Changes in regulatory T cells following antiretroviral therapy in HIV infection and the impact of regulatory T cells on HIV replication

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ABSTRACT

Changes in regulatory T cells following antiretroviral therapy in HIV infection and the impact of regulatory T cells on HIV replication

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The role and dynamics of circulating regulatory T cells (Tregs) during human immunodeficiency virus (HIV) infections remain unclear. Therefore, this study aimed to evaluate the frequency of Tregs and related factors in HIV-infected patients, as well as the impact of Tregs on HIV replication.

The frequency of T cell subsets, including Tregs ($CD4^+CD25^{high}FoxP3^+$ cells), and the production of cytokines were investigated in 17 antiretroviral therapy (ART)-naïve patients and 20 patients with viral suppression who received ART for > 3 months; 13 patients were sequentially sampled before and after ART. Factors correlated with the frequency of Tregs, and differences in T cell subsets and cytokines during ART were analyzed. The TZM-bl assay was used to measure the reduction in relative light units (RLU) of HIV-1 Tat-regulated Luc reporter gene expression after infection with Env-pseudotyped virus in Treg and non-Treg co-culture conditions.

Longitudinal comparison of the sequential samples of 13 patients revealed that the frequency of CD4 T cells and naïve T cells significantly increased, whereas that of effector memory CD8 T cells significantly decreased after ART. No difference was observed in

cytokine concentrations before and after ART, except for a significant decrease in INF-α2 after ART. In ART-naïve patients, the frequency of Tregs was significantly negatively correlated with CD4 T cell (r= –0.595, p=0.012) and CD8 T cell counts (r= –0.517, p=0.034). However, linear regression model estimated that only the CD4 T cell count was negatively associated with Treg frequency in ART-naïve patients (β-coefficient= –0.511, p=0.039). No factor correlated with the frequency of Tregs was found in patients with viral suppression after ART. In the T cell culture model, the degree of RLU reduction increased with the increasing number of Tregs; when more than 10^4 Tregs were added, the reduction in RLU was greater in wells containing Tregs than in those containing the same number of non-Treg cells.

An inverse relationship between Treg frequency and CD4 T cell count was observed in ART-naïve patients in this study; however, this relationship disappeared with successful ART. Notably, Tregs may play a role in controlling HIV replication in infected cells. Therefore, both aspects of Tregs must be considered when designing strategies for Treg modulation to treat HIV infections.

Key words : human immunodeficiency virus, regulatory T cell, antiretroviral therapy

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I. INTRODUCTION

Human immunodeficiency virus (HIV) causes a chronic infectious disease associated with progressive depletion of CD4 T lymphocytes and defective HIV-specific T-cell responses leading to acquired immunodeficiency syndrome (AIDS).[1,2] In addition to the decreased lymphocyte count, HIV infection also results in defective function of various immune cells and abnormal activation of the immune system.[3] Highly active antiretroviral therapy (HAART) can suppress viral replication and restore the CD4 T cells by mitigating symptoms caused by immunodeficiency, thus increasing life expectancy.[4,5] However, HIV is not completely eradicated by HAART; during therapy, the virus can persist as a latent infection in the form of low levels of viremia (below the detection limit) or as a silent provirus in resting cells. Various immunological abnormalities, including chronic immune activation, persist even when viral replication is successfully inhibited by HAART.

Regulatory T cells (Tregs) account for 5% of peripheral CD4 T cells and are

characterized by high expression levels of CD25 (α chain of IL-2 receptor), low levels of CD127 (α chain of IL-7 receptor) and low levels of the master transcriptional regulator forkhead box P3 (FoxP3), which is important for controlling immunosuppressive function.[6,7] Tregs control immune tolerance and regulate excessive immune responses during infection.[8] However, Treg can also contribute to disease progression through inappropriate downregulation of protective immune response. This dual nature of Tregs has also been observed during HIV infections. Thus, Tregs can be both beneficial—by suppressing generalized chronic T cell activation—and detrimental, by reducing HIV-specific responses, T cell proliferation, and cytokine production that contribute to viral persistence.[9] Furthermore, it is difficult to elucidate the changes in Tregs and their impact on immune response or viral replication following the initiation/maintenance of antiretroviral therapy (ART).

Tregs are a relatively scarce cell population in human blood, and their absolute number continues to decline during HIV infection. Therefore, characterizing the function and specificity of Tregs in HIV-infected individuals remains challenging. Conflicting data have been published regarding the suppressive capabilities and effect of Treg on HIV infection[10] Analyzing T cell subsets in samples obtained from HIV-infected patients, as well as ex vivo expansion and modulation of these Tregs can potentially help us better understand the impact of Tregs in HIV infection.

In this study, we aimed to investigate changes in T cell subsets, including Tregs, and plasma cytokine levels as a function of ART and factors associated with the frequency of Tregs in HIV-infected patients. We also aimed to evaluate the effect of Treg modulation on HIV replication using an *in vitro* T cell culture model.

II. MATERIALS AND METHODS

1. Study population and samples

This was a single-center prospective study conducted at a 2,500-bed tertiary care hospital in South Korea. Adult patients with HIV infection (confirmed by western blotting) who underwent regular follow-ups at the infectious disease clinic between August 2013 and September 2014, were included in this study. The exclusion criteria were as follows: patients with conditions that may cause non-HIV-related CD4 decline, such as hematologic malignancies, or those receiving CD4-lowering medications. The researchers did not intervene in the treatment of HIV, and ART was provided in accordance with national and international guidelines, per the decision of physicians.

Whole blood samples were collected from all participants at the time of enrollment. For participants enrolled prior to ART initiation, blood samples were collected once at enrollment and then sequentially at three months, six months, or more after treatment initiation. Blood samples at each time point were separated into plasma and peripheral blood mononuclear cells (PBMCs), and stored in a liquid nitrogen tank. Data on demographics, HIV diagnosis, CD4 T cell count, HIV viral load, medical history, history of opportunistic diseases and co-infections, ART, and treatment progress were also collected. For the purpose of this study, virologic suppression was defined at a confirmed HIV RNA level <400 copies/ml.

Informed consent was obtained from all the participants. This study was approved by the Institutional Review Board (IRB) of Severance Hospital, and the study procedure was designed in compliance with ethical considerations for prospective studies.

