

Increased Regulatory T-Cell Percentage Contributes to Poor CD4⁺ Lymphocytes Recovery: A 2-Year Prospective Study After Introduction of Antiretroviral Therapy

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Background. The primary aim of this study was to determine the impact of regulatory T cells (Tregs) percentage on immune recovery in human immunodeficiency virus (HIV)-infected patients after antiretroviral therapy introduction.

Methods. A 2-year prospective study was conducted in HIV-1 chronically infected naive patients with CD4 count <500 cells/mm³. Regulatory T cells were identified as CD4⁺CD25^{high}CD127^{low} cells among CD4⁺ lymphocytes. Effect of Treg percentage at inclusion on CD4 evolution overtime was analyzed using a mixed-effect Poisson regression for count data.

Results. Fifty-eight patients were included (median CD4 = 293/mm³, median Treg percentage = 6.1%). Percentage of Treg at baseline and CD4 nadir were independently related to the evolution of CD4 absolute value according to time: (1) at any given nadir CD4 count, 1% increase of initial Treg was associated with a 1.9% lower CD4 absolute value at month 24; (2) at any given Treg percentage at baseline, 10 cell/mm³ increase of CD4 nadir was associated with a 2.4% increase of CD4 at month 24; and (3) both effects did not attenuate with time. The effect of Treg at baseline on CD4 evolution was as low as the CD4 nadir was high.

Conclusions. Regulatory T-cell percentage at baseline is a strong independent prognostic factor of immune recovery, particularly among patients with low CD4 nadir.

Keywords. ART; immune response; immunological failure; nadir CD4; T-regulatory cells.

The mechanisms sustaining the absence of immune recovery in human immunodeficiency virus (HIV)-infected patients upon effective antiretroviral therapy

(ART) remain elusive [1]. In most studies, 20 to 30 percent of patients are concerned by a discordant response to ART, depending on the definition of the immunological failure [2–5]. In patients with abnormal T lymphocytes CD4⁺ (CD4) count, despite virological success, an increased mortality has been shown and cardiovascular and malignant pathologies are frequently observed [6–9]. Regulatory T cells (Tregs) are known to modulate the host response to viral infections [10, 11], but their role in HIV pathogenesis is still controversial [12, 13].

Some recent cross-sectional studies highlighted the potential deleterious role of these cells in case of suboptimal gain of CD4 count [14–18]. All included patients with many years of suppressive ART and compared the clinical and immunologic characteristics of immune

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responders (ie, with appropriate CD4 recovery) with nonresponders. Therefore, it was unknown whether the elevated Treg percentage observed in nonresponders was a cause or a consequence of the disease duration and CD4 loss before ART introduction. To date, no data are available concerning the impact of Treg on the evolution of CD4 count during the 2 years after ART introduction.

The aim of this study was to prospectively analyze whether Treg percentage measured at ART introduction could be independently associated with lack of immune recovery after 2 years.

MATERIALS AND METHODS

Population

We performed a 2-year prospective cohort study including chronically HIV-1-infected naive patients with CD4 count $<500/\text{mm}^3$ who started ART between January 2011 and March 2012. Patients were included the first day of ART and observed for 2 years. The frequency of follow-up visits and immunovirological control was based on French recommendation [19]. Patients with oncohematological diseases, cancer, and/or recent history of chemotherapy or treatment with hydroxyurea were not included. The following patients were excluded during the 2-year follow-up: (1) patients lost to follow-up; (2) patients experiencing virological failure (ie, 2 successive HIV RNA above 50 cp/mL) [19]; (3) patients with lack of follow-up (ie, less than 4 biological measurements per year) to minimize the possibility of a viral load rebound between 2 measurements impacting the CD4 recovery. CD4, Treg (determined as $\text{CD4}^+\text{CD25}^+\text{CD127}^-$ cells), and viral load were measured at baseline and were prospectively recorded for 1 year after ART introduction. During the second year of follow-up, only CD4 count and viral load were prospectively recorded. Written informed consent was obtained from every subject, and local Ethical Committee approval was received for the study.

Routine Laboratory Assessments

Viral loads were determined after extraction of 0.6 mL plasma using the Abbott Real Time HIV-1 assay under the dedicated m2000rt version 4.0 m2000rt software 4.0 package (quantitation limit 40 HIV RNA copies/mL).

