CD4+CD25+CD127 regulatory cells play multiple roles in maintaining HIV-1 p24 production in patients on long-term treatment: HIV-1 p24-producing cells and suppression of anti-HIV immunity

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S U M M A R Y
Background: A major question when attempting to eradicate and treat HIV-1 infection is how to reactivate latent proviruses. Stimulating HIV-1-specific cytolytic T lymphocytes (CTL) has been shown to facilitate the elimination of the latent viral reservoir after viral reactivation. Regulatory T (Treg) cells are known to be capable of lowering both HIV-specific immunoreactions and general immune activation during HIV-1 infection. It was hypothesized that the depletion of Treg cells could increase the HIV-1-specific cytolytic T lymphocyte response and reactivate HIV-1 p24 production.

Methods: Treg cells were isolated by isolation kit according to the surface marker of Treg cells. Real-time PCR method was used to quantify HIV-1 DNA. P24 antigens in the cell culture supernatant was done by ELISA. Cells activation and HIV specific HIV-1 CD8+ T cells were analyses using a FACS Calibur flow cytometer and CELLQUEST software.

Results: This study included both HIV-infected patients who were antiviral treatment-naïve and patients with sustained viral responses to antiretroviral therapy (ART) for 1 or 5 years. It was found that the HIV-DNA levels in Treg cells were approximately 10-fold higher than those in non-Treg CD4+ cells and that the depletion of Treg cells could enhance the frequency of HIV-1-specific CTL and immune activation after 5 years of effective ART.

Conclusions: CD4+CD25+CD127 regulatory cells play multiple roles in maintaining HIV-1 p24 production in long-term ART patients. Treg cells may be a target for eliminating the latent HIV reservoir after effective long-term ART.

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1. Introduction

Since the discovery of HIV, many classes of antiviral drugs that can limit viral replication to an undetectable level have been developed. These effectively prevent the occurrence of AIDS-related symptoms in infected individuals. Although current antiretroviral therapies can control viral replication, the persistence of latent HIV is a major obstacle to eradicating the virus from an infected individual. In latently infected cells, the integrated provirus is transcriptionally silent,1–3 but is able to produce replication-competent viruses upon activation.4,5 Because of the persistence of a viral reservoir,6,7 life-long antiretroviral therapy (ART) is required, which raises concerns about the adverse effects over decades of therapy, the development of resistance, and the financial burden of long-term treatment. Accordingly, strategies to eradicate HIV-1 from infected individuals are urgently needed.

Efforts have been focused on the possibility of using pharmacological interventions to ‘purge’ latent viruses. However, early studies using interleukin 2 (IL-2) alone or in combination with anti-CD3 antibodies to reactivate latent HIV-1 failed to eliminate the reservoir.1,8–12 Recent studies have focused on identifying small molecules that are able to reactivate latent virus.13 Two US Food and Drug Administration (FDA)-approved drugs, valproic
acid\textsuperscript{14} and suberoylanilide hydroxamic acid (SAHA),\textsuperscript{15–17} can reactivate latent virus in primary cell models and in cells from infected individuals. However, clinical studies of valproic acid, which possesses histone deacetylase (HDAC) inhibitor activity, have failed to show a consistent effect in eliminating the latent reservoir.\textsuperscript{18–23} Although these data indicate that latent HIV proviruses can be forced out of transcriptional silencing, evidence that latently infected resting CD\textsuperscript{+} T cells would be killed upon exposure to reactivating agents is still lacking both in vitro and in vivo.\textsuperscript{23,24} Nonetheless, Shan et al. found that the stimulation of HIV-1-specific cytotoxic T lymphocyte (CTL) responses prior to viral reactivation may be essential for eradicating latent viruses.\textsuperscript{1}

