Increased Turnover of FoxP3^{high} Regulatory T Cells Is Associated With Hyperactivation and Disease Progression of Chronic HIV-1 Infection

Shaojun Xing, PhD,† Junliang Fu, MD, PhD,† Zheng Zhang, MD, PhD,† Yingying Gao, MD,† Yanmei Jiao, MD, PhD,‡ Fubiao Kang, MD,† Jiyuan Zhang, PhD,† Chunbao Zhou, PhD,† Hao Wu, MD, PhD,‡ and Fu-Sheng Wang, MD, PhD*†*

Objectives: To characterize the homeostasis of CD4$^+$FoxP3^{high} regulatory T cells (Treg) and its association with immune hyperactivation in the disease progression of chronic HIV-1 infection.

Design: Treg proliferation and apoptosis markers were determined and the relation to disease progression and Treg activation was analyzed.

Methods: Fifty-six HIV-1–infected highly active antiretroviral therapy (HAART)–naive subjects and 17 HAART-treated subjects were enrolled. Proliferation and apoptosis of Treg from peripheral blood were evaluated by intracellular Ki-67 and active caspase-3 or surface Annexin-V staining. T-cell activation markers, CD38 and HLA-DR, were simultaneously monitored. The effects of in vitro TCR (T cell receptor) stimulation on proliferation, apoptosis, and activation of Treg were determined from both HIV-1–infected subjects and healthy controls.

Results: HIV-1–infected patients displayed increased Treg turnover status indicated by higher expression of proliferation marker Ki-67 and apoptosis marker active caspase-3 and Annexin-V. Turnover level of Treg was positively associated with disease progression and immune hyperactivation. In vitro TCR stimulation increased the turnover level of Treg. The HAART treatment decreased the turnover and activation levels of Treg in complete responders.

Conclusions: Turnover level of Treg was increased in HIV-1–infected subjects, which is associated with immune hyperactivation and the disease progression, and may serve as a surrogate marker to predict HIV-1 disease progression.

Key Words: pathogenesis, homeostasis, regulatory T cells, FoxP3, HIV-1

(*J Acquir Immune Defic Syndr* 2010;54:455–462)

INTRODUCTION

HIV-1 infection in humans often results in altered T-cell homeostasis,[1] which is characterized by the rapid turnover of both CD4$^+$ and CD8$^+$ T cells. Recent studies have shown that this rapid turnover of T cells mainly include the increased proliferation and apoptosis of T cells, thus leading to the decreased half-life of T cells in chronic HIV-1–infected individuals.[1,2] To date, the mechanisms underlying the rapid turnover of T cells remain unknown for this disease.

Immune activation is the hallmark of chronic HIV-1 infection and has been demonstrated to predict the disease progression of chronic HIV-1 infection[3,4] and the efficiency of highly active antiretroviral therapy (HAART) treatment.[5] Some lines of evidence have shown that the excessive activation induced by chronic HIV-1 infection can consequently lead to rapid turnover of T cells,[6,7] which was proposed to further result in exhaustion and deletion of lymphocytes and contribute to development of AIDS,[8,9] Other findings suggest that the high turnover rates of lymphocytes from HIV-1–infected individuals are caused by either virus-specific T-cell activation or non-specific "bystander" activation.[9]

CD4$^+$CD25^{high}FoxP3^{high} regulatory T cells (Treg) play an important role in the maintenance of immunological tolerance to both self and foreign antigens by suppressing aggressive T-cell responses.[10] However, the immune modulation function of Treg during chronic HIV-1 infection remains debatable.[11] In some studies, Treg were found to suppress specific immune responses to HIV-1 ex vivo, which would suggest that they are detrimental to the host.[12–15] On the other hand, some studies reported that Treg can also slow down the disease progression by suppressing immune activation.[16–19] Notably, when compared with conventional CD4$^+$ T cells, Treg showed higher turnover rates in vivo in healthy subjects without any overt immune activation.[20,21] However, it remains unknown whether Treg turnover level and homeostasis are altered as a result of host immune hyperactivation during chronic HIV-1 infection.

Received for publication January 4, 2010; accepted April 22, 2010.

From the *Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China; †Research Center of Biological Therapy, Beijing Institute of Infectious Diseases, Beijing 302 Hospital, Beijing, China; and ‡Department of Infectious Diseases, Beijing You-An Hospital Affiliated to Capital Medical University, Beijing, China.

Supported by grants from the National Grand Program on Key Infectious Disease (2008ZX10001-006 and 2008ZX10001-002) and supported in part by grants from the National Key Basic Research Program of China (2006CB504205), the National Natural Science Foundation of China (30801040), and Beijing Municipal of Science and Technology Major Project (D0906003040591).

The authors S.X. and J.F. contributed equally to this work.

The authors have no financial conflict of interest.

Correspondence to: Fu-Sheng Wang, MD, PhD, Research Center of Biological Therapy, Beijing 302 Hospital, Beijing100039, China (e-mail: fswang@public.bta.net.cn).

To address the issues mentioned above, this study investigated the homeostasis of peripheral Treg during chronic HIV-1 infection and its relationship with immune hyperactivation. In addition, we found that antiviral therapy is able to temper the activation of Treg and their turnover level. Therefore, our study not only identifies the associations between immune activation and the homeostasis of Treg but also provides a novel understanding of the immune pathogenesis mediated by Treg.