Cellular Immunophenotyping

The absolute count of CD4 and complementary expression of cell surface were assessed on fresh whole EDTA-anticoagulated peripheral blood and measured on a FC500 flow cytometer using the FC500 software version 2 (Beckman Coulter, Hialeah, FL). Lymphocytes were stained with monoclonal antibodies according to manufacturer's recommendation: energy-coupled dye-labeled anti-CD4, phycoerythrin-labeled anti-CD127, PC5-labeled anti-CD25 (Beckman Coulter, Hialeah, FL). Regulatory T cells were determined as $\text{CD25}^+\text{CD127}^-$ cells among total CD4^+ lymphocytes, and results are expressed

as percentages of $\text{CD4}^+\text{CD25}^+\text{CD127}^-$ cells out of CD4^+ lymphocytes. A minimum number of 2000 CD4^+ cellular events was recorded.

Statistical Analysis

Variables were described with medians, first and third quartiles for continuous variables, and counts and percentages for categorical variables. Correlations between CD4 count and Treg were explored with Spearman tests. Impact of Treg percentage on CD4 count evolution along the first 2 years after ART introduction was studied using a mixed-effect Poisson regression. All of the prospectively recorded CD4 measurements per patient were used in the model. Time was introduced as third-order polynomial. Random intercept, slope, and quadratic term for time were included to allow for subject-specific initial CD4 value, slope, and curvature of CD4 evolution through time. Age at treatment onset and nadir of CD4 (ie, the lowest value of CD4 measured before ART introduction) were considered as main confounding variables of Treg percentage and thus were studied. Nonadjusted models were fitted before mutual adjustment. Effect sizes were represented with 95% confidence interval as multiplicative factor on CD4 cell count. Interactions of Treg and CD4 nadir, respectively, with time were tested to detect time-dependent slope of those variables on CD4 count. Interaction between Treg percentage and CD4 nadir was also tested to detect the different effect of one according to the level of the other one. Those interactions were kept in the model when *P* value reached 10%. To illustrate the model, we considered patients according to the level of immunosuppression (nadir $\text{CD4} <$ or $>350/\text{mm}^3$) and Treg percentage ($<$ or $>9\%$). These values approximately correspond to the top quartile of participants based on the CD4 nadir and Treg percentage distribution (Table 1). Results were considered to be significant when *P* values were less than 5%. Analyses were performed with R software (software 3.0.2, 2013; R Development Core Team, R: A Language Environment for Statistical Computing, Vienna, Austria).

RESULTS

Population Description

One hundred one patients were included, and 43 patients were excluded during the follow-up (Figure 1). Finally, 58 patients were observed for 26.2 months (interquartile range [IQR], 24.5–27.3). Characteristics of the population are described in Table 1. Six patients were Centers for Disease Control and Prevention (CDC) Stage C (10%), 10 CDC B (17%), and 42 CDC A (73%). Median zenith viral load was 4.7 (IQR, 4.4–5.2). Half of the patients were infected with HIV-1 B subtype viruses ($n = 30$). Only 2 patients were coinfecting with a hepatitis virus (hepatitis B virus [HBV] = 1, HCV = 1). All patients received a nucleoside reverse-transcriptase inhibitor's backbone

Table 1. Baseline Characteristics of the 58 Naive HIV-1-Infected Patients Studied

Variable	N or Median	% or 1st–3rd Quartiles
Man (%)	47	(81)
Men who have sex with men (%)	41	(70)
Age at baseline, in year (IQR)	39.9	(31.2–47.7)
CDC stage C (%)	6	(10)
CD4 nadir, cells/mm ³ (IQR)	267	(150–325)
Viral Load, log ₁₀ cp/mL (IQR)	4.4	(3.8–5)
Protease inhibitor base regimen (%)	48	(83)
Time to viral indetectability, in days (IQR)	103	(61–168)
Duration of follow up, in months (IQR)	26.2	(24.5–27.3)
CD4, cells/mm ³ (IQR)	293	(161–375)
Treg, cells/mm ³ (IQR)	17,5	(12.2–22)
Treg, % of CD4 (IQR)	6.1	(4.6–8.78)

Abbreviations: CDC, Centers for Disease Control and Prevention; HIV, human immunodeficiency virus; IQR, interquartile range; N, number.

(tenofovir-emtricitabine or abacavir-lamivudine) combined with (i) a protease inhibitor’s based regimen (atazanavir, darunavir or lopinavir, all boosted with ritonavir; n = 48, 82%), or with (ii) efavirenz (n = 5, 9%), or with (iii) raltegravir (n = 5, 9%).