CD\textsuperscript{4+}CD\textsuperscript{25+}CD\textsuperscript{127} regulatory T (Treg) cells have been shown to play an important role in maintaining immunological tolerance to both self and foreign antigens by suppressing aggressive T cell responses.\textsuperscript{25–28} Several studies have reported that during HIV infection, Treg cells contribute to HIV-specific immune dysfunction by limiting HIV-specific immunoreaction,\textsuperscript{29–31} and other studies have indicated that Treg cells reduce immune activation.\textsuperscript{25,32,33} The majority of studies involving patients who were HIV-infected long-term non-progressors (LTNPs) or elite controllers, have reported decreased Treg cell percentages in peripheral blood\textsuperscript{34–37} and rectal mucosa\textsuperscript{38} compared with regular progressors.\textsuperscript{25} Treg cells are susceptible to HIV infection both in vitro\textsuperscript{38–41} and in vivo.\textsuperscript{42–43} Of note, it has been reported that HIV-DNA-harboring cells appear to be more abundant in Treg cells than in non-Treg cells in patients on prolonged ART.\textsuperscript{44} Similar results have been found in the mucosa of simian immunodeficiency virus (SIV)-infected macaques.\textsuperscript{25,45} These data suggest that Treg cells constitute a part of the viral reservoir.

It is important to determine whether the depletion of Treg cells would assist in the eradication of HIV-1 production. It was hypothesized that the depletion of Treg cells would contribute to elevating the frequency of HIV-specific CTL and HIV-1 production, which may benefit latent viral eradication in patients on prolonged ART. To assess this hypothesis, treatment-naïve HIV-infected patients and HIV-infected patients who had received ART for 1 or 5 years with sustained viral responses were studied. It was found that Treg cells constitute an important factor in HIV-1 p24 production and that the depletion of Treg cells enhanced the frequency of HIV-1-specific CTL immune activation, and the depletion of p24 expression after 5 years of effective ART treatment.

2. Materials and methods

2.1. Patients

Thirty adults who were chronically infected with HIV-1 (cared for by the Center for Infectious Diseases, Beijing You’an Hospital, Capital Medical University) were enrolled in this study. No enrolled patient was co-infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). The patients were divided into three groups: 10 AIDS patients who were ART-naïve (Table 1), 10 patients who had received ART for 1 year (ART1) and who exhibited persistent viral suppression with plasma HIV-1 RNA levels below 50 copies/ml after 12 weeks of ART (Table 1), and 10 patients who had received ART for 5 years (ART5) and who exhibited persistent viral suppression with plasma HIV-1 RNA levels below 50 copies/ml after 12 weeks of ART (Table 1). The ART regimen included two nucleoside reverse-transcriptase inhibitors (NRTIs) plus one non-nucleoside reverse-transcriptase inhibitor (NNRTI). CD\textsuperscript{4+} T cell numbers were <200 cells/μl before ART treatment for the ART-treatment patients.

The exclusion criteria included pregnancy, active tuberculosis (TB; defined as suspected TB or being in the first 2 months of anti-TB therapy), and moribund status.\textsuperscript{32} The study protocol was approved by the Beijing You’an Ethics Committee, and written informed consent was obtained from each subject. All of the HIV-infected subjects were serologically identified as having the

Table 1

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ART, antiretroviral therapy; M, male; F, female.
human leukocyte antigen (HLA)-A2+ genotype, which was further confirmed by PCR.46

2.2. Primers, probes, and M13 bacteriophages containing target sequences were used as standards for real-time PCR (qPCR)

The primer and probe sequences for the qPCR assays were optimized and designed to be as similar as possible to both previous reports.17,45 The chemokine receptor CCR5 was chosen as a surrogate for estimating the number of cells, because each cell contains only a single copy of CCR5, as shown previously.49,4

2.3. qPCR quantification of HIV-1 DNA and cellular CCR5

Total DNA from the target cells was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). qPCR was performed in a 25-μl volume containing 2.5 μl DNA target, 12.5 μl Gene Expression Master Mix (Applied Biosystems, 4369016), 1 μM primers, and 0.2 μM probe. The reaction consisted of the following conditions using an ABI 7500 PCR machine (Applied Biosystems): 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min (40 cycles). A single round of qPCR was sufficient for total HIV-1 DNA and the CCR5 gene using the reaction conditions indicated previously.50 For each run, a standard curve was created in a 7-log-unit range by 1:10 serial dilutions of the M13 bacteriophage standard containing the appropriate gene; all samples were performed in duplicate.

2.4. Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque density gradient centrifugation of heparinized blood samples. CD4+CD25+CD127 Treg cells were isolated from PBMCs by CD4 negative selection followed first by CD127 negative selection and then by CD25 positive selection using a CD4+CD25+CD127dim/– isolation kit (Milteny Biotech) with MidiMACS and MiniMACS separator units (Milteny Biotech), according to the manufacturer’s instructions. The purity of CD4+CD25+CD127dim/– Treg cells was >90% for each subject group, as determined by flow cytometry analysis (Figure 1a).