METHODS

Subjects

Fifty-six HIV-1–infected individuals were enrolled in our study, including 8 long-term nonprogressors (LTNP, who had a duration of infection of more than 10 years, a peripheral $CD4^+$ T-cell count exceeding 500 cells/μL, no use of antiretroviral drugs, and a plasma viral RNA concentration maintained below 500 copies/mL), 30 typical progressors (TP, who were antiviral treatment naive and had typical progressive disease, peripheral $CD4^+$ T-cell counts 200∼500 cells/μL, and levels of plasma viral RNA exceeding 1000 copies/mL), and 18 AIDS patients (who had an AIDS-defining condition according to the World Health Organization classification, including progressive decline in peripheral $CD4^+$ T-cell counts < 200 cells/μL and plasma viral RNA ≥ 1000 copies/mL without receiving antiviral treatment, present or previous opportunistic infections). In addition, 12 complete responders (CRs) to HAART (who exhibited effective viral suppression with plasma HIV-1 RNA below 500 copies/mL) and 5 nonresponders (NRs) to HAART (who showed failure to suppress viral replication) were also enrolled in the study. The HAART regimen included 2 nucleoside reverse transcriptase inhibitors plus 1 nonnucleoside reverse transcriptase inhibitor. Eighteen age-matched and gender-matched uninfected healthy subjects were employed as healthy control (HC). The study protocol was approved by the Ethics Committee of our unit, and written informed consent was obtained from each subject.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation from heparinized blood samples. $CD4^+CD25^{high}CD127^{low}$ Treg were isolated from PBMCs by CD4 negative selection followed by CD25 positive selection, using a $CD4^+CD25^+CD127^{dim/-}$ T-cell isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) with MidiMACS and MiniMACS separator units according to the manufacturer's instructions. The purity of $CD4^+CD25^{high}CD127^{low}$ Treg was $> 90\%$ for each subject group, as determined by flow cytometric analysis. Isolated $CD4^+CD25^{high}CD127^{low}$ Treg were incubated with 5 μM CFSE (5- and 6- carboxyfluorescein diacetate succinimydyl ester) for 10 minutes at 37°C in phosphate-buffered saline containing 0.1% BSA. Labeling was quenched with RPMI1640 containing 10% fetal calf serum on ice for 5 minutes, and cells were washed twice with phosphate-buffered saline. Then CFSE-labeled $CD4^+CD25^{high}CD127^{low}$ Treg were seeded at 2×10^6 cells per milliliter in RPMI 1640 containing 10% fetal calf serum in the presence of 2×10^6 cells per milliliter feeder cells (CD4-depelted PBMCs) with or without anti-CD3 (1μg/mL) stimulation for 48 hours.

Flow Cytometric Analysis

The monoclonal antibody (mAb) FoxP3 (Clone: PCH101) was purchased from eBiosciences (San Diego, CA). All other monoclonal antibodies (mAbs) including Ki-67-FITC/PE, active caspase-3-PE, CD38-APC, HLA-DR-PerCP, CD4-PE-Cy7 were purchased from Becton Dickinson (San Jose, CA). For FoxP3 staining, the cells were surface stained and then permeabilized and fixed using eBioscience fix/perm (eBiosciences) according to the manufacturer's instructions. After permeabilization, the cells were incubated with anti-FoxP3 mAbs for 30 minutes. For active caspase 3 staining, the cells were permeabilized and fixed using fix/perm (BD Pharmingen, San Diego, CA), then active caspase-3 staining was performed according to the manufacturer's instructions. Acquisition of data was performed using Becton Dickinson FACSCalibur for 4-color staining protocols or using a Becton Dickinson FACSAria cytometer for 5-color protocols. At least 20,000 $CD4^+$ T cells were acquired per sample. Gating was performed on positively stained samples in comparison with that stained with matched isotype control. Data were analyzed using the FlowJo program v5.7.2 (Tree Star, Ashland, OR).

Apoptosis Assay

Apoptosis was determined by labeling with Annexin-V as described previously.[22] Briefly, freshly isolated PBMCs or cultured T cells were first incubated with mAbs to surface antigens; after washing, cells were suspended in calcium (Ca^{2+})-binding buffer and incubated with 5 μL of Annexin-V for 15 minutes at room temperature and then immediately acquired on a FACSCalibur.

Plasma HIV-1 RNA Monitoring

The 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) was used to quantify HIV-1 RNA levels in plasma samples in our laboratory according to a previously described protocol.[22] The cut-off value for positive detection was 500 copies per milliliter.

Statistical Analysis

Data analysis was performed with SPSS version 13.0 for Windows software (SPSS Inc, Chicago, IL) and expressed as mean ± standard deviation for percentages. The statistical significance of differences between the 2 groups was determined using the nonparametric Mann–Whitney U test. Multiple comparisons were performed with the nonparametric Kruskal–Wallis H test applied with Bonferroni step down (Holm) correction. The Wilcoxon signed ranks test was used for the 2 related samples test. Spearman correlation analysis was performed between 2 parameters. $P < 0.05$ was considered to represent a significant difference.

RESULTS

Rapid Turnover of Treg Increases With Disease Progression During Chronic HIV-1 Infection

In general, Ki-67 is used as a surrogate marker for T-cell proliferation in HIV-1 infection[4,23–25], whereas active caspase-3

is a key effector kinase during apoptosis[26,27] and is often used as an intracellular marker of cellular apoptosis.[28] In this study, $CD4^+CD25^{high}FoxP3^{high}$ T cells were defined as Treg. First, we monitored intracellular Ki-67 and active caspase-3 expression on Treg (Fig. 1A). In addition, Annexin-V staining was performed to measure the apoptosis status of $CD4^+$ $CD25^{high}CD127^{low}$ Treg (Fig. 1B). As shown in Figures 1C–E, $CD25^{high}FoxP3^{high}$ Treg displayed higher levels of Ki-67, caspase-3, and Annexin-V expression than those of $CD4^+CD25^{low}FoxP3^{low}$ and $CD4^+CD25^{low/-}FoxP3^-$ T cells (non-Treg) regardless of disease status, indicating that Treg have increased turnover level when compared with non-Treg. And HIV-1–infected subjects displayed a significant increase in expression of Ki-67, caspase-3, and Annexin-V molecules on both Treg and non-Treg compared with HCs (Fig. 1C–E). Furthermore, there were higher levels of Ki-67, caspase-3, and Annexin-V expression in Treg from AIDS patients than TP and LTNP, and all these markers were also significantly elevated in TP compared with LTNP (Fig. 1C–E). Although the progressive elevation of these markers occurred in both Treg and non-Treg in these HIV-1–infected subjects, more significant changes were observed in Treg. These data indicate that chronic HIV-1 infection leads to a rapid turnover level in overall $CD4^+$ T cells, particularly for Treg, indicated by high levels of proliferation and apoptosis, and this turnover level is further accelerated with the disease progression.