Correlation Between CD4, Regulatory T Cell, and Viral Load at Antiretroviral Therapy Introduction, M12 and M24

At ART introduction, there was a significant positive correlation between Treg percentage and HIV viral load measured at ART introduction (Spearman $\rho = 0.27$; $P = .04$). On the contrary, there was a significant negative correlation between Treg percentage and CD4 nadir count ($R = -0.43$; $P < .001$) and between Treg percentage and CD4 count ($R = -0.47$; $P < .001$).

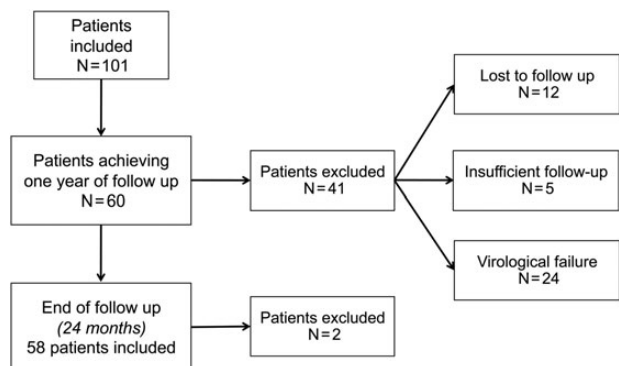


Figure 1. Study flow chart. Inclusion criteria: chronically HIV-1 infected patients; no history of HAART; TL CD4 count <500/mm³; no onco-haematological diseases, cancer, and/or recent history of chemotherapy or treatment with Hydroxyuree. Insufficient follow up: <4 CD4 T lymphocytes measurements per year.

At month 12, the correlation between CD4 count and Treg percentage was lost ($R = -0.33$; $P = .06$).

Impact of Regulatory T-Cell Percentage on CD4 Reconstitution

In the unadjusted models, both percentage of Treg at ART introduction and CD4 nadir were statistically related to the CD4 absolute value at baseline and during follow-up. On average, 1% increase of Treg at baseline was associated with 4.2% (95% confidence interval [CI], 5.9–2.5, $P < .001$) decrease of CD4 absolute value. Similarly, patients with 10/mm³ increase of CD4 nadir had on average 5.7% higher CD4 (95% CI, 5.0–6.4; $P < .001$). There was no significant effect of age on CD4 reconstitution.

In the adjusted model, both Treg percentage at baseline and CD4 nadir were related to the evolution of CD4 absolute value overtime. At any given nadir CD4 count, for each 1% of Treg more at ART introduction, a 1.9% (95% CI, 2.9–0.8, $P < .001$) lower CD4 absolute value is expected at month 24. Likewise, at any given Treg percentage at baseline, a 10/mm³ higher CD4 nadir was associated with 2.4% (95% CI, 1.8–3.0, $P < .001$) higher CD4 absolute value at month 24. For both variables, the effect on CD4 does not significantly attenuate over the 2 years of the follow-up ($P > .05$; Table 2). Regulatory T cell percentage at baseline has different effect size according to the CD4 nadir, ie, it decreased when CD4 nadir increased: the impact of 1% increase of Treg on CD4 evolution was 0.14% lower for each additional 10/mm³ increase of CD4 nadir (Table 2).

Figure 2 shows that CD4 nadir >350/mm³ is a strong prognostic factor of immunological recovery (there were no patients with CD4 nadir >350/mm³ and Treg percentage >9%). But among patients with CD4 nadir <350/mm³, patients with Treg percentage <9% at baseline had a better immunological recovery than patients with Treg >9% (Figure 2A and C).

Table 2. Effect Sizes From Adjusted Model Including Interactions With Time, Treg, and T Lymphocytes CD4⁺ (CD4) Nadir on CD4 Absolute Value

Variable	Effect Size	95% CI	P Value
Treg at baseline (per additional percent)	0.981	.971–.992	<.001
Interaction with time (per month)	1	.999–1.001	.192
CD4 nadir (per 10/mm ³)	1.024	1.018–1.03	<.001
Interaction with time (per month)	0.999	.999–1.00	.072
Interaction of Treg with CD4 nadir (per additional percent of Treg and 10/mm ³ CD4 nadir)	1.001	1.001–1.002	<.001

Abbreviations: CI, confidence interval; Treg, regulatory T cell.