2. Cell immunophenotyping and sorting

T cell subsets were determined by six-color flow cytometry using a fluorescence-activated cell sorter (FACS; BD Biosciences, San Jose, CA, USA). Aliquots of cryopreserved PBMCs were thawed, washed twice in Dulbecco's phosphate-buffered saline, and stained for viability at room temperature using a Live/Dead Fixable Aqua Dead Cell Staining Kit (Invitrogen). The cells were surface-stained with the following directly conjugated human antibodies: FITC-conjugated anti-CD3 (BioLegend, San Diego, CA, USA), PE-conjugated anti-CD4, APC/Cy7-conjugated anti-CD8, APC-conjugated anti-CD45RA, and perCP/Cy5.5-conjugated anti-CCR7, APC-conjugated CD25 (BD Pharmingen, San Diego, CA, USA), APC-conjugated anti-Foxp3 (eBioscience, San Diego, CA, USA), and PE-conjugated anti-PD-1 (BD Biosciences). Data were analyzed using BD FACSuite (BD Biosciences).

For this study, $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells were considered as CD4 T cells and CD8 T cells, respectively. $CD3^+/CD45RA^+/CCR7^+$ cells were considered naïve T cells, $CD3^+/CD45RA^-/CCR7^+$ cells as central memory T cells, and $CD3^+/CD45RA^-/CCR7^-$ cells as effector memory T cells. The $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cell fractions were analyzed separately. In addition, $CD3^+/CD4^+/CD25^{high}/FOXP3^+$ cells were considered Tregs.

3. Plasma cytokine measurement

Cytokine levels were measured using the ProCarta Human Cytokine Immunoassay Kit (Affymetrix, Santa Clara, CA, USA). Interleukin (IL)-1β, IL-4, IL-6, IL-12, IL-15, IL-17,

tumor necrosis factor (TNF)-α, interferon (INF)-α, INF-γ, and monocyte chemoattractant protein (MCP)-1 were quantified using a Luminex 200 System software (Luminex Corporation, TX, USA).

4. Treg modulation of HIV replication in T cell culture models

We performed a TZM-bl assay to measure the effect of Tregs on HIV replication and determined it as the degree of reduction in HIV-1 Tat-regulated firefly luciferase (Luc) reporter gene expression after a single round of infection with Env-pseudotyped viruses using the method reported by Montefiori et al.[11-13]

Tregs ($CD3^+/CD4^+/CD25^{high}$) and conventional T cells (Tcons; $CD3^+/CD4^+/CD25^-$) were purified from the PBMCs of an HIV-infected patient by magnetic activated cell sorting (MACS). Purified Tregs and Tcons were cultured (1×10^5 cells/well) in 96-well U-bottomed plates. Tregs were cultured in the presence of 100 U/ml recombinant human (rh) IL-2 (HumanZyme; ProteinTech Group, Inc.) at 37°C and 5% CO_2, and counted after 3, 5, and 7 days of culture.

Env-pseudotyped viruses were prepared by transfecting 293T/17 cells (3×10^6 cells in 15 ml growth medium, in a T-75 culture flask) with 5 μg of rev/env expression plasmid (pNL4-3-deltaE-EGFP) and 10 μg of env-deficient HIV-1 backbone vector (pSG3Δenv). The pseudovirion-containing culture fluid was collected from the flasks, filtered through a 0.45 μm filter, aliquoted, and stored at –80°C in 1 ml sterile screw-capped cryogenic vials.

Env-pseudotyped virus stocks were titrated by performing serial five-fold dilutions (for a total of 11 dilutions) in 96-well culture plates: columns 1–11 were used for the dilution

series and column 12 was used for the controls. Freshly trypsinized TZM-bl cells (10^4 cells/100 µl) were added to each well and incubated for 48–72 h. Then, 100 µl of culture fluid was removed from each well, and an equal volume of Luc reporter gene system reagent (Britelite; PerkinElmer) was added to each well. After incubation for 2 min at room temperature, 150 µl of cell lysate was transferred to 96-well black solid plates for measuring the luminescence using a luminometer. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using Reed–Muench formula.[14] Wells with relative light units (RLU) >2.5 times of the background were considered positive for the calculation.[11]

Using another 96-well template, Dulbecco's modified Eagle medium (DMEM) was added to all wells in column 1 (cell control; 150 µl) and columns 2–6 (100 µl; column 2 is the virus control). In columns 3 and 4, Tregs were added in numbers starting at 10^5 cells and decreasing 0.1 fold from rows A to F. Similarly, Tcons were added to columns 5 and 6 from rows A to F. Env-pseudotyped viruses were thawed and diluted in complete DMEM to a concentration of 4,000 $TCID_{50}$/ml. Approximately 50 µl of Env pseudovirions (200 $TCID_{50}$) was added to all wells in columns 2 to 6, rows A to F. Freshly trypsinized TZM-bl cells were prepared at a density of 1×10^5 cells/ml in growth medium and 100 µl of cell suspension (10^4 cells) was added to each well in columns 1 to 6 from rows A to F. Plates were covered and incubated for 48 h at 37°C and 5% CO_2. Culture medium (100 µl) was removed from each well, and the same volume of Luc reporter gene system reagent (Britelite; PerkinElmer) was added to each well. After incubation for 2 min at room temperature, 150 µl of cell lysate was transferred to 96-well black solid plates for luminescence measurement. The percentage reduction in RLU for each sample was calculated by measuring the difference in mean RLU between the test wells (cells + Tregs

or Tcons + pseudovirions) and the cell control wells, and dividing this result by the difference in mean RLU between the virus control and cell control wells, subtracting from 1, and multiplying by 100.

5. Statistical analysis

Nonparametric tests were performed for all data. Comparisons between the two groups were made using the Mann–Whitney U test for continuous values and using chi-squared and Fisher's exact tests for categorical values. Correlations were determined using Spearman's rank correlation coefficients. Univariate and multivariate linear regression analyses were performed to determine the correlates of Tregs frequency. Each variable was first tested using univariate linear regression analysis; variables with $p < 0.1$ in the univariate analysis were then used in multivariate analysis. The Wilcoxon signed-rank test was used for paired comparisons. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS software version 22 (IBM Corp., Armonk, NY, USA), and GraphPad Prism V8.0 (GraphPad Software, San Diego, CA, USA).