2.5. Cell culture

PBMCs (1 × 10⁶), PBMCs depleted of Treg cells (1 × 10⁶), and Treg cells (2.5 × 10⁵) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10⁵ M 2-mercaptoethanol. After incubation at 37 °C in a 5% CO₂ atmosphere for 72 h, the cells were washed, stained, and analyzed.

2.6. Flow cytometry analysis

All antibodies were purchased from BD Pharmingen (San Diego, CA, USA), except anti-HIV-1 gag p24 KC57-FITC, which was from Beckman Coulter (Fullerton, CA, USA), and phycoerythrin (PE)-labeled HLA-A2 pentamer complexes loaded with the HIV-1 gag p17 epitope (77–85, S19, SLYNTVATL), which were from Prommune (Oxford, UK). PBMCs were isolated from freshly heparinized blood by Ficoll–Hypaque centrifugation (Pharmacia, Uppsala, Sweden).

After 72 h of cell culture, the detection of CD4 cell activation was performed by immunolabeling with PE-anti-HLA-DR, FITC-anti-CD38, PerCP-anti-CD3, and APC-anti-CD4.

For the staining of virus-specific CD8+ T cells, the cells were simultaneously stained after 72 h of culture with anti-CD3-Percp, anti-CD8-allophycocyanin, pentamer-PE, or the corresponding isotype. For the assessment of intracellular p24 gag expression in CD4+ T cells, the cells were harvested after 72 h of cell culture and were first stained with PerCP-anti-CD3 and APC-anti-CD4, and then fixed and stained using the Fix and Perm kit (Caltag) and anti-p24 KC7-FITC antibody, as described previously.51,52 After staining, four-color flow cytometry analyses were performed using a FACSCalibur flow cytometer and CELLQUEST software (Becton-Dickinson, San Jose, CA, USA).

2.7. ELISA

Detection of p24 antigens in the cell culture supernatant was done by ELISA. In brief, 96-well MaxiSorp plates (Nunc, Denmark) were coated with 0.5 mg/ml of anti-Gag p24 antibodies in 15 mM Na₂CO₃, 35 mM NaHCO₃ (pH 9.7), overnight at 4 °C. The plates were blocked in phosphate-buffered saline (PBS) containing 2% (w/v) skimmed milk, and sera were added in serial dilutions. Specific antibodies were detected following incubation with rabbit anti-human IgG1 or IgG2a conjugated to horseradish peroxidase (Zymed/Invitrogen). Development and absorbance reading was done as described above.

2.8. Assays for CD4 T cell counts and plasma HIV-1 RNA levels

T cell counts were determined by three-color flow cytometry using anti-CD3-APC, anti-CD4-FITC, and anti-CD8-PE monoclonal antibodies (BD Bioscience San Diego, CA, USA). The analysis was performed using a BD FACSCount flow cytometer in accordance with the Chinese Center for Disease Control and Prevention (CDC) guidelines. Plasma HIV RNA was quantified by real-time PCR (Roche, Germany). The detection sensitivity of this assay is 40 copies/ml.

Figure 1. HIV-DNA levels are higher in Treg cells than in non-Treg CD4+ cells. (a) Dot plots of flow cytometry results obtained for purified CD4+CD25+CD127 regulatory cells. (b) Comparison of HIV-DNA levels (per 10⁶ cells) between Treg and non-Treg CD4+ cells in treatment-naïve, ART1, and ART5 patients.
2.9. Statistical analysis

Comparisons were performed using non-parametric independent sample tests, and all reported p-values were two-sided and considered significant at $p < 0.05$. All data were analyzed using SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. HIV persistence in Treg cells

To determine whether Treg cells are an important site of HIV persistence, the HIV-DNA levels in Treg and non-Treg CD4+ cells from direct material from 30 HIV-infected patients (10 treatment-naïve, 10 ART1, and 10 ART5 patients) were examined. The results showed a higher level of HIV-DNA in Treg cells than in non-Treg CD4+ cells in all three groups of patients (Figure 1b, $p < 0.001$), with an approximately 10-fold higher HIV-DNA level in Treg cells than in non-Treg cells (Figure 1b). Further, the intracellular HIV-1 p24 levels in PBMCs, PBMCs depleted of Treg cells, and Treg cells after 72 h of cell culture and the levels of p24 antigen in the cell culture supernatant were examined. It was found that there were more p24 antigens in Treg cells than in the other cell types (Figure 2a–c), as well as in Treg cell culture supernatant (Figure 2d, e).