Rapid Turnover of Treg is Associated With Immune Activation During HIV-1 Infection

To investigate the association between activation level and turnover level in Treg in these HAART-naive HIV-1–infected subjects, we detected CD38 and HLA-DR expression on Treg. We found that there was a significant inverse correlation between $CD4^+$ T-cell counts and percentages of CD38 ($r = -0.470$; $P = 0.024$) and HLA-DR ($r = -0.487$; $P = 0.019$) on Treg (Fig. 2A). In addition, there was a positive correlation between plasma viral load with CD38 expression ($r = 0.439$; $P = 0.036$) and HLA-DR expression of Treg ($r = 0.449$; $P = 0.032$) (Fig. 2B). These data suggested that activation of Treg increased with disease progression. Notably, our study showed that a vast majority of Ki-67^+ and active caspase-3^+ Treg displayed an activation phenotype with high CD38 and HLA-DR expression (Fig. 2C, D). Further analysis showed that CD38 and HLA-DR expression on Treg was positively correlated with their increased turnover level in

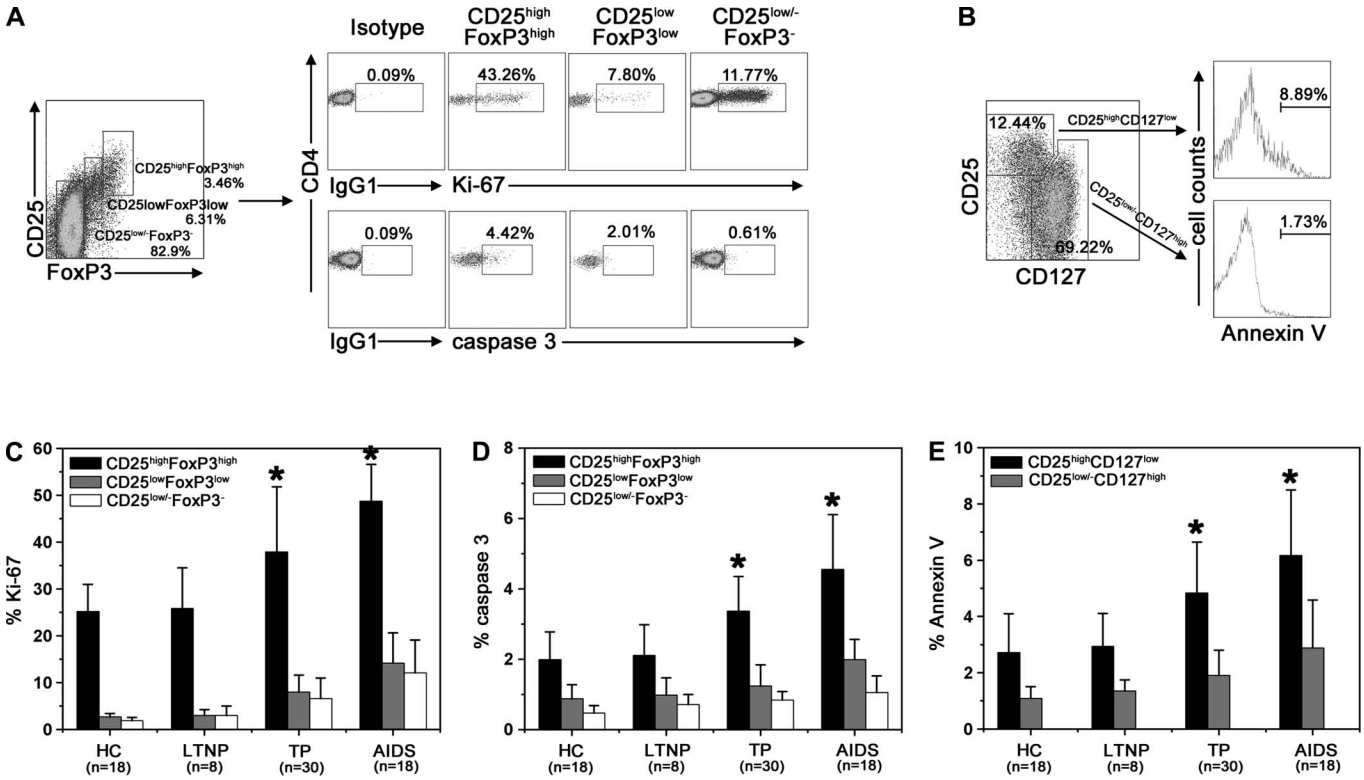

FIGURE 1. Proliferation and apoptosis of Treg increase with disease progression during chronic HIV-1 infection. Representative flow cytometric plots show higher Ki-67 and active caspase-3 expression on $CD4^+CD25^{high}FoxP3^{high}$ Treg (A), and increased Annexin-V expression on $CD4^+CD25^{high}CD127^{low}$ Treg (B) compared with non-Treg in HIV-1–infected subjects. Treg expressed significantly increased Ki-67 (C), active caspase-3 (D) and Annexin-V (E) compared with non-Treg in all patient groups and HCs. Expressions of Ki-67, active caspase-3, and Annexin-V on Treg of TP patients were significantly higher than LTNP patients and HCs, and AIDS patients were much higher than TP patients. There was no significant difference between LTNP patients and HCs. Case numbers for each group are given in Table 1. Data are shown as mean ± SD. *$P < 0.05$.