III. RESULTS

1. Study population and baseline characteristics

A total of 24 participants were enrolled in this study, and 48 blood samples were collected during the study period. Seventeen participants were enrolled prior to ART initiation, and seven participants were enrolled during receiving ≥3 months of ART. Of the 17 ART-naïve participants, 16 had at least one post-ART follow-up sample collection, and 13 had viral suppression levels <400 copies/ml. The mean number of sample collections per participant was 2.3 in the ART-naive group. Seven participants enrolled during ART had confirmed viral suppression confirmed in their blood samples. We analyzed 17 ART naive samples and 20 virally suppressed samples. (Figure 1) In cases with multiple virally suppressed samples from the same participant, the sample with the longest time since ART initiation was selected.

The baseline characteristics of the study participants are shown in Table 1. The median age was 27 and 31 years, and the percentages of male participants were 94.1% and 90.0% in the ART-naïve and virally suppressed patients, respectively. Body mass index and underlying comorbidities did not differ between the two groups. The median viral load at baseline was 49,200 copies/ml in ART-naïve patients, whereas it was below the detection limit (<20 copies/ml) in virally suppressed patients after a median of 169 days of ART. All virally suppressed patients were receiving ART at the time of sampling, most of whom were on protease inhibitor-based regimens (45%), followed by non-nucleotide reverse transcriptase inhibitor-based regimens (35%) and integrase strand transfer inhibitor-based regimens (20%). The median CD4 T cell count was significantly lower in ART-naïve patients than in virally suppressed patients (226 vs. 466, p=0.002), and the incidence of

AIDS-defining illness, AIDS diagnosis, and co-infection with other pathogens was higher in the ART-naïve group; however, this difference was not statistically significant.

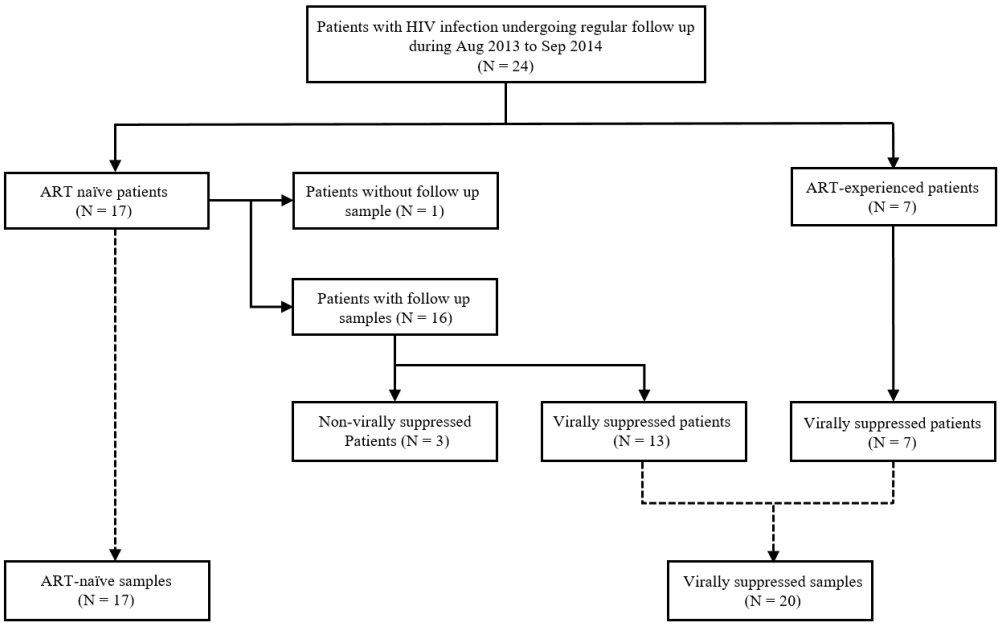

Figure 1. Flow chart of study participants and sample collection. Virological suppression was defined at a confirmed HIV RNA level <400 copies/ml. HIV, human immunodeficiency virus; ART, antiretroviral therapy.

Table 1. Baseline characteristics of study participants with HIV infection.

Variables	ART-naïve patients (N=17)	Virally suppressed patients[1] (N=20)		
		Total (N=20)	Enrolled before ART (N=13)	Enrolled during ART (N=7)
Demographics				
Age (year)	27 [25–41.5]	31 [25.3–50.3]	28 [23.5–47]	38 [30–51]
Male	16 (94.1)	18 (90.0)	12 (92.3)	6 (85.7)
BMI (kg/m^2)	21.7 [20.6–23.5]	21.8 [18.7–24.2]	21.8 [19.8–24.3]	22.1 [17.6–23.8]
Alcohol use	9 (52.9)	10 (50.0)	7 (53.8)	2 (28.6)
Current smoker	10 (58.8)	9 (45.0)	8 (61.5)	2 (28.6)
Baseline comorbidities				
Hypertension	0 (0)	0 (0)	0 (0)	0 (0)
Diabetes Mellitus	0 (0)	2 (10.0)	0 (0)	2 (28.6)
Dyslipidemia	0 (0)	1 (5.0)	0 (0)	1 (14.3)
Chronic kidney disease	1 (5.9)	1 (5.0)	1 (7.1)	0 (0)
Malignancy	0 (0)	1 (5.0)	0 (0)	1 (14.3)
Chronic HBV carrier	0 (0)	0 (0)	0 (0)	0 (0)
Previous TB	1 (5.9)	1 (5.0)	0 (0)	1 (14.3)
HIV infection				
Viral load at sampling (copies/ml)	49200 [19600–348000]	<20 [<20–54]	26 [<20–109]	<20 [<20–<20]
CD4 count at sampling (/mm^3)	226 [65.5–446]	466 [300–663]	410 [311–667]	638 [256–664]
ART duration at sampling (day)	0 [0–0]	169 [115–1437]	168 [102–179]	1875 [163–1980]
ART at sampling	0 (0)	20 (100)	13 (100)	7 (100)
NRTI + NNRTI	0 (0)	7 (35.0)	6 (46.2)	1 (14.3)
NRTI + PI	0 (0)	9 (45.0)	5 (38.5)	4 (57.2)
NRTI + INI	0 (0)	4 (20.0)	2 (15.4)	2 (28.6)
AIDS	8 (47.1)	4 (20.0)	2 (15.4)	2 (28.6)
AIDS defining illness	5 (29.4)	3 (15.0)	1 (7.7)	2 (28.6)
Co-infection	7 (41.2)	3 (15.0)	2 (15.4)	1 (14.3)
Active TB	1 (5.9)	1 (5.0)	0 (0)	1 (14.3)
Latent TB	1 (6.7)	3 (37.5)	1 (50.0)	2 (33.3)

Candidiasis	4 (23.6)	0 (0)	0 (0)	0 (0)
Pneumocystis jirovecii	2 (11.8)	0 (0)	0 (0)	0 (0)
Others	3 (17.7)	1 (5.0)	1 (7.1)	0 (0)

Continuous variables are presented as medians [interquartile ranges], and categorical variables are presented as numbers (percentages).

