HIV specific Regulatory T cells are associated with higher CD4 cell counts in Primary Infection.

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Abstract

Objective: Expansion of Regulatory T (Treg) cells has been described in chronically HIV-infected subjects. We investigated whether HIV-suppressive Treg could be detected during primary HIV infection (PHI).

Methods: Seventeen patients diagnosed early after PHI (median: 13 days; 1-55) were studied. Median CD4 cell count was 480 cells/µl (33-1306) and plasma HIV RNA levels ranged between 3.3 to 5.7 log_{10} cp/mL. Suppressive capacity of blood purified CD4^+ CD25^+ was evaluated in a co-culture assay. Fox-p3, IL-2 and IL-10 were quantified by RT-PCR and intra-cellular staining of ex vivo and activated CD4^+ CD25^{high} T cells.

Results: The frequency of CD4^+ CD127^{low} CD25^{high} T cells among CD4 T cells was lower in PHI compared to chronic patients (n=19). They exhibited a phenotype of memory T cells and expressed constitutively FoxP3. Similarly to chronic patients, Treg from PHI patients inhibited the proliferation of PPD and HIV p24 activated CD4^+ CD25^- T cells. CD4^+ CD25^{high} T cells from PHI patients responded specifically to p24 stimulation by expressing IL-10. In untreated PHI patients, the frequency, as well as HIV-specific activity of Treg decreased during a 24-month follow up. A positive correlation between percentages of Treg and both CD4 cell counts and the magnitude of p24-specific suppressive activity at diagnosis of PHI was found.

Conclusions: Our data showed that HIV drives Treg since PHI and that these cells persist throughout the course of the infection. A correlation between the frequency of Treg and CD4 T cell counts suggest that these cells may impact on the immune activation set point at PHI diagnosis.

Key words: PHI, Treg, IL-10, HIV-specific CD4 T cells, immune activation
Introduction

Primary HIV infection (PHI) is characterized by high levels of viral replication followed by induction of HIV-specific CD4 and CD8 T cell immune responses [1-4]. Studies have shown that the magnitude of these immune responses determines the subsequent course of infection [5-7]. However, these responses are ineffective at eradicating the virus, and the chronic infection characterized by a gradual loss of CD4 T cells leads to AIDS without therapy in the majority of patients.

A relationship between T cell activation, CD4 T cell decline and clinical outcome has been shown in the chronic phase of the infection [8-11]. Several clinical studies have also demonstrated that the virological and immunological events that occur during PHI are strongly predictive of disease progression [12, 13]. These reports support the hypothesis that HIV causes CD4 T cell depletion as a consequence of generalized T-cell activation [14, 15]. This was confirmed in a prospective study conducted in acutely infected adults showing that “the immune activation set point” established early in HIV infection determines the rate of CD4 T cell loss over time [16].

Regulatory T cells (Treg) finely regulate immune responses and cellular activation [17]. Treg cells including CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells were reported to influence the outcome of various infections [18]. CD4<sup>+</sup>CD25<sup>+</sup> Treg cells suppress antigen-specific CD4 and CD8 responses and also control inappropriate or exaggerated immune activation induced by pathogens [19, 20]. We, and others, have reported that regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells can suppress HIV-specific effector CD4 and CD8 T-cell responses in chronically HIV-infected patients [21, 22]. We have found in chronically infected patients that HIV antigens triggered the proliferation of virus-specific Treg [23]. However, the influence of Treg in HIV infection remains unclear [24]. Treg can limit immune activation and viral replication but may dampen poly-functional adaptive immune responses against viral antigens [23, 25-28].

In the present study we investigated whether Treg cells could be detected in early phases of HIV infection. For this, a comparison of Treg frequency, phenotype and function in patients studied at early and chronic stages of HIV infection was undertaken. The effects of in vitro stimulation with HIV antigen of purified Treg from patients diagnosed at the primary HIV infection (PHI) were studied. In a clinical stand point we looked at the correlation between Treg frequency and CD4 T cell counts and plasma HIV RNA values at PHI. The impact of combined antiretroviral therapy initiation during PHI on Treg function was studied in a 24-months longitudinal follow up of subjects.
Patients and Methods

This is a prospective study conducted in two clinical sites in France. To be enrolled subjects must have had evidence of acute or recent HIV infection as defined by a negative or weakly reactive HIV antibody enzyme immunoassay with less than 3 bands on HIV Western Blot and detectable plasma HIV RNA. Subjects were offered to participate to this study before physician’s decision of c-ART initiation based on usual criteria and physician’s judgment. Chronic HIV-infected patients included in this study have received c-ART for at least one year and exhibited CD4 cell counts above 500 cells/mm$^3$ and plasma HIV viral load below 50 copies/ml. Fresh blood samples were collected on EDTA tubes and processed within 3 hours after they were drawn. All patients provided written informed consent; the study was approved by the ethical committee of Hôpital Européen Georges Pompidou, Paris, France.