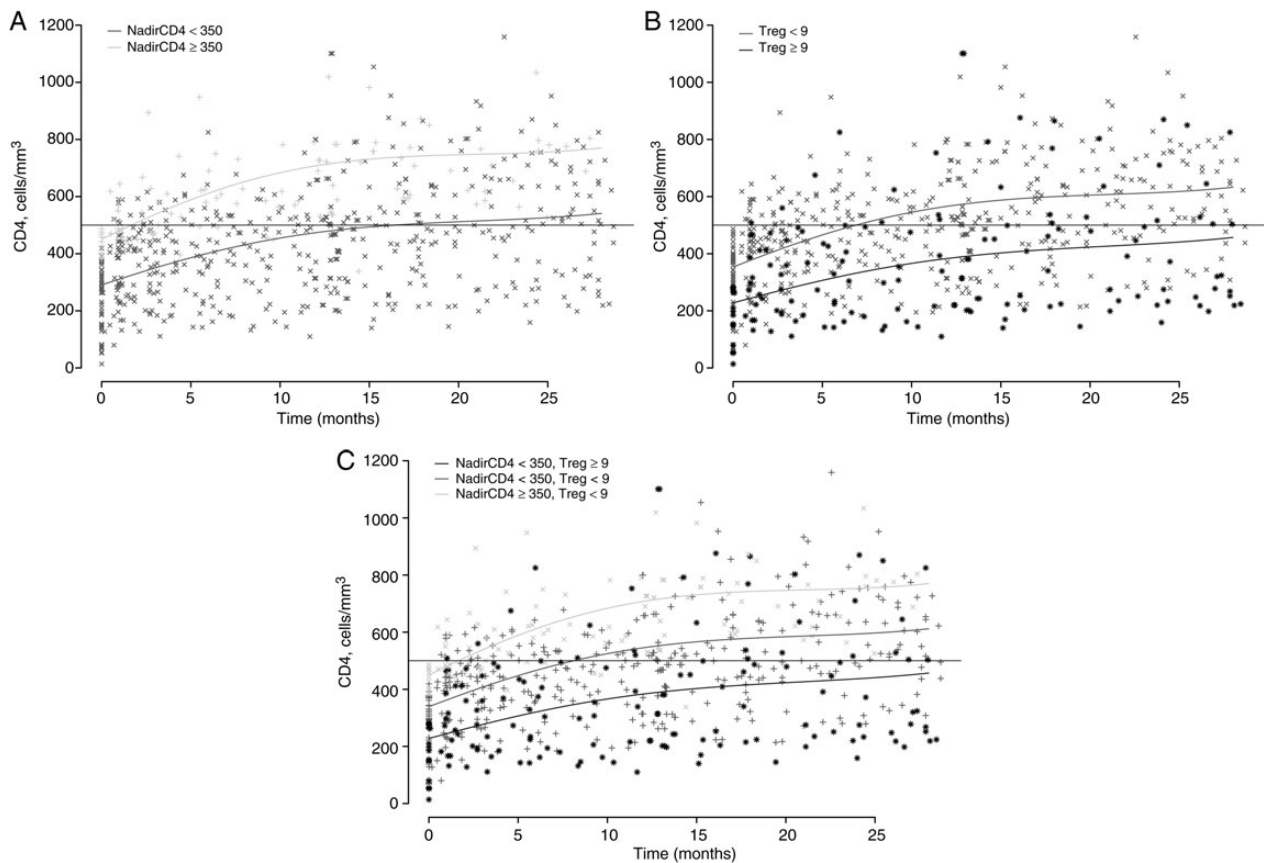


Figure 2. CD4⁺ T lymphocyte count evolution in the 58 antiretroviral therapy-naïve human immunodeficiency virus-1-infected patients during the 2 years after highly active antiretroviral therapy introduction. Black and gray crosses represent the CD4 value of patients; black and gray curves represent the median CD4 value in each group according to the following: (1) CD4 nadir (nadir <350/mm³: n = 47, nadir >350/mm³: n = 11) (A); (2) percentage of regulatory T cells (Tregs) at baseline (Treg <9%: n = 45, Tregs >9%: n = 13) (B). (3) CD4 nadir and Treg percentage (nadir >350/mm³ and Treg <9%: n = 11; nadir <350/mm³ and Treg >9%: n = 13, nadir <350/mm³ and Treg <9%, n = 34) (C).

DISCUSSION

In Western countries, the discordant immunological response to ART is a very frequently encountered problem and is associated with increased risk of death or cardiovascular and malignant pathologies [2–5]. In this context, CD4 nadir is known as an important prognostic factor of CD4 count reconstitution [17, 20]. Here, we extend these results by describing, in a prospective cohort study, that Treg percentage, as CD4 nadir, was as an independent predictor of low CD4 recovery despite virological success. However, we found an interaction between Treg percentage and CD4 nadir, which showed that the effect of Treg percentage was attenuated for increasing values of CD4 nadir.