[1] Virological suppression was defined at a confirmed HIV RNA level <400 copies/ml.

HIV, human immunodeficiency virus; ART, antiretroviral therapy; BMI, body mass index; HBV, hepatitis B virus; TB, tuberculosis; NRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; INI, integrase strand transfer inhibitor; AIDS, acquired immunodeficiency syndrome.

2. T cell subsets and plasma cytokine profiles in HIV-infected patients

Differences in T cell subsets and cytokines between ART-naïve and virally suppressed HIV-infected patients are shown in Table 2. The median CD4 T cell count and frequency were 226 (11.2%) and 466 (23.8%) in ART-naïve and virally suppressed patients, respectively. Frequency of naïve T-cells ($CD3^+/CD45RA^+/CCR7^+$) was higher in patients with viral suppression (16.7% vs. 29.0%), whereas the frequencies of effector memory T cells ($CD3^+/CD45RA^-/CCR7^-$; 41.5% vs. 31.7%) and $PD\text{-}1^+$ CD4 T cells ($CD3^+/CD4^+PD\text{-}1^+$; 2.4% vs. 0.9%) were lower in patients with viral suppression. Frequency of Tregs was 6.8% in ART-naïve patients and 8.2% in patients with viral suppression.

When analyzing samples from 13 participants with serial pre-ART and post-ART (receiving at least 3 months of ART and having viral suppression), the median ART duration between samples was 168 days, and the CD4 T cell count increased significantly from 235 to 410 (p=0.02) with a decrease in viral load (70,800 copies/ml vs. 26 copies/ml, p=0.001). CD8 T cell count and frequency did not differ between pre-ART and virally suppressed samples. The frequency of naïve T cells was significantly increased in virally suppressed samples (12.0% vs. 29.1%, p=0.039). Both CD4 and CD8 naïve T cell frequencies increased after ART, but the increase in CD8 naïve T cell frequency was statistically significant (7.4% vs. 9.1%, p=0.009). The changes in the frequency of total central memory T cells and effector memory T cells during ART were not significant, however, the frequency of effector memory T cells decreased numerically during ART (41.5% vs. 33.8%, p=0.101) and the decrease in CD8 effector memory T cell frequency was statistically significant (35.6% vs. 21.2%, p=0.013). The median Treg frequency

increased from 4.7% to 8.8% and the PD-1^{+} CD4 T cell frequency decreased from 4.4% to 0.9% during ART, both changes were not statistically significant.

Among plasma cytokines, the levels of most pro-inflammatory cytokines, such as IL-1β, IL-2, IL-6, TNF-α, MCP-1, INF-α2, and INF-β, were numerically lower in virally suppressed samples compared to those in ART-naïve samples. Among them, only the decrease in INF-α2 level was statistically significant in patients with serial samples (42.6 pg/ml vs. 8.6 pg/ml, p=0.039). IL-15 and IL-17 were both absent in ART-naive and virally suppressed patients. The levels of anti-inflammatory cytokines, such as IL-4, IL-10, and IL-12, did not differ significantly according to virological control status.

Table 2. Differences in T cell subsets and cytokines between ART-naïve and virally suppressed patients with HIV infection.

Variables	ART-naïve patients (N=17)	Virally suppressed patients[1] (N=20)	Patients with serial samples (N=13)		
			before ART	virally suppressed (during ART)	p-value
Baseline characteristics					
Age (year)	27 [25–41.5]	31 [25.3–50.3]	28 [24.5–47]	28 [23.5–47]	0.953
Viral load (copies/ml)	49200 [19600–348000]	<20 [<20–54]	70800 [27100–532000]	26 [<20–109]	0.001
ART duration (day)	0 [0–0]	169 [115–1437]	0 [0–0]	168 [102–179]	0.001
T cell subsets					
CD4 count (/mm^3)	226 [65.5–446]	466 [300–663]	235 [85.5–443.5]	410 [311–667]	0.02
CD4 %[2]	11.2 [6.8–16.0]	23.8 [18.8–35.5]	11.2 [6.8–16.3]	22.2 [17.2–27.9]	0.004
CD8 count (/mm^3)	854 [461–1488]	621 [509–1194]	912 [539.5–1807]	803 [528–1260]	0.507
CD8 %[2]	50.1 [42.9–62.4]	41.5 [33.2–48.3]	50.8 [36.5–62.4]	43.5 [38.0–49.0]	0.075
Naïve T cell %[2]	16.7 [7.0–20.8]	29.0 [16.6–38.9]	12.0 [5.9–20.8]	29.1 [16.6–36.2]	0.039
Naïve CD4 %[2]	2.7 [1.5–9.4]	11.8 [6.8–20.7]	2.7 [1.5–9.5]	9.7 [7.8–21.0]	0.101
Naïve CD8 %[2]	7.4 [5.2–10.3]	10.1 [7.0–18.8]	7.4 [3.2–8.6]	9.1 [7.0–18.2]	0.009
Central memory T cell %[2]	12.4 [8.1–16.4]	13.1 [9.1–22.7]	13.0 [8.7–17.9]	11.9 [8.6–17.4]	0.917
Central memory CD4 %[2]	9.4 [4.6–12.6]	10.9 [7.8–19.8]	9.4 [4.9–15.0]	8.1 [6.7–14.0]	0.753
Central memory CD8 %[2]	2.1 [1.4–2.9]	1.8 [0.9–2.5]	2.1 [1.4–3.4]	1.9 [0.9–2.4]	0.311
Effector memory T cell %[2]	41.5 [32.3–59.7]	31.7 [14.2–42.2]	41.5 [34.5–60.3]	33.8 [14.9–41.2]	0.101
Effector memory CD4 %[2]	2.2 [1.0–4.4]	3.9 [0.9–11.9]	1.9 [1.0–4.4]	3.8 [0.7–13.8]	0.196
Effector memory CD8 %[2]	35.4 [24.7–52.3]	17.1 [8.2–28.9]	35.6 [24.4–55.0]	21.2 [10.5–27.9]	0.013
Regulatory T cell %[3]	6.8 [3.8–16.1]	8.2 [1.9–11.3]	4.7 [3.8–7.7]	8.8 [0.3–13.2]	0.917
$CD3^+/CD4^+/PD\text{-}1^+$ %[2]	2.4 [1.6–5.8]	0.9 [0.5–6.0]	4.4 [1.8–10.2]	0.9 [0.6–6.2]	0.279
Cytokines					
IL-4 (pg/ml)	2.7 [0.9–6.1]	2.1 [0.5–3.1]	2.7 [0.7–5.9]	2.2 [1.4–3.5]	0.753
IL-10 (pg/ml)	5.8 [4.7–13.2]	3.5 [2.9–5.7]	5.8 [4.7–9.7]	4.5 [3.2–8.3]	0.116
IL-1β (pg/ml)	3.4 [1.6–6.0]	2.3 [0.9–3.3]	3.4 [1.4–5.8]	2.4 [1.7–3.3]	0.701
IL-6 (pg/ml)	16.1 [8.0–25.8]	8.7 [4.5–14.9]	16.0 [7.3–18.5]	10.2 [8.5–16.8]	0.552

