reconstitution. However, effective treatment does not result in a full recovery of all the different subsets of helper and cytotoxic T lymphocytes [4]. Following initiation of effective ART, there is an early redistribution of CD4+ cells from lymph nodes into circulation. These cells are mainly activated and are memory cells. Subsequently, there is a gradual recovery of naive CD4+ T cells. Immune reconstitution includes recovery of antigen-specific responses to opportunistic infections [5].

Tregs play a key role in blunting what would otherwise be uncontrolled T cell responses. The effect of HIV on Tregs is complex. Early on, Tregs may blunt the anti-HIV response of cytotoxic T cells [6]. In advanced HIV infection, CD4+ cells are depleted. Along with this, we and others have demonstrated an overall depletion of regulatory T cell numbers, but a variable increase in the proportion of cells expressing a regulatory T cell phenotype [7]. Depletion of regulatory cells may predispose patients to hypersensitivity reactions to medications and to inflammatory conditions including the immune reconstitution inflammatory syndrome [8].

The increased proportion of regulatory cells in advanced HIV disease may reflect an ongoing regulatory response to immune activation [9]. Treatment with effective combination ART is associated with restoration of the number of CD4+ cells and restoration of the immune response [2]. However, as noted in one study, the effect on Tregs is poorly defined with limited normalization [10]. We hypothesized that effective ART would lead to improvement in Treg proportion along with the restoration of CD4+ cell counts. To test this hypothesis, we prospectively analyzed fresh peripheral blood mononuclear cells (PBMC) from ART naive patients in Lima, Peru, who were initiating ART. We noted that effective ART (as measured by a decrease in HIV RNA <400 copies/mL) was associated with a gradual normalization of CD4+ cells and the proportion of T cells with a regulatory T-cell phenotype.

Methods

Patients were recruited from the national program “Programa de Tratamiento de Gran Actividad” (TARGA) and the antiretroviral program “Cohorte de Virus de Inmunodeficiencia Humana y SIDA” (COVIHS) at the Instituto de Medicina Tropical Alexander von Humboldt Universidad Peruana Cayetano Heredia (IMTAvH –UPCH) in Lima, Peru. During this period, antiretroviral drugs were only supplied to patients with
CD4$^+$ cell counts $<$200 cells/mm$^3$, but none of the patients had an active opportunistic infection at the time of initiation to therapy. All patients included in this study were treated with 2 nucleoside reverse transcriptase inhibitors plus a non-nucleoside reverse transcriptase inhibitor. Patients about to initiate ART were asked to consent to serial blood draws to test for flow cytometry analysis and immunological studies of PBMC. For consenting patients, blood draws were performed at baseline previous to initiation of ART and then 2, 4, 8, 12, 24, 36, and 48 weeks afterwards. Data on viral loads, and ART were obtained from the medical records. Eighteen consecutive patients achieving HIV RNA $<$400 copies/mL (the lower limit of the assay performed in Lima) and providing serial blood samples were included in this analysis. All participants signed a written informed consent form prior to enrollment in the study. The Institutional Review Board (Comité Institucional de Ética) of the Universidad Peruana Cayetano Heredia in Lima, Peru approved the study protocol and consent forms.

Flow Cytometry
PBMCs were isolated from heparinized blood by density gradient centrifugation (Ficoll-Hypaque GE Healthcare, USA). Regulatory cells were defined by staining for CD4$^+$, CD25$^+$, and FoxP3$^+$. PBMCs were first stained using peridinin-chlorophyll-protein (PerCP) anti-CD4 and phycoerythrin-conjugated (PE) anti-CD25 monoclonal antibodies (BD Biosciences). After fixing and permeabilizing, the cells were stained for intracellular FoxP3 using a fluorescein-isothiocyanate (FITC)-conjugated anti-FoxP3 monoclonal antibody (clone ECH 101, eBiosciences). Recent data suggest that FoxP3 may be increased transiently during T cell activation and additional surface markers may be needed to differentiate activated T cells expressing FoxP3 from true Tregs. A 4-color panel for Tregs was added during the course of the study to include staining for CD127 as follows: CD25-FITC, CD127-PE, CD4-PerCP and Foxp3-Allophycocyanin (APC). Cells were analyzed using a FACS caliber flow cytometer (Beckton Dickinson). Tregs were identified as CD25$^+$ and FoxP3$^+$ cells among CD4$^+$ cells within the lymphocyte gate. Absolute CD4$^+$ cell counts were performed using a 4-color single-platform staining of whole blood cells (anti–CD3-FITC, CD4-PE, CD45 PerCP, and CD8 APC). Flow cytometry analysis used FlowJo software, version 8.5 (Tree Star). The standardization of this assay in normal subjects and comparison of HIV-infected patients in different CD4$^+$ cell count strata has been previously described [7].