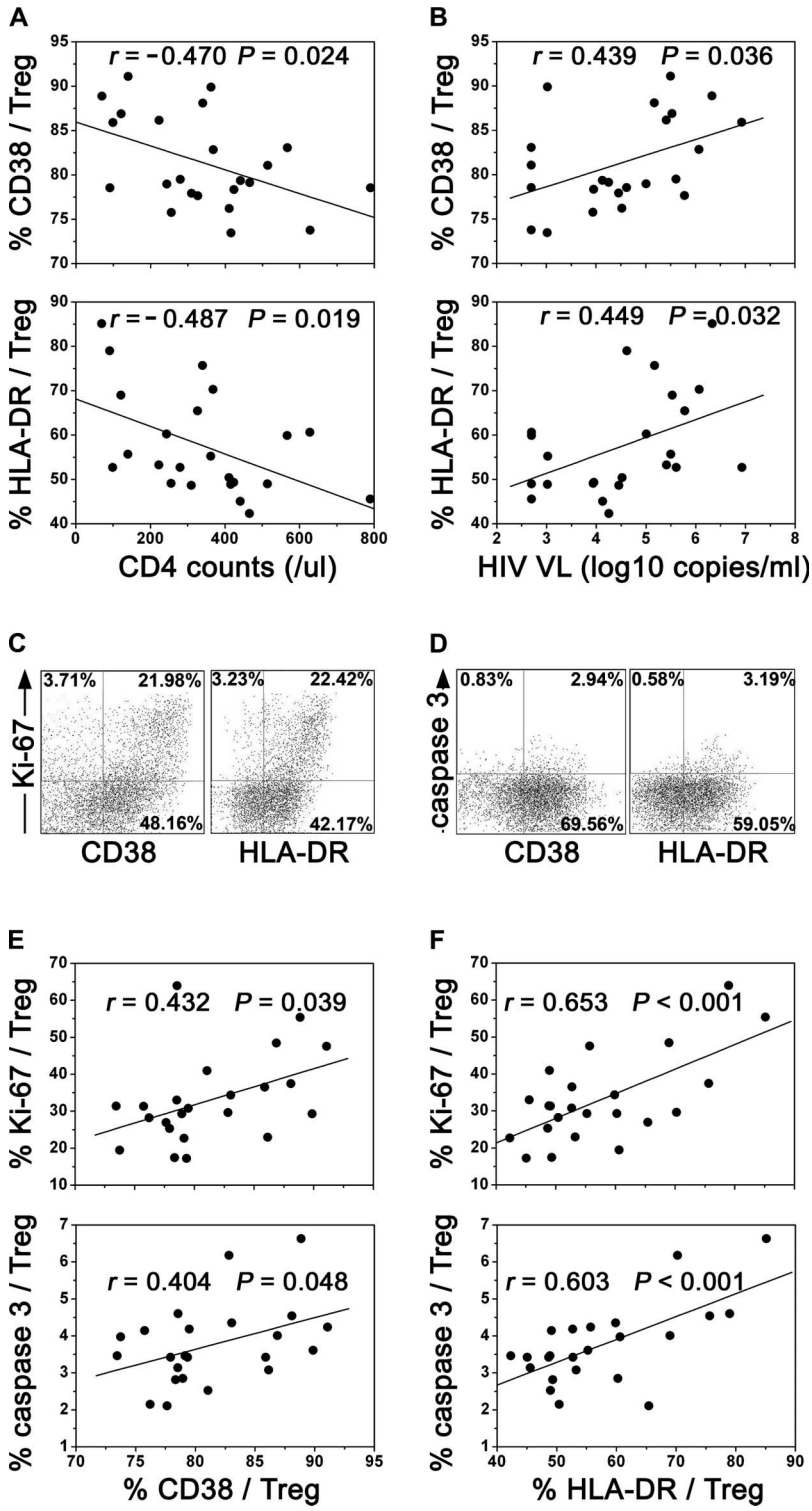

FIGURE 2. Treg exhibit increased activation, proliferation and apoptosis markers during chronic HIV-1 infection. CD38 and HLA-DR expression on $CD4^{+}FoxP3^{high}$ Treg correlated with CD4 counts (A) and viral load (B). Representative data from one HIV-1–infected subject (CD4 T cells absolute counts: 420 cells/μL; plasma viral load: 1320 copies/mL) showed that most Ki-67^{+} (C) and active caspase-3^{+} (D) Treg express CD38 and HLA-DR (cells in the figures were gated on $CD4^{+}FoxP3^{high}$ T cells). CD38 (E) and HLA-DR (F) expression on Treg associated with Ki-67 and active caspase-3 expression on Treg (n = 23, all subjects were HAART naive).

these HIV-1–infected subjects. We found that CD38 and HLA-DR expression on Treg was positively correlated with expression of the proliferation marker Ki-67 ($r = 0.432$; $P = 0.039$ and $r = 0.653$; $P < 0.001$, respectively; Fig. 2E upper panel and Fig. 2F upper panel) and apoptosis markers active caspase-3 expression on Treg ($r = 0.404$; $P = 0.048$ and $r = 0.603$; $P < 0.001$, respectively; Fig. 2E lower panel and Fig. 2F lower panel). The comprehensive correlation analysis indicated that the rapid turnover of Treg was associated with immune hyperactivation in the HIV-1–infected subjects.