Figure 2. There is more p24 in Treg cells than in non-Treg cell culture supernatant. (a) Representative plots of the percentage of p24 in the PBMC, non-Treg PBMC, and Treg cells after 72 h of culture. Comparison of p24 levels in PBMC, non-Treg PBMC, and Treg cells from (b) ART1 and (c) ART5 patients after 72 h of culture. Comparison of p24 levels in cell culture supernatant of PBMC, non-Treg PBMC, and Treg cells from (d) ART1 and (e) ART5 patients after 72 h of culture. Patients are numbered P11 through P30.
3.2. Depletion of Treg cells facilitates CD4+ T cell activation

Because Treg cells can suppress immune activation, whether the depletion of Treg cells facilitates CD4+ T cell activation after certain periods of ART was next examined (Figure 3a). The results indicated that the depletion of Treg cells induced CD4+ T cell activation in all patients after 72 h of cell culture, regardless of the treatment period (Figure 3b–d). These results support the notion that Treg cell depletion facilitates CD4+ T cell activation.

3.3. Depletion of Treg cells increases the frequency of HIV-specific CD8+ T cells with prolonged ART

Because depletion of Treg cells in HIV-infected individuals would induce immune cell activation, whether the depletion of Treg cells would increase the frequency of HIV-specific CTL after different time periods of ART and thereby improve the control of viral persistence was investigated (Figure 4a). As shown in Figure 4b, four of the 10 treatment-naive patients exhibited an elevated frequency of HIV-specific CTL after depletion of Treg cells, whereas the other six patients exhibited a reduced frequency of HIV-specific CTL after depletion of Treg cells after 72 h of cell culture. The frequencies of HIV-specific CTL were elevated in six of the 10 ART1 patients and all of the ART5 patients upon the depletion of Treg cells after 72 h of cell culture (Figure 4c, d).

4. Discussion

An increasing body of evidence has demonstrated that Treg cells can reduce both HIV-specific immunoreactions29,31 and immune activation during HIV-1 infection.25,32,33 It has also been demonstrated that stimulating HIV-1-specific CTLs and reactivating latent HIV-1 may be essential for the successful eradication of the latent viral reservoir.1 Accordingly, it was hypothesized that the depletion of Treg cells may be beneficial for reducing the HIV-1 DNA levels and decreasing HIV-1 p24 production. In this study, the results showed that the HIV-DNA levels were approximately 10-fold higher in Treg cells than in non-Treg CD4+ cells and that the depletion of Treg cells increased CD4+ cell activation after 5 years of ART. Because HIV should exist primarily as a latent viral reservoir after 5 years of sustained viral response to ART, these findings of higher levels of p24 antigen in Treg cells and in Treg cell culture supernatants indicate that Treg cell depletion may reduce the viral reservoir. Therefore, Treg cell depletion may facilitate the elimination of latent HIV by reducing the viral reservoir and elevating the frequency of HIV-specific CD8+ T cells. Although the results of the present study demonstrate that Treg cells can increase the level of CD4+ cell activation, these data do not indicate that Treg cell depletion can reactivate latent virus. Further studies should be performed to confirm whether Treg cell depletion can reactivate latent virus.

In addition, it has become increasingly clear that the severity of AIDS is associated with chronic immune hyperactivation. Because the immune activation was high in the non-ART and ART1 patients, the depletion of Treg cells may have caused an enhancement of this excessive immune activation.23 However, in the 5-year ART patients, HIV had been suppressed for a very long time, and the excessive immune activation had been controlled to some extent. These results are consistent with those of previous reports that have shown that a low Treg response may contribute to both viral control and generalized immune activation in HIV controllers.35–37,53 Treg cells are Foxp3-positive cells. Bettelli et al. initially described Foxp3 as functionally interacting with and inhibiting the transactivation of the transcription factors nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB).54 Several groups have reported changes in the histone acetylation of certain promoters regulated by Foxp3.38,55,56 Thus, the mechanism by which Treg cell depletion could reactivate latent HIV needs further study.