IL-12 (pg/ml)	8.8 [7.0–19.5]	6.4 [4.5–14.9]	8.8 [7.0–18.5]	6.6 [5.6–13.4]	0.421
TNF-α (pg/ml)	29.5 [13.5–19.5]	15.6 [1.9–27.3]	29.5 [10.2–37.2]	21.2 [13.6–29.5]	0.917
IL-17 (pg/ml)	0 [0–0.5]	0 [0–1.0]	0 [0–0.5]	0 [0–3.1]	0.441
MCP-1 (pg/ml)	75.5 [40.8–99.8]	50.3 [37.8–69.5]	75.8 [52.8–100.4]	45.8 [35.8–75.7]	0.249
IL-15 (pg/ml)	0 [0–0]	0 [0–0]	0 [0–0]	0 [0–0]	0.655
INF-α2 (pg/ml)	36.8 [3.0–104.6]	8.6 [1.3–20.9]	42.6 [6.5–105.4]	8.6 [6.6–22.4]	0.039
INF-β (pg/ml)	8.6 [2.4–12.6]	2.3 [0–8.6]	5.0 [0.6–17.9]	2.5 [1.0–15.2]	0.328
INF-γ (pg/ml)	12.2 [12.2–33.3]	14.2 [1.3–27.4]	12.2 [6.1–4-0.5]	14.8 [9.7–27.4]	0.753

Continuous variables are presented as medians [interquartile ranges].

[1] Virological suppression was defined at a confirmed HIV RNA level <400 copies/ml.

[2] Percentage of peripheral blood mononuclear cells.

[3] Percentage of CD4 T cells.

Naïve T cells, $CD3^+/CD45RA^+/CCR7^+$; central memory T cells, $CD3^+/CD45RA^-/CCR7^+$; effector memory T cells, $CD3^+/CD45RA^-/CCR7^-$; regulatory T cells, $CD3^+/CD4^+/CD25^{high}/FoxP3^+$; HIV, human immunodeficiency virus; ART, antiretroviral therapy; IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; INF, interferon.

3. Correlates of Treg frequency in HIV-infected patients

The relationship between Treg frequency and other T cell subsets or plasma cytokines was evaluated in ART-naïve and virally suppressed HIV-infected patients (Table 3). As shown by Spearman's rank correlation coefficient (r), Treg frequency was moderately negatively correlated with CD4 T cell ($r = –0.595$, $p = 0.012$) and CD8 T cell ($r = –0.517$, $p = 0.034$) counts in ART-naïve patients. Linear regression model estimated that only CD4 T cell count was negatively associated with Treg frequency in ART-naïve patients (β coefficient= –0.511, p= 0.039) (Table 4, Figure 2). However, this correlation was not observed in virally suppressed patients. The frequency of other T cell subsets or cytokine levels did not correlate with Treg frequency in either ART-naïve or virally suppressed patients.

Table 3. Correlates of Treg frequency in ART-naïve and virally suppressed patients.

Variables	ART-naïve patients (N=17)		Virally suppressed patients[1] (N=20)	
	Correlation coefficient (r)	p-value	Correlation coefficient (r)	p-value
Age (year)	0.207	0.424	0.399	0.081
Viral load (copies/ml)	–0.346	0.174	0.205	0.387
CD4 T cell count (/mm^3)	–0.595	0.012	–0.218	0.356
CD4 T cell %[2]	–0.366	0.148	0.096	0.686
CD8 T cell count (/mm^3)	–0.517	0.034	–0.353	0.126
CD8 T cell %[2]	–0.167	0.522	–0.095	0.691
$CD3^+/CD45RA^+/CCR7^+$ (Naïve T cell) %[2]	–0.063	0.811	–0.176	0.458
$CD3^+/CD4^+/CD45RA^+/CCR7^+$ (Naïve CD4) %[2]	–0.287	0.264	–0.174	0.462
$CD3^+/CD8^+/CD45RA^+/CCR7^+$ (Naïve CD8) %[2]	0.164	0.528	–0.105	0.659
$CD3^+/CD45RA^-/CCR7^+$ (Central memory T cell) %[2]	0.017	0.948	0.016	0.947
$CD3^+/CD4^+/CD45RA^-/CCR7^+$ (Central memory CD4) %[2]	–0.037	0.888	–0.017	0.945
$CD3^+/CD8^+/CD45RA^-/CCR7^+$ (Central memory CD8) %[2]	0.265	0.304	0.134	0.574
$CD3^+/CD45RA^-/CCR7^-$ (Effector memory T cell) %[2]	–0.036	0.892	0.218	0.356
$CD3^+/CD4^+/CD45RA^-/CCR7^-$ (Effector memory CD4) %[2]	0.079	0.764	0.095	0.691
$CD3^+/CD8^+/CD45RA^-/CCR7^-$ (Effector memory CD8) %[2]	–0.054	0.837	0.137	0.565
$CD3^+/CD4^+/PD\text{-}1^+$ %[2]	–0.063	0.811	0.227	0.336
IL-4 (pg/ml)	0.197	0.449	–0.034	0.887
IL-10 (pg/ml)	0.218	0.4	–0.074	0.757
IL-1β (pg/ml)	0.291	0.257	–0.101	0.673
IL-6 (pg/ml)	0.233	0.368	–0.215	0.363
IL-12 (pg/ml)	–0.096	0.715	–0.076	0.75
TNF-α (pg/ml)	0.282	0.272	–0.183	0.439
IL-17 (pg/ml)	–0.252	0.329	0.042	0.859
MCP-1 (pg/ml)	–0.029	0.911	–0.14	0.556
IL-15 (pg/ml)	–0.128	0.625	0.219	0.354
INF-α2 (pg/ml)	–0.006	0.981	–0.038	0.874
INF-β (pg/ml)	0.238	0.359	0.168	0.479
INF-γ (pg/ml)	0.214	0.41	–0.245	0.297