Data Analysis
Analysis was performed using independent 2-sample $t$ test with pooled standard deviation (unequal sample size, unequal variance) comparing the proportion of CD4$^+$ cells that express CD25$^+$ and FoxP3$^+$ from HIV-infected patients to healthy donors at different time points before and during highly active antiretroviral therapy (HAART). Correlation between both flow cytometry methods used to characterize Tregs was determined by Spearman correlation analysis.

Results
Eighteen consenting patients were started on HAART and achieved viral loads $<$400 copies/mL. The demographic data, baseline CD4$^+$ cell count, viral load, and antiretroviral regimen are presented in Table 1. The median baseline viral load was 125,582 copies/mL (range, 5400–540,000 copies/mL (Table 1). The median time to viral load $<$400 copies/mL was 3 months (range, 2–6 months). During the first year, the overall CD4$^+$ cell count increased in each patient.

At baseline, the median CD4$^+$ cell count was 75 cells/mm$^3$ and the proportion of CD4$^+$ cells that were CD25$^+$ FoxP3$^+$ ranged from 3.2% to 41.2%, median 13.25%. After initiation of HAART, there was an initial rapid increase in the CD4$^+$ cell count followed by subsequent gradual increase of nearly 200 cells/mm$^3$ by week 48 of therapy (Figure 1A). The initial increase in CD4$^+$ cells was accompanied by a transient increase in the proportion of CD4$^+$ cells that were CD25$^+$ FoxP3$^+$ (Figure 1B). However, over time, as HIV RNA decreased, the proportion of cells that were CD25$+$ FoxP3$^+$ decreased to a median 5.1%,
which is similar to that in normal controls as we previously reported (Figure 1B). The changes in absolute number of regulatory cells paralleled the proportion of cells with regulatory markers (Figure 1C). Most CD25\(^+\) FoxP3\(^+\) CD4\(^+\) cells were in the CD127lo-neg region (median, 97.75%; range, 86.20%–99.5%, Figure 1D). The median proportion of CD4\(^+\) cells expressing CD25 and FoxP3 was 5.0% (range, .3%–18.9%). When FoxP3 was excluded from the analysis the median proportion of CD4\(^+\) cells that were CD25\(^+\) CD127lo-neg was higher (10.15%; range, 3.0%–30.5%). However, we found a good correlation between both definitions (Spearman r = .81, P < .0001, Figure 1E).

Discussion

In this study, we examined a cohort of ART-naive HIV-infected patients successfully initiating HAART for expression of markers of Tregs. Over time, the proportion of CD4\(^+\) cells that expressed this regulatory phenotype gradually decreased to normal levels in this cohort of patients responding to therapy. Among those who did not achieve HIV RNA below the limits of detection, the response of Tregs was variable. Some patients demonstrated significant decreases in the proportion of Tregs, but most Tregs did not change significantly or remained elevated. Thus, the changes noted were associated with viral suppression.
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These data contrast with Lim et al who suggested little to no normalization of Treg numbers in a small group of patients initiating ART [11]. There are several possible explanations for this discrepancy. First, not all of the patients studied by Lim and colleagues had sustained virologic responses. Thus, their results may have reflected incomplete viral suppression. Along with this, the investigators noted ongoing lymphocyte activation at one of their sites, also suggesting incomplete viral suppression. Lim et al used a slightly different method for identification of regulatory cells (ie, cells that were CD4+CD25+CD127lo). However, in our hands, there was a close correlation of the proportion of regulatory cells using the different methods, but this is unlikely to explain the differences. Finally, in our study, the cell phenotype was studied on fresh cells, whereas Lim et al worked with cryopreserved cells. We have compared expression of both CD4+CD25+CD127lo on nonpermeabilized cells and CD4+CD25+FoxP3+ on permeabilized cells before and after cryopreservation. In both cases, expression of regulatory markers was markedly altered by cryopreservation mainly with loss of expression (median decrease of 61%, range +15% to −78%, Sattui S, de la Flor C, Sanchez C et al submitted for publication). Interestingly, Lim et al noted lower overall levels of expression of the regulatory markers at baseline comparable to those in our studies. Thus, the effects of cryopreservation may have initially blunted their ability to detect differences in expression of regulatory markers [12]. Our results parallel those of Weiss et al, who studied patients that were interrupting therapy. Weiss et al noted that treatment interruption led to increased proportions of CD4+ cells expressing a regulatory phenotype [13].

Interestingly, we noted an increased proportion of Tregs at the beginning of ART, with a gradual decrease to normal proportions. This may be driven by redistribution of Tregs from lymphoid tissue to peripheral blood. HIV induces Tregs to migrate and accumulate in peripheral and mucosal lymphoid tissues [14]. Effective ART changes the proportion of T cell subset in lymphoid tissue [15]. Specifically, Treg proportion in lymphoid tissue decreased after effective ART. This change may be reflected in the peripheral blood [15].

Our main goal is to describe a time-dynamic normalization of Treg proportion after an effective ART. The functional significance of altered regulatory T cell numbers (decreased) and proportions (increased) is not known. However, the normalization of these numbers noted here provides further support for the ability of ART to reverse the immunopathology noted in advanced AIDS.

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