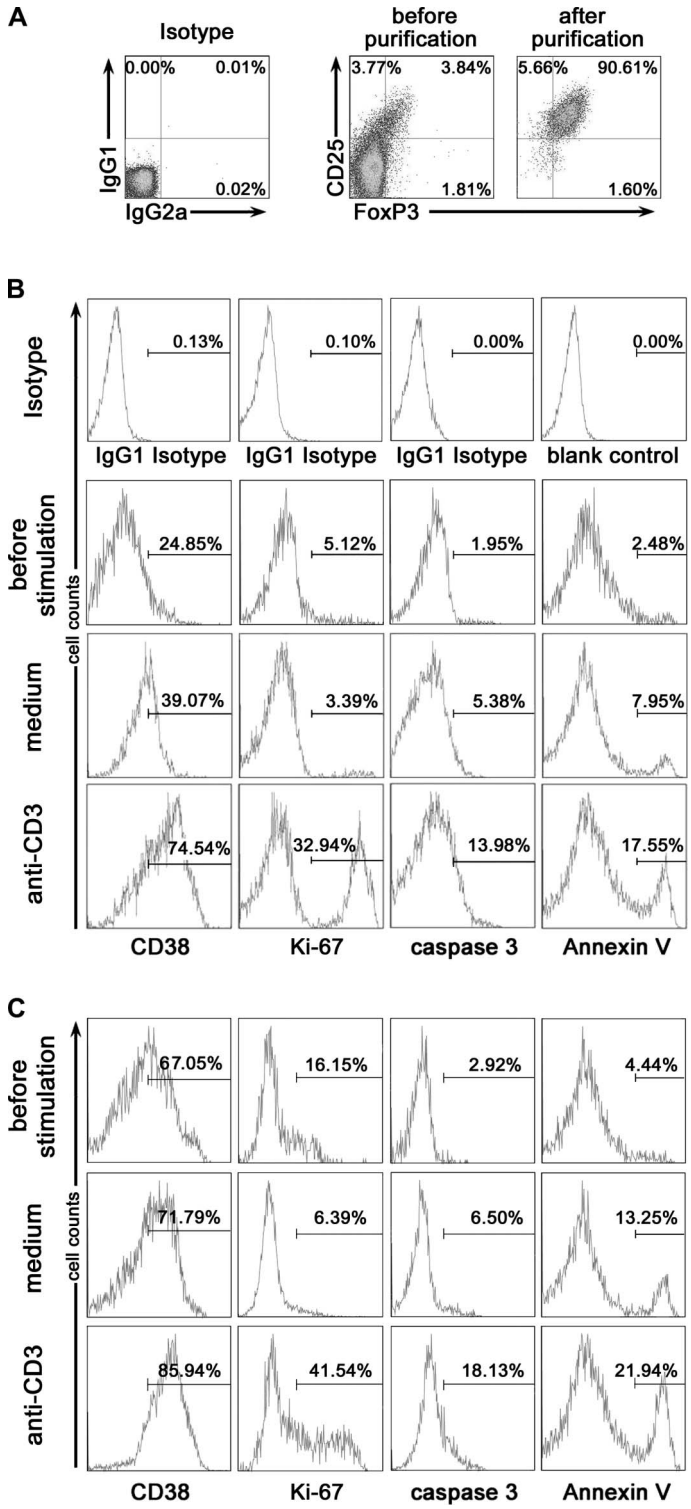

FIGURE 3. Anti-CD3 activation induces higher frequency of Ki-67, active caspase-3, and Annexin-V on Treg from HCs and HIV-1–infected subjects. A, The representative figure showed that the purity of $CD4^+$ $CD25^{high}FoxP3^+$ Treg from 1 HC was >90%. CFSE-labeled $CD4^+CD25^{high}CD127^{low}$ Treg were cultured for 48 hours in the presence of 2×10^6 cells per milliliter feeder cells (CD4-depelted PBMC) with or without 1 μg/mL anti-CD3. Annexin-V staining was performed at 24 hours and directly detected ex vivo. CD38, Ki-67, and active caspase-3 expression on Treg and apoptosis of Treg were significantly upregulated after anti-CD3 stimulation compared with Treg cultured in medium in HC (B) and HIV-1–infected subject (CD4 T cells absolute counts: 411 cells/μL; plasma viral load: 33200 copies/mL) (C). The data are representative of 3 independent experiments with similar results.

In Vitro TCR-Induced Hyperactivation is Accompanied by a Rapid Turnover of Treg

Considering the correlations observed above, we proposed that increased Treg turnover level may result from hyperactivation in HIV-1 infection. To validate this hypothesis, purified $CD4^+CD25^{high}CD127^{low}$ Treg (The purity of $CD4^+CD25^{high}FoxP3^+$ Treg was > 90%, Fig. 3A) from HCs and HIV-1 patients were stimulated with or without anti-CD3 antibodies for 48 h in vitro in the presence of feeder cells. The data showed that anti-CD3 stimulation could upregulate CD38

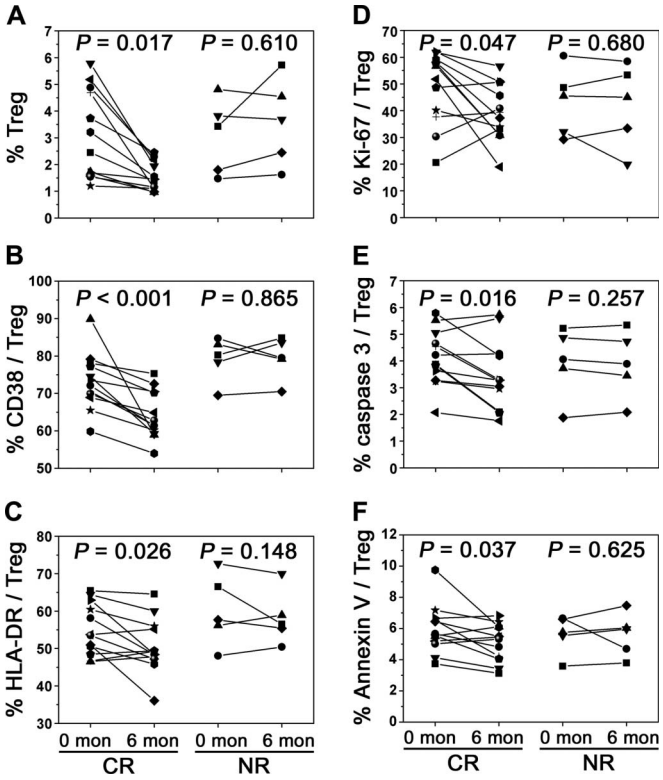

FIGURE 4. Viral suppression by antiviral therapy significantly reduces activation and proliferation and apoptosis markers of Treg. A, Treg frequency decreased after 6 months of HAART treatment in CRs but not in NRs. B and C, HAART treatment reduced CD38 and HLA-DR expression on Treg in CRs but not in NRs. D, E, and F, Ki-67, active caspase-3 on Treg, and apoptosis of $CD4^+CD25^{high}CD127^{low}$ Treg were significantly decreased in CRs but not in NRs. Data are shown as mean ± SD. *$P < 0.05$. CRs (n = 12); NRs (n = 5).