[1]Virological suppression was defined at a confirmed HIV RNA level <400 copies/ml.
[2] Percentage of peripheral blood mononuclear cells
HIV, human immunodeficiency virus; ART, antiretroviral therapy; IL, interleukin; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; INF, interferon.

Table 4. Independent correlates of Treg frequency in ART-naïve patients. (N=17)

Variables	β-coefficients	p-value
Age (year)	-0.475	0.101
Viral load (copies/mL)	-0.122	0.617
CD4 T cell count (/mm3)	-0.511	0.039
CD8 T cell count (/mm3)	-0.495	0.089

Treg, regulatory T cell; ART, antiretroviral therapy

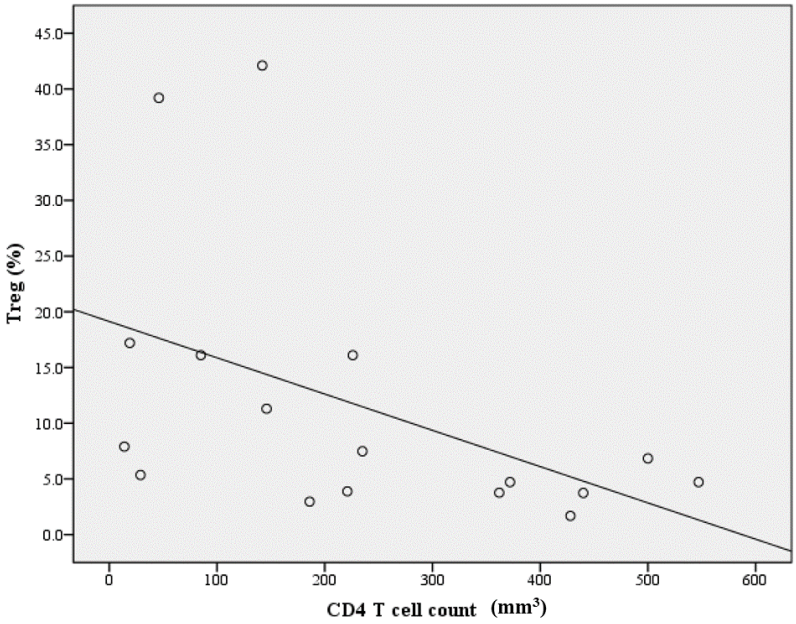

Figure 2. Spearman correlation between CD4 T cell count and Treg frequency in ART-naïve patients. Treg; regulatory T cell, ART; antiretroviral therapy.

4. Effects of Treg modulation on HIV replication in T cell culture models

The reduction in RLU of HIV-1 Tat-regulated Luc reporter gene expression after infection with the Env-pseudotyped virus in the TZM-bl assay was measured under Treg- and Tcon-containing conditions. When 100 Tregs (or Tcons) were added to each well, RLU reduction in the Treg-containing well was 0.82%, whereas no RLU reduction was observed in the Tcon-containing well. As the number of cells added increased, the degree of RLU reduction increased in both the Treg and Tcon-containing wells; however, when 10^4 and 10^5 cells were added, the reduction in RLU was greater in the Treg-containing wells than in the Tcon-containing wells (3.11% vs. 1.27% with 10^4 cells, and 4.39% vs. 2.13% with 10^5 cells) (Figure 3).

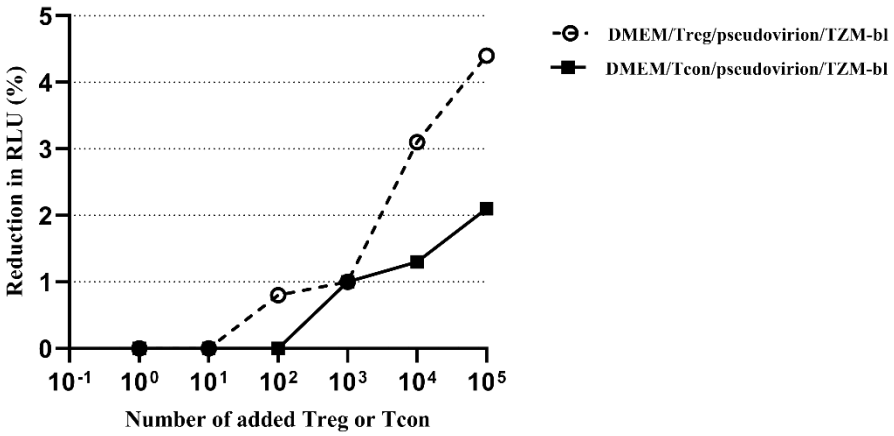

Figure 3. Reduction in relative light unit (RLU) of HIV-1 Tat-regulated firefly luciferase reporter gene expression after Env-pseudotyped virus infection in TZM-bl assay, depending on the presence of Tregs or Tcons. RLU, relative light unit; Treg, regulatory T cells ($CD3^+CD4^+CD25^{high}/FoxP3^+$), Tcon; Treg not containing conventional CD4 T cells ($CD3^+/CD4^+/CD25^-$); DMEM, Dulbecco's modified Eagle medium.