In this study, it was found that the depletion of Treg cells elevated the frequency of HIV-1-specific CTL, which is consistent with previous publications. A notable report showed that HIV-specific CD8+ T cells carrying protective HLA alleles (HLA B*27 and B*57) were able to evade Treg suppression,57 which suggested that the suppressive activity of Treg cells may be a major mechanism that impairs the development of protective effector responses. It

Figure 3. Depletion of Tregs enhances the activation of CD4+ T cells in patients on prolonged ART. (a) Representative plots of CD38 and HLA-DR expression in CD4+ T cells. Changes in the ratios of the percentages of HLA-DR+CD38+ T cells over total CD4+ cell counts in PBMCs (dots on the left) and depleted Treg cells (dots on the right) in (b) treatment-naive, (c) ART1, and (d) ART5 patients. The y-axis represents the ratios of the percentage of HLA-DR+CD38+ T cells over the total CD4+ cell counts. The patients are numbered P1 through P30.
was also reported that a high perforin-to-Foxp3 ratio was associated with non-progressive disease.\textsuperscript{58} Other reports have shown the ex vivo depletion of Tregs from PBMCs or lymphoid cell suspensions to enhance T cell responses to HIV or SIV antigens.\textsuperscript{29,30,58–62} Furthermore, the stimulation of HIV-1-specific CTLs and the reactivation of latent HIV-1 may be essential for the successful eradication of the latent viral reservoir.\textsuperscript{1} In this study, it was found that Treg cells are important HIV-1 p24-producing cells and the depletion of Treg cells may increase the frequency of HIV-1-specific CTLs and reactive CD4+ cells, thereby enhancing the eradication of the latent viral reservoir. In addition, because only the HLA-A2-positive phenotype can be detected by flow cytometry, cells from HLA-A2-positive phenotype patients were examined by flow cytometry and the CTL detected; however, this does not exclude the possibility that individuals with other HLA phenotypes respond in the same way.

It was found that the HIV-DNA levels were higher in Treg cells than in non-Treg CD4+ cells, which is consistent with the report that HIV-DNA-harboring cells appeared to be more abundant in the Treg subset than among non-Treg cells in patients on prolonged ART.\textsuperscript{44} It was found that the HIV-DNA levels were 10-fold higher in Treg cells than in non-Treg CD4+ cells; in a previous report, this.

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\caption{Depletion of Treg cells increases the frequency of HIV-specific CD8+ T cells in patients on prolonged ART. (a) Representative plots of HIV-specific CD8+ T cells. Changes in the ratios of the percentages of HIV-specific CD8+ T cells over total CD8+ cell counts in PBMCs (dots on the left) and depleted Treg cells (dots on the right) in (b) treatment-naive, (c) ART1, and (d) ART5 patients. The y-axis represents the ratios of the percentage of HIV-specific CD8+ T cells over total CD8+ cell counts. The patients are numbered P1 through P30.}
\end{figure}
difference ranged from 1.5- to 8-fold higher. This variation may be due to racial differences and the different surface markers used. Because the results of the present study, along with those reported in a number of previous publications, suggest that Treg cells could constitute part of the viral reservoir, the depletion of Treg cells may reduce the size of this viral reservoir. The latent reservoir is established at the earliest stage of infection. Furthermore, ART in acutely infected macaques increases SIV-specific viral responses and the capacity of the immune response.53 The transient depletion of Treg cells in transgenic mice reactivated virus-specific CD+ T cells and reduced the retroviral set points.54 Thus, early ART in combination with Treg cell depletion may help preserve immune responses and eliminate the viral reservoir; however, this hypothesis requires further study for confirmation.

In conclusion, CD4+CD25+CD127 regulatory cells play multiple roles in maintaining HIV-1 p24 production in long-term ART patients. The results of this study indicate that the depletion of Treg cells may facilitate the elimination of HIV-1 p24 production by depleting those cells that produce p24 and by increasing the frequency of HIV-specific CD8+ T cells. Whether Treg cell depletion can reactivate latent virus needs further examination. Indeed, Treg cells may be a good target for strategies to eliminate the latent HIV reservoir.

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Conflict of interest: The authors declare that they have no competing interests.

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