expression on $CD4^+CD25^{high}CD127^{low}$ Treg compared with medium treatment only (Fig. 3B left panel and Fig. 3C left panel). Along with elevated activation, proliferation marker Ki-67 expression and apoptosis marker active caspase-3 of Treg were simultaneously increased after 48 hours incubation (Fig. 3B middle panel and 3C middle panel). In addition, Annexin-V positive staining also increased after 24 hours stimulation (Fig. 3B right panel and Fig. 3C right panel). These data indicated that anti-CD3–induced activation can promote the turnover level of Treg and also supported the notion that HIV-1 infection induced immune hyperactivation may increase the turnover level of Treg.

Viral Suppression by Antiviral Treatment Decrease the Frequency and Turnover Level of Treg

The effect of HAART treatment on the activation and homeostasis of Treg remains unknown.[29–31] We found that the frequency of peripheral Treg in CRs significantly decreased after 6 months of HAART treatment compared with before treatment (3.137 ± 1.660% vs. 1.547 ± 0.536%, $P = 0.017$) but unchanged in NRs (3.066 ± 1.405% vs. 3.602 ± 1.630%, $P = 0.610$) (Fig. 4A). Along with the reduction of Treg frequency in these responders, CD38 and HLA-DR expression on Treg also decreased after HAART treatment (73.264 ± 7.560% vs. 64.286 ± 6.462%, $P < 0.001$ for CD38; 55.144 ± 6.910% vs. 50.662 ± 7.381%, $P = 0.026$ for HLA-DR, respectively) (Fig. 4B, C). Meanwhile, the NRs displayed unaltered expression of CD38 and HLA-DR expression on Treg (79.188 ± 5.938% vs. 79.514 ± 5.610%, $P = 0.865$; 60.214 ± 9.557% vs. 58.252 ± 7.242%, $P = 0.148$, respectively) (Fig. 4B, C). More importantly, we found that turnover level of Treg also significantly decreased in responders but not in NRs. Expressions of the proliferation marker Ki-67 and apoptosis marker active caspase-3 on Treg were significantly reduced in these CRs, respectively, after 6 months of HAART treatment (48.688 ± 13.558% vs. 39.051 ± 10.590%, $P = 0.047$ for Ki-67; 4.141 ± 1.051% vs. 3.460 ± 1.285%, $P = 0.016$ for active caspase-3) (Fig. 4D, E). By contrast, the NRs still maintained high Treg turnover level during the 6-month treatment period (43.180 ± 12.787% vs. 42.016 ± 15.584%, $P = 0.680$ for Ki-67; 3.950 ± 1.305% vs. 3.898 ± 1.253%, $P = 0.257$ for active caspase-3) (Fig. 4D and 4E). In addition, the apoptosis of Treg indicated by Annexin-V staining also decreased after HAART treatment in the CRs

TABLE 1. Characteristics of Subjects in the Study

Subjects	HC	LTNP	TP	AIDS	CR to HAART	NR to HAART
Cases (n)	18	8	30	18	12	5
Age (yrs)	36 (28–50)	38 (35–61)	44 (32–59)	42 (30–55)	40 (30–59)	45 (40–55)
Gender (m/f)	10/8	3/5	16/14	10/8	7/5	3/2
CD4 counts (cells/μL)	NA	609 (507–796)	302 (192–496)	146 (82–196)	171 (71–348)*	214 (107–335)*
					358 (195–606)†	263 (115–385)†
Plasma HIV RNA (copies/mL)	NA	<500	114150 (1240–6,033,019)	153457 (50,700–8,555,187)	127,261 (25,257–1,617,993)*	288,388 (81049–754,608)*
					<500†	312,322 (3117–711,064)†

Data are expressed as the median (range), unless otherwise stated.
*Baseline data before HAART treatment.
†After 6 months of HAART treatment.
NA, not applicable.

(5.945 ± 1.572% vs. 5.026 ± 1.296%, $P = 0.037$), but not in NRs (5.113 ± 1.193% vs.5.3936 ± 1.398%, $P = 0.625$) (Fig. 4F). These data indicate that efficient antiviral therapy significantly decrease the hyperactivation and turnover level of Treg in HIV-1–infected subjects.

DISCUSSION

Previous studies demonstrated that Treg, largely expanded in HIV-1 infection, can inhibit immune responses to HIV-1 and thus favor the pathogen persistence.[12–15] However, little information is available regarding the homeostasis of Treg and relevant mechanisms in chronic HIV-1 infection. The present study demonstrated that Treg displayed rapid turnover level, which are associated with immune hyperactivation and disease progression of HIV-1 infection. HAART treatment efficiently decreased the Treg turnover level in a cohort of CRs but not in NRs. These data characterized the homeostasis of Treg in HIV-1–infected subjects and highlighted a novel role of Treg in HIV-1–induced immune pathogenesis.