IV. DISCUSSION

Tregs play a complex role in retroviral infections, including HIV infection, and the balance of beneficial and detrimental effects of Tregs may change between the acute and chronic phases of infection.[9] Kinetic studies have shown that Tregs begin to expand within weeks of retroviral infection compared to healthy controls.[15] In chronic infection, many studies have consistently reported that overall Treg numbers decrease, similar to other CD4 subsets, but with relative sparing or increased frequency.[9] Mechanisms promoting the expansion of Tregs during HIV infection are not clearly understood, but several proposed mechanisms spare Tregs relative to other CD4 T cell subsets in chronic infection. The increased survival of HIV-infected Tregs might be attributed to this finding. HIV-infected human Tregs remain largely in the reservoir and have been reported to be less sensitive to T cell receptor re-stimulation than $CD4^{+}CD25^{-}$ T cells, suggesting a relative resistance to activation-induced cell death.[16] The permissiveness of Tregs to HIV replication may also be reduced, in part as a result of FoxP3-mediated inhibition of HIV-1 transcription.[17,18] The sparing of Treg frequency may also be explained by increased conversion of peripheral conventional CD4 T cells to peripheral Tregs upon induction of HIV-exposed plasmacytoid dendritic cells (DCs).[19]

In this study, Treg frequency did not change significantly before and after ART. This may be due to the relatively higher initial CD4 counts and lower initial Treg frequencies of ART-naïve participants in this study compared to HIV patients analyzed in previous studies, which may have prevented meaningful ART-related changes.

The inverse relationship between Treg frequency and CD4 T cell count in ART-naïve patients may be understood in terms of the role of Tregs in HIV infection. Increased

immune activation is a hallmark of HIV infection, and Tregs have been shown to regulate the immune system to suppress inflammation and viral spread by activated T cells.[10] In vitro studies have shown that in addition to limiting the number of susceptible cells, Tregs can limit the infection of conventional CD4 T cells through DC-CD4 T cell immunological synapses.[20] Therefore, Tregs may help prevent the deleterious pathogenic consequences of HIV infection by controlling the immune activation status of virus-producing cells by inducing them to enter a quiescent state. However, by forcing infected T cells into a quiescent state, Tregs promote the generation of a latent viral reservoir,[21] which is the ultimate obstacle to curing HIV. In addition, Tregs have been reported to be more susceptible to HIV than conventional CD4 T cells and act as an important viral reservoir; replication-competent viruses have been reactivated from Tregs even after long-term ART.[22,23] Meanwhile, experimental studies have reported that Tregs suppress CD8 T cell proliferation and effector functions.[24] Thus, disruption of the cell-mediated immune response against HIV inhibits viral clearance in infected individuals, likely leading to an increased viral replication.[10] This Treg-mediated suppression is maintained during chronic infection and contributes to the exhausted phenotype of CD8 T cells.[25] Many studies have reported that in contrast to progressors, HIV controllers and long-term non-progressors exhibit Treg levels similar or even lower than healthy donors.[26-28] For these reasons, increased Treg frequency is a hallmark of advanced HIV infection and inflammation.

Most studies showed an inverse relationship between Treg frequency and CD4 T cell count[27,29,30], and only ART was reported to partially abolish the effect of HIV infection on abnormal Treg frequency.[31-34] In this study, consistent with other studies, an inverse

relationship between Treg frequency and CD4 T cell count was also observed in ART-naïve participants, but this relationship disappeared with successful ART.

Importantly, in vivo studies using the human IL-2/diphtheria toxin fusion protein (Ontak) to deplete Tregs in simian immunodeficiency virus (SIV)-infected control macaques reported that after >75% depletion of Treg frequencies, major CD4 T cell activation occurred, leading to the reactivation of latent SIV. However, Treg depletion also significantly increases SIV-specific CD8 T cell frequencies, resulting in the rapid clearance of reactivated virus.[35-37] These results suggest that Treg depletion is a plausible strategy for HIV cure by reducing the reservoir while enhancing effective cell-mediated immune responses.

However, the beneficial effects of Tregs should also be considered when applying this strategy. In addition to reducing non-specific generalized inflammation, Tregs have been suggested to reduce HIV replication in several types of target cells, including CD4 T cells.[38] The underlying mechanism for controlling HIV replication in conventional activated T cells has been reported to be dependent on direct contact between Tregs and conventional CD4 T cells.[39] Moreno-Fernandez et al. showed in an *in vitro* study that Tregs reduced the level of HIV infection of conventional T cells in a dose-dependent manner.[38] Purified Tregs and conventional T cells from healthy donors were activated separately, and conventional T cells were infected with HIV and cultured with or without Tregs. Co-culture of conventional T cells and Tregs at a 1:1 ratio resulted in the inhibition of conventional T cell infection.[38] Similarly, in the T cell culture model in this study, co-culture of Tregs with TZM-bl cells showed less HIV replication in TZM-bl cells than in co-culture of non-Treg T cells with TZM-bl cells, and this effect was shown in a dose-

dependent manner in co-cultured Tregs. Tregs have high intracellular levels of cyclic adenosine monophosphate (cAMP), a known inhibitor of T cell growth, differentiation, and proliferation and can transfer cAMP to conventional T cells through gap junctions.[40,41] Moreno-Fernandez et al. concluded that Tregs inhibit conventional T cell activation via a mechanism involving cAMP, which in turn reduces HIV infection.[38]

In this study, the frequency of naïve T cells increased significantly, whereas that of effector memory T cells decreased after ART. Changes in CD4 T cell frequencies were not significant; however, changes in CD8 T cell frequencies were significant. The frequency of central memory T cells was not significantly different before and after ART for either CD4 or CD8 T cells. These changes in T cell subsets have also been reported in other studies.[3,42-44] During progressive HIV infection, naïve T cells are preferentially targeted, resulting in a marked decrease in their frequency.[45] The process of immune recovery in ART-treated patients induces a sustained increase in naïve T cells.[46] The decrease in effector memory T cell subsets, which may play an important role in the direct killing of target cells, may result from the migration of these cells from the blood to lymphoid tissues or from the decrease in HIV antigens after ART.[47]