Cell isolation

CD4 T lymphocytes were purified from whole blood using RosetteSep CD4 enrichment and CD8 depletion antibody cocktail (Stem Cell Technologies, Vancouver BC, Canada). CD4 T cells (> 90 % purity) were incubated with CD25 magnetic beads (Miltenyi Biotech) allowing the purification of CD4$^{+}$CD25$^{+}$ (20µL per 10$^7$ cells) or CD4$^{+}$CD25$^{+}$ populations (2µL per 10$^7$ cells). The CD4$^{+}$CD25 or CD4$^{+}$CD25$^{+}$ high populations were subsequently separated using 2 passages on magnetic columns. This generally resulted in obtaining higher than 85 % purity of cell populations. Monocytes (> 90% purity) obtained by plastic adherence, were used as antigen-presenting cells.

Flow cytometric analysis

Phenotyping of the CD4$^{+}$CD25$^{+}$ and CD4$^{+}$CD25$^{+}$ cell subsets was performed on fresh whole blood samples using four-colour direct flow cytometry. Monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC) were used for immunostaining (All purchased by BD Biosciences, Le Pont Claix, France): anti-CD25-FITC, anti-CD4-PerCP, anti-CD3-APC, anti-CD45RO-APC, anti-CD25-APC, anti-CD25-PE, anti-HLA-DR-PE, anti-CD45RA-PE, anti-CD127-PE, anti-CD40L-PE, anti-CD122-PE, anti-CD95-PE, anti-CD69-PE, anti-CD103-PE and anti-CTLA-4-PE as previously described [23]. For staining of Foxp3, the cells were fixed and permeabilized using a fixation/permeabilization kit according to the manufacturer’s protocol. Lymphocytes were stained with AlexaFluor 488 anti-human Foxp3 (PCH101, eBioscience, San Diego, CA). Isotype-matched controls were used in all staining experiments. The production of IL-10 and IL-2 by anti-CD3/anti-CD28 or p24 activated CD4$^{+}$CD25$^{+}$ and CD4$^{+}$CD25$^{+}$ high purified cells was assessed as previously described [23].
Analyses were performed using FACSscalibur™ and CellQuest™ software (Becton Dickinson, San Jose, California, USA) on at least 10,000 events. Gating was restricted to the population of lymphocytes according to their light scattering properties.

**Proliferation and suppression assays**

The different subpopulations (CD4+CD25- cells and CD4+CD25+ cells) were assessed for their proliferative capacities in response to polyclonal stimulation and to recall antigens and p24 protein as previously described [23]. Antigen tested were: 5 µg/ml purified tuberculin (PPD, Statens Seruminstitut, Copenhagen, Denmark), 5 µg/ml p24 protein (Protein Science Corp., Meriden, Connecticut, USA) or 5 µg/ml of cytomegalovirus (CMV) antigen (Bio Whittaker Europe), in combination with soluble anti-CD28 mAb. For direct suppression assays, CD4+CD25+ lymphocytes were incubated for 5 days in the presence of p24 or PPD either alone or with varying numbers of CD4+CD25+ cells resulting in a suppressor : responder ratios of 0/1, 1/1, 1/2 and 1/10 in a final amount of 5x10^5 cells/well. Percentage of inhibition was calculated as follows: 1 - (mean cpm of co-culture wells divided by mean cpm of CD4+CD25- cells cultured alone) x 100. Cell proliferation was assessed using 0.5 µCi [3H] thymidine (Amersham Pharmacia, Uppsala, Sweden) incorporation. Stimulation indices were calculated by dividing the mean cpm of stimulated cells by the mean cpm of unstimulated cells.

**RNA isolation and real time quantitative RT-PCR**

Total RNA from 5x10^6 purified CD4+CD25- and CD4+CD25<sup>high</sup> cells either non stimulated or after 48h stimulation in the presence of plate-bound anti-CD3 and soluble anti-CD28 or p24 antigen, was purified as previously described [23]. Quantitative PCR was performed in a LightCycler System (Roche diagnostics) using a SYBR Green PCR kit from Roche Diagnostics. The cDNA input for each population was normalized to obtain equivalent signals with Splicing Factor 3A1 (SF3A1) used as housekeeping gene. Primers used were:

**SF3A1:** Forward: TGGCAAGTAAAGCACGGATGGAAACTGA  
Reverse: GTAGTAAGCCAGTGTTGGGAATCTTTG

**Foxp3:** Forward: TCCAAATACGGACACCCCCTTTCA  
Reverse: ACAGCCCCCTTCGCTCCTCCA

**IL-2:** Forward: TGCTGGATTTACAGATGATTTG
Reverse: TGAGCATCCTGGTGAGTTTG

HL-10; Forward: AGTCTGAGAACAGCTGCACCCACTTC
Reverse: GGGCATCACCTCCTCCAGGTAA

Statistical analysis

Data are expressed as mean ± S.D for percentages and median and ranges for absolute values. Statistical comparisons were performed using Mann Whitney rank sum test. Analysis of correlation was assessed using the Spearman Rank Test for non parametric data. All results were conducted using Prism GraphPad Ver5 (Graphpad Software, San Diego, CA, USA). Significance was considered for value of $P \leq 0.05$. 
Results:

Phenotypic characteristic of circulating CD4^+ CD25^{high} T cells in PHI patients

Seventeen patients diagnosed early after PHI (median: 13 days; 1-55 days) were studied. Median CD4 cell count was 480 cells/µL (33-1306) and plasma HIV RNA levels ranged between 3.3 to 5.7 log_{10} cp/mL. Since the level of immune activation in non-treated PHI patients may hamper discrimination between Treg and activated CD4 T cells that may also express CD25 and FoxP3 molecules, we evaluated the percentages of cells expressing high levels of CD25. These cells exhibit lower levels of CD127 molecules and higher FoxP3 expression as compared to CD4^+ CD25^− and CD4^+ CD25^{low} T cells as shown in Figure 1A and B for a representative PHI patient. Analysis of the cohort of PHI and c-ART treated chronic patients (n=19) showed that the median percentages of CD4^+ CD25^{high} T cells were 2.2% and 5.3% in PHI and chronic patients, respectively (p<0.05) (Figure 1C).

We investigated whether these CD4^+ CD25^{high} T cells might exhibit other phenotypic features of Treg previously characterized in chronic HIV-infected patients [23]. As shown in figure 1D, analysis of CD4^+ CD25^{high} from 13 PHI patients showed that these cells express characteristics of Treg. As compared to CD4^+ CD25^−, CD4^+ CD25^{high} exhibit a phenotype of memory T cells since 65% (median) express CD45RO molecule (P<0.05) with around 21% co-expressed the CD45RA marker. Forty-five percent of CD4^+ CD25^{high} express CD122, the β chain of the IL-2 receptor, as compared to 2% of CD4^+ CD25^− (P<0.001). Fifty and 25% of CD4^+ CD25^{high} express HLA-DR and CD40L as compared to 5% (P<0.01) and less than 1% (P<0.001) of CD4^+ CD25^−, respectively. Finally, a higher proportion of CD4^+ CD25^{high} compared to CD4^+ CD25^− T cells express antigens like CD69 (20%) (P<0.001), CD103 (17%) (P<0.001) and iCTLA-4 (18%) (P<0.05). We next confirmed that purified CD4^+ CD25^{high} T cells isolated from PHI patients express high levels of FoxP3 transcripts (figure 3B). Globally, these results show that CD4^+ CD25^{high} express phenotypic features of Treg. Moreover, they did not differ significantly from Treg characterized in chronic HIV patients [23].

Peripheral CD4^+ CD25^+ T cells from PHI patients are hypo-responsive to polyclonal and antigen-specific stimulation.

In order to determine whether CD4^+ CD25^+ circulating T cells in PHI patients exhibit functional characteristics of Treg, we assessed their ability to proliferate in response to recall antigens including PPD, CMV and p24 protein. Since functional experiments required a higher number of cells we performed these assays using CD4^+ CD25^+ that contained a high proportion of CD4^+ CD25^{high} T cells (ranging from 75-85%) (not shown), as previously
reported [23]. In the presence of anti-CD3 and soluble anti-CD28 mAbs, CD4+CD25- and CD4+CD25+ displayed the same proliferative capacity (Figure 2A). Similarly to CD4+CD25+ isolated from chronic patients, CD4+CD25+ from PHI patients did not proliferate in the presence of either tubercul in, CMV, or p24 antigens (Figure 2A) as compared to CD4+CD25+ cells from PHI and chronic patients (figure 2A). These results demonstrate that CD4+CD25+ isolated in PHI patients exhibit in vitro proliferative characteristics of regulatory T cells.