Treg, defined as $CD4^+CD25^{high}FoxP3^{high}$ T cells in this study since FoxP3 is a reliable marker of Treg,[32,33] show a memory and activation status in both healthy adults[10] and HIV-1–infected subjects.[34] In this study, we found that Treg display a rapid turnover level indicated by the increased expression of Ki-67 (a proliferation marker[29]) and active caspase-3 and phenotypic Annexin-V staining (apoptosis markers) ex vivo in HIV-1–infected subjects. This finding is consistent with previous observations in healthy adults in whom Treg were also replenished by rapid turnover of memory population in vivo.[20] Notably, this rapid turnover of Treg was found to be associated with disease progression of HIV-1 infection in this study. On the one hand, the turnover level of Treg was progressively increased in TP subjects and AIDS patients but was maintained at relatively low levels in LTNP. On the other hand, it positively associated with expression of activation markers, CD38 and HLA-DR, which are also recognized markers for HIV disease progression.[3,35] This difference in the turnover level of Treg may reflect the variation in immune responses between LTNP and TP.[36] Thus, this study characterized the homeostasis of Treg in HIV-1–infected subjects and extends the notion that the turnover level of Treg can serve as a marker of disease progression in HIV-1 infection.

The factors that lead to the rapid turnover status of Treg in HIV-1 infection are still unclear. Previous studies have demonstrated that immune activation might play a key role in acceleration of T cell turnover during HIV-1 infection.[1] Our data supported the notion that the turnover status of Treg in TP and AIDS patients is significantly higher than that in LTNP subjects, suggesting that the host's immune status might largely influence the Treg status. This hypothesis was further supported by the associations between immune hyperactivation induced by HIV-1 infection and the rapid turnover of Treg in the present study. We found that Treg activation was inversely associated with $CD4^+$ T-cell counts and positively with plasma HIV-1 viral load. More important, there is a positive correlation between activation and turnover level of Treg of HIV-1–infected subjects. This association was observed at the cellular level because we determined that Treg highly expressing Ki-67 and active caspase-3 also expressed higher levels of activation marker CD38 and HLA-DR in HIV-1–infected subjects. In addition, in vitro TCR stimulation can also efficiently activate Treg and simultaneously induce high proliferation and apoptosis levels in Treg. These findings raise the possibility that persistent immune hyperactivation may lead to rapid turnover status of Treg during chronic HIV-1 infection.

In this longitudinal study, we found Treg frequency was significantly decreased after 6 months effective HAART therapy. These data were consistent with study of Lim et al[37] and our previous study.[22] However, some studies reported that HAART treatment did not alter or even increased the frequency of Treg.[30,31,38,39] The discrepancy may result from the differences in identification of Treg, follow-up time, and baseline characterization of enrolled patients.[30,31,37–39] In addition to the decreased frequency of Treg, we also found that the turnover level of Treg was significantly decreased in patients undergoing effective HAART treatment along with recovery of activation level of Treg and $CD4^+$ T-cell counts but not in NRs to HAART. Notably, hyperactivation may not be the only cause for accelerated turnover of Treg during HIV-1 infection. In fact, Treg themselves are susceptible to HIV-1 infection as a result of high level activation and rapid turnover,[28,40] which may contribute to the alteration of Treg homeostasis. And cytokines may influence the homeostasis of Treg.[41–43] Thus, future studies should further dissect the factors that influence Treg homeostasis in HIV-1 infection.

Previous studies have shown that Treg are depleted,[17] and their function impaired in progressive HIV-1–infected subjects[14,16] and simian immunodeficiency virus–infected rhesus macaques.[44] However, Treg can still inhibit HIV-1–specific $CD4^+$ and $CD8^+$ T-cell responses in patients with lower levels of plasma viremia and higher CD4: CD8 T cell ratios.[12,45] Combined with these reports, our research indicated that high levels of activation and rapid turnover level of Treg may be responsible for Treg dysfunction during chronic HIV-1 infection.

In conclusion, our study characterized the homeostasis of Treg in HIV-1–infected subjects and found that the rapid turnover of Treg correlated with immune hyperactivation and disease progression of chronic HIV-1 infection. HAART treatment could decrease the activation and turnover level of Treg in CRs. This study further clarified the role of Treg in immune pathogenesis of chronic HIV-1 infection and may have implications for medical management of the disease.

REFERENCES

1. Douek DC, Picker LJ, Koup RA. T cell dynamics in HIV-1 infection. *Annu Rev Immunol*. 2003;21:265–304.
2. Hellerstein M, Hanley MB, Cesar D, et al. Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. *Nat Med*. 1999;5:83–89.
3. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis*. 1999;179:859–870.
4. Hazenberg MD, Otto SA, van Benthem BH, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*. 2003;17:1881–1888.
5. Benito JM, Lopez M, Lozano S, et al. Differential upregulation of CD38 on different T-cell subsets may influence the ability to reconstitute $CD4^+$