Our study had several limitations. First, the sample size available for this study is quite small to show meaningful differences in Tregs and plasma cytokine levels before and after ART. Second, the timing of sample collection after ART was inconsistent and the median duration of ART was 169 days. An accurate comparison would require the analysis of sequential samples collected at regular intervals over a longer period after ART. Furthermore, in the in vitro T cell culture model experiment, Tregs and Tcons were purified from the PBMCs of only one HIV-infected patient. Therefore, it is difficult to

generalize the results of this study. Additionally, when Tregs and Tcons were purified using MACS, additional flow cytometry was not performed to determine the purity of the isolates. Finally, when Tregs or Tcons were co-cultured with TZM-bl cells, it is possible that some of the injected pseudoviruses infected Tregs or Tcons rather than the TZM-bl cells. This may have contributed to the decrease in the RLU measured in TZM-bl cells as the number of Tregs and Tcons increased. To confirm this, the env-pseudotyped viral load in added Tregs or Tcons should be measured, which was not performed in this study. Despite these limitations, this study is significant because it is the first in Korea to identify changes in the frequency of Tregs in patients with HIV before and after ART. While we were able to confirm that a high Treg frequency is associated with a detrimental role in chronic HIV infection, as shown in many studies, we were also able to confirm the ambivalent and complex aspects of Tregs in HIV infection using the T cell culture model and TZM-bl assay.

V. CONCLUSION

The critical role of Tregs in the pathogenesis and control of HIV makes them interesting targets for manipulation in the search for a cure. Overall, Tregs play a detrimental role in chronic HIV infections by inhibiting specific immune responses, thereby reducing the immunological control over HIV-infected cells and causing uncontrolled viral infections. Treg depletion directly targeted at a small fraction of the reservoir—which may inhibit reactivation of the virus and boost cell-mediated immune responses—may be a desirable therapeutic strategy for HIV cure research. However, the Treg-mediated reduction in HIV replication could play a beneficial role, particularly during early HIV infections when effector immune cells are not yet activated, or by limiting infection to sites of high virus activation. For instance, one might consider modulating an increase in Tregs to prevent HIV infection in HIV-exposed uninfected individuals, including preventing vertical transmission of HIV-1.[48,49] However, early interference with the Treg suppressive function could exacerbate HIV infection.[50] Therefore, careful consideration should be given to the appropriate timing for using a Treg depletion strategy.

In conclusion, an inverse relationship between Treg frequency and CD4 T cell count was observed in ART-naïve patients, which disappeared with successful ART. In contrast, it was also revealed in the TZM-bl assay that Tregs may play a role in inhibiting HIV replication in infected cells. Therefore, both aspects of Tregs need to be considered when designing Treg modulation strategies for the treatment of HIV infection. More detailed analyses are required to understand the changes in Treg frequency, phenotype, and tissue distribution as a function of HIV infection phases.

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ABSTRACT (IN KOREAN)

HIV 감염에서 항레트로바이러스 치료에 따른 조절 T 세포의 변화 및 조절 T 세포가 HIV 복제에 미치는 영향

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사람면역결핍바이러스(HIV) 감염에서 조절 T 세포(Treg)의 역할과 그 역학은 아직 명확하게 밝혀지지 않았다. 이 연구는 HIV에 감염된 환자에서 항레트로바이러스 치료(ART)에 따른 Treg의 빈도 변화 및 Treg의 빈도와 관련된 인자를 평가하고 Treg이 HIV 복제에 미치는 영향을 평가하기 위해 수행되었다. ART 경험이 없는 환자 17명과, 3개월 이상 ART를 받고 HIV가 억제된 환자 20명을 대상으로 하였다. 연구 대상자의 혈액 샘플에서 Treg을 포함한 여러 T 세포 하위 집합의 빈도와 사이토카인 레벨을 측정하였고, Treg의 빈도와 관련된 요인을 확인하였다. 이 중 13명의 환자는 ART 전후에 순차적으로 샘플이 채취되었고, ART에 따른 Treg 및 T 세포 하위 집합의 빈도 변화를 분석하였다. T 세포 배양 모델에서 TZM-bl 세포에 Env-pseudovirus를 감염시킨 후, Treg 혹은 Treg이 아닌 일반 T 세포와 공동 배양 조건에서 HIV-1 Tat-regulated Luc reporter 유전자 발현의 상대적 광단위(RLU)를 측정하였다.

13명의 환자를 대상으로 ART 전후의 샘플을 순차적으로 수집하여 분석한 결과, ART 후 CD4 T 세포와 naïve T 세포의 빈도는 유의하게 증가한 반면, effector memory CD8 T 세포의 빈도는 유의하게 감소하였다. 사이토카인에 관해서는 ART 후 INF-α2가 유의하게 감소한 것을 제외하고 ART 전후 다른 사이토카인 레벨의 의미있는 차이는 없었다. ART를 받지 않은 환자에서, Treg의 빈도와 CD4 T 세포 수 ($r= -0.595$, $p= 0.012$) 및 CD8 T 세포 수($r= -0.517$, $p= 0.034$) 간에 유의한 음의 상관관계가 확인되었다. 선형 회귀 모델에서는

ART를 받지 않은 환자에서 CD4 T 세포 수만이 Treg 빈도와 음의 상관관계가 있는 것으로 확인할 수 있었다. (β-coefficient= -0.511, p= 0.039). 그러나 ART 후 바이러스가 억제된 환자에서는 Treg 빈도와 상관관계가 있는 요인이 없었다. T 세포 배양 모델에서는 추가되어 배양된 Treg의 수가 증가할수록 RLU 감소 정도가 증가했으며, 10^4개 이상의 Treg이 추가되었을 때 같은 양의 Treg이 아닌 일반 T 세포가 추가된 경우보다 RLU 감소가 더 크게 측정되었다.

이 연구에서는 ART를 받지 않은 환자에서 Treg 빈도와 CD4 T 세포 수 사이의 음의 상관관계가 확인되었지만, ART를 받고 바이러스가 억제되면 이러한 관계는 확인되지 않았다. 반면에 Treg은 표적 세포에서 HIV의 복제를 조절하는 역할을 할 수 있음을 확인하였다. HIV 감염을 치료하기 위한 Treg 조절 전략을 설계할 때는 이러한 HIV 감염에서 Treg의 양면적인 특성을 모두 고려해야 한다.

핵심되는 말: 사람면역결핍바이러스, 조절 T 세포, 항레트로바이러스 치료