Recent cross-sectional observational studies described an association between Treg percentage and immunological nonresponse to highly active ART (HAART). They included patients under suppressive ART for many years and compared clinical and immunological characteristics of immunological responders versus nonresponders. This clearly suggests that a high

Treg percentage could be an important factor associated with immunological discordant response to ART. However, it was argued that the differences observed in Treg percentage between the 2 groups (patients with immune recovery vs nonresponders) in such studies only reflect differences in CD4 nadir. In fact, a strong negative correlation between Treg percentage, CD4 count, and CD4 nadir has been frequently reported in viremic untreated patients [14, 21–24]. Thus, in other words, the complex pattern of immune dysfunctions (including increased Tregs) observed in patients with incomplete immune reconstitution could be the consequence of low CD4 nadir, not the cause [16]. This is an important limitation to these previous cross-sectional studies [14–18]. To our knowledge, only 1 prospective study evaluated the impact of Tregs in immune response to ART. Its results suggest a possible prognostic value of a high Treg percentage in immunological nonresponder patients ($r^2 = 0.17$, $P < .05$) [25]. However, in this study, patients were observed during 1 year but have been on suppressive ART for many years (at least 2 years), which strongly limits

the interpretation of the results. More importantly, it has to be highlighted that the definition used in the literature for the immunological response to ART is highly variable. The different classic outcomes such as slope of CD4 absolute value evolution overtime [14], fixed absolute value objective, or CD4 gain at end of follow up [26–28] could be impacted by CD4 count at ART introduction and by the duration of follow-up (few months or many years). In the current study, CD4 reconstitution was analyzed using a nonlinear model. Using the mixed-effect Poisson regression for count data model used here, we were able to investigate the role of Treg and CD4 nadir on immune reconstitution without misclassifying patients in groups of immunological responder or inadequate responder. Every CD4 value recorded during the prospective follow-up was used in the model. This allowed analysis of interaction between CD4 absolute value evolution, CD4 nadir, and Treg percentage at baseline with time. Using this model, we describe a negative impact of a high Treg percentage at ART introduction on immunological response, thereby confirming results of previous cross-sectional studies [14–18]. More importantly, we highlighted that this effect does not attenuate with time.

We believe that our results are of major importance. First, Treg percentage should now be used to detect patients at risk of immunological nonresponse to ART, ie, with CD4 nadir less than $350/\text{mm}^3$ and Tregs of more than 9% (Figure 2). On the contrary, it is probable that patients with high CD4 nadir (whatever the Treg percentage), or with a low Treg percentage at ART introduction, have a high probability of achieving a normal reconstitution of CD4 count. Such high-risk patients (CD4 nadir $<350/\text{mm}^3$ and Treg percentage $>9\%$ – 10%) should be preferentially included in therapeutics assays, which are conducted to improve the immune response.

Second, these results raise the question of the interest of directly targeting Treg, in complement with ART, in patients with low nadir and high Treg percentages. However, the direct pathogenic role of this CD4 subset in the immunological nonresponse is not yet proven. Pion et al [29] have shown that HIV infection down regulates Foxp3 expression in Treg, which is followed by the loss of their suppressive capacity and alterations in suppressive cytokine secretion pattern. We can hypothesize that once virological suppression is obtained under ART, free-viruses/uninfected Treg could disrupt CD4 homeostasis. This has been suggested by others [15]. It would now be interesting to perform in vitro functional assays of Treg from aviremic patients under long-term ART, to compare results from immunological responders versus patients with a persistently low CD4 repopulation. If a deleterious effect is confirmed in aviremic treated patients, new therapeutics strategies targeting Treg should be elaborated, as already proposed in cancer [30, 31].

Our study has some limitations. In particular, we did not observe a statistical relationship between age and immunological response. Indeed, previous studies found that increasing age is

associated with a smaller CD4 gain [4, 32]. This can probably be explained in the current study by a lack of statistical power due to the relatively low duration of ART and the low number of patients studied.

CONCLUSIONS

In conclusion, in this population of HIV-infected naive patients, a high Treg percentage measured at ART introduction was associated with a lower CD4⁺ T cell recovery during the 2 following years. The percentage of Tregs could be used in clinical practice in association with CD4 nadir to detect patients at risk for immunological nonresponse to ART.

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Author contributions. J. S., T. F., G. M., F. V., and J. D. designed the study, performed immunological laboratory assessment, statistical analysis, and the writing of the manuscript. C. M. and F. P. performed immunological laboratory assessment. D. M. B. performed statistical analysis and the writing of the manuscript. J. S., T. F., L. C., P. M., and C. C. included patients.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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