T cells under successful highly active antiretroviral therapy. *J Acquir Immune Defic Syndr*. 2005;38:373–381.
6. Douek DC, Betts MR, Hill BJ, et al. Evidence for increased T cell turnover and decreased thymic output in HIV infection. *J Immunol*. 2001;167: 6663–6668.
7. Mohri H, Perelson AS, Tung K, et al. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J Exp Med*. 2001;194:1277–1287.
8. Sieg SF, Bazdar DA, Lederman MM. S-phase entry leads to cell death in circulating T cells from HIV-infected persons. *J Leukoc Biol*. 2008;83: 1382–1387.
9. Grossman Z, Meier-Schellersheim M, Sousa AE, et al. $CD4^+$ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat Med*. 2002;8:319–323.
10. Sakaguchi S. Naturally arising $CD4^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531–562.
11. de St Groth BF, Landay AL. Regulatory T cells in HIV infection: pathogenic or protective participants in the immune response? *AIDS*. 2008;22:671–683.
12. Weiss L, Donkova-Petrini V, Caccavelli L, et al. Human immunodeficiency virus-driven expansion of $CD4^+CD25^+$ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*. 2004;104:3249–3256.
13. Aandahl EM, Michaelsson J, Moretto WJ, et al. Human $CD4^+$ $CD25^+$ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol*. 2004;78:2454–2459.
14. Kinter AL, Hennessey M, Bell A, et al. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med*. 2004;200:331–343.
15. Montes M, Lewis DE, Sanchez C, et al. $Foxp3^+$ regulatory T cells in antiretroviral-naive HIV patients. *AIDS*. 2006;20:1669–1671.
16. Tsunemi S, Iwasaki T, Imado T, et al. Relationship of $CD4^+CD25^+$ regulatory T cells to immune status in HIV-infected patients. *AIDS*. 2005; 19:879–886.
17. Eggena MP, Barugahare B, Jones N, et al. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol*. 2005; 174:4407–4414.
18. Card CM, McLaren PJ, Wachihi C, et al. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis*. 2009;199: 1318–1322.
19. Ndhlovu LC, Loo CP, Spotts G, et al. FOXP3 expressing $CD127^{lo}$ $CD4^+$ T cells inversely correlate with $CD38^+$ $CD8^+$ T cell activation levels in primary HIV-1 infection. *J Leukoc Biol*. 2008;83:254–262.
20. Vukmanovic-Stejic M, Zhang Y, Cook JE, et al. Human $CD4^+$ $CD25^{hi}$ $Foxp3^+$ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest*. 2006;116:2423–2433.
21. Akbar AN, Vukmanovic-Stejic M, Taams LS, et al. The dynamic co-evolution of memory and regulatory $CD4^+$ T cells in the periphery. *Nat Rev Immunol*. 2007;7:231–237.
22. Jiao Y, Fu J, Xing S, et al. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8(+) T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology*. 2009;128:e366–e375.
23. Sachsenberg N, Perelson AS, Yerly S, et al. Turnover of $CD4^+$ and $CD8^+$ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. *J Exp Med*. 1998;187:1295–1303.
24. Hazenberg MD, Stuart JW, Otto SA, et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood*. 2000;95:249–255.
25. Orendi JM, Bloem AC, Borleffs JC, et al. Activation and cell cycle antigens in $CD4^+$ and $CD8^+$ T cells correlate with plasma human immunodeficiency virus (HIV-1) RNA level in HIV-1 infection. *J Infect Dis*. 1998;178:1279–1287.
26. Yue FY, Kovacs CM, Dimayuga RC, et al. Preferential apoptosis of HIV-1-specific $CD4^+$ T cells. *J Immunol*. 2005;174:2196–2204.
27. Herbeuval JP, Grivel JC, Boasso A, et al. $CD4^+$ T-cell death induced by infectious and noninfectious HIV-1: role of type 1 interferon-dependent, TRAIL/DR5-mediated apoptosis. *Blood*. 2005;106:3524–3531.
28. Jiang Q, Zhang L, Wang R, et al. $FoxP3^+CD4^+$ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2-/-gammaC-/- mice in vivo. *Blood*. 2008;112:2858–2868.
29. Bi X, Suzuki Y, Gatanaga H, et al. High frequency and proliferation of $CD4^+$ $FOXP3^+$ Treg in HIV-1-infected patients with low CD4 counts. *Eur J Immunol*. 2009;39:301–309.
30. Gaardbo JC, Nielsen SD, Vedel SJ, et al. 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 anti-retroviral therapy. *Clin Exp Immunol*. 2008;154:80–86.
31. Epple HJ, Loddenkemper C, Kunkel D, et al. Mucosal but not peripheral $FOXP3^+$ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood*. 2006;108: 3072–3078.
32. Roncador G, Brown PJ, Maestre L, et al. Analysis of FOXP3 protein expression in human $CD4^+CD25^+$ regulatory T cells at the single-cell level. *Eur J Immunol*. 2005;35:1681–1691.
33. Wang J, Ioan-Facsinay A, van der Voort EI, et al. Transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells. *Eur J Immunol*. 2007;37:129–138.
34. Rallon NI, Lopez M, Soriano V, et al. 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*. 2009;155:35–43.
35. Liu Z, Cumberland WG, Hultin LE, et al. Elevated CD38 antigen expression on $CD8^+$ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than $CD4^+$ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol*. 1997;16:83–92.
36. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity*. 2007;27:406–416.
37. Lim A, Tan D, Price P, et al. Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. *AIDS*. 2007;21: 1525–1534.
38. Kolte L, Gaardbo JC, Skogstrand K, et al. Increased levels of regulatory T cells (Tregs) in human immunodeficiency virus-infected patients after 5 years of highly active anti-retroviral therapy may be due to increased thymic production of naive Tregs. *Clin Exp Immunol*. 2009; 155:44–52.
39. Andersson J, Boasso A, Nilsson J, et al. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol*. 2005;174:3143–3147.
40. Zaunders JJ, Ip S, Munier ML, et al. Infection of $CD127^+$ (interleukin-7 receptor+) $CD4^+$ cells and overexpression of CTLA-4 are linked to loss of antigen-specific CD4 T cells during primary human immunodeficiency virus type 1 infection. *J Virol*. 2006;80:10162–10172.
41. Zhang H, Chua KS, Guimond M, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of $CD4^+CD25^+$ regulatory T cells. *Nat Med*. 2005;11:1238–1243.
42. Harnaha J, Machen J, Wright M, et al. Interleukin-7 is a survival factor for $CD4^+$ $CD25^+$ T-cells and is expressed by diabetes-suppressive dendritic cells. *Diabetes*. 2006;55:158–170.
43. Vang KB, Yang J, Mahmud SA, et al. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern $CD4^+Foxp3^+$ regulatory T cell development. *J Immunol*. 2008;181:3285–3290.
44. Pereira LE, Villinger F, Onlamoon N, et al. Simian immunodeficiency virus (SIV) infection influences the level and function of regulatory T cells in SIV-infected rhesus macaques but not SIV-infected sooty mangabeys. *J Virol*. 2007;81:4445–4456.
45. Kinter AL, Horak R, Sion M, et al. CD25(+) Regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8(+) T cells in vitro. *AIDS Res Hum Retroviruses*. 2007;23:438–450.