Peripheral CD4+CD25+ from PHI patients suppress CD4 T cell proliferation in response to recall antigens and HIV proteins

We investigated the potential suppressive effect of CD4+CD25+ isolated from PHI patients and chronic patients. As shown in figure 2B, addition of increasing numbers of CD4+CD25+ T cells resulted in a similar dose-dependent inhibition of the proliferation of CD4+CD25+ to tubercul in and p24 protein in PHI (n=9) and chronic patients (n=6). At a ratio CD4+CD25+/CD4+CD25- of 1/4, CD4+CD25+ from PHI and chronic patients inhibited by 37% and 46% in median autologous CD4+CD25+ proliferation to PPD, respectively. At the same ratio the response to p24 antigens was inhibited by 50% and 52% by CD4+CD25+ purified from PHI and chronic patients, respectively (figure 2B). This suppressive effect was up to 75% when cells were mixed at a 1/1 ratio.

Analysis of cytokine expression by CD4+CD25high T cells from PHI patients in response to HIV antigens

We have previously demonstrated that Treg isolated from chronic patients treated with α-ART exhibited a specific bias towards HIV antigens by producing either IL-10 or expressing TGF-β transcripts [23]. Therefore, we were interested to investigate whether such cells could be detected among PHI patients. We have focused our analysis on purified CD4+CD25high T cells from 6 patients at early time after diagnosis.

Following polyclonal stimulation, the mean frequency of CD4+CD25high and CD4+CD25+ T cells producing IL-2 increases significantly from 2.3 +/− 0.65 % and 0.6 +/− 0.06 % to 15.5+/−4.1 % and 7.8 +/− 1 % respectively (Figure 3A). In the same conditions, mean percentages of CD4+CD25high and CD4+CD25+ producing IL-10 increased from 3.4 +/− 0.6 % and 1.9 +/− 0.4 % to 22.3+/−5.8 % (P<0.05) and 6.2 +/− 1.2% (P<0.05), respectively. HIV specific stimulation induces a greater percentage of CD4+CD25high IL-2+ cells (14.7+/−4.4%) than CD4+CD25IL-2- cells (0.9+/−0.1%) (P<0.001). Similarly, in the same stimulation conditions, the frequency of CD4+CD25highIL-10+ cells was higher (16.2 +/− 3.5 %) than CD4+CD25IL-10- (11.1 +/− 0.16 %) (P<0.01). Altogether, these results indicate the presence of p24 specific T cells producing IL-2 and IL-10 among CD4+CD25high isolated from PHI patients.
Next, we investigated the level of mRNA transcripts of Foxp3, IL-2 and IL-10 at baseline and after stimulation in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. Samples from 5 PHI and 3 chronic patients were studied. As shown in figure 3B, CD4⁺CD25⁺ cells express higher levels of FoxP3 transcripts that remained stable following polyclonal and p24 stimulation. HIV specific stimulation induced a significant increase of IL-10 mRNA levels in CD4⁺CD25⁺ (P<0.05), but not in the CD4⁺CD25⁻ subsets.

**Longitudinal follow up of the suppressive capacity of CD4⁺CD25⁺ isolated from PHI patients.**

Next, we followed longitudinally up to 24 months the suppressive capacity of Treg isolated from 6 PHI patients that did not initiate c-Art based on clinical, immunological and physician’s decisions. Follow up showed that percentages of CD4⁺CD25⁺ dropped from 4.27 ± 0.76 % at diagnosis to 1.82 ± 0.52 % at months 6 (not shown) and 24 (figure 4A). We analyzed the suppressive activity of CD4⁺CD25⁺ T cells from these PHI patients throughout the follow up. CD4⁺CD25⁺ isolated at times of PHI diagnosis (month 0), and at months 6 and 24 were mixed at a 1/4 ratio to autologous CD4⁺CD25⁻ T cells in the presence of either tuberculin or p24 antigens. Interestingly, the suppression of CD4⁺CD25⁺ proliferation in response to tuberculin by CD4⁺CD25⁺ did not vary over time and ranged from 29 ± 2.3 % at diagnosis (month 0) to 47 % ± 11.2 % at month 24 (P=NS). In contrast, suppressive activity of CD4⁺CD25⁻ in response to p24 decreased from 59 ± 8.2 % at diagnosis to 27.4 ± 5.8 % at month 6 (P<0.05) and remained stable thereafter (23 ± 10 % at month 24) (Figure 4B).

**Correlation between frequency of CD4⁺CD25⁺ and CD4 T cell counts at diagnosis of PHI.**

In our prospective cohort of PHI patients, 9 of them initiated c-Art following the diagnosis of HIV infection based on clinical and immunological considerations. Median of CD4 T cell counts of patients who initiated c-Art was significantly lower than that of patients who remained untreated during the follow up (352 cells/µl and 561 cells/µl, respectively; P<0.05) whereas plasma HIV RNA did not differ significantly (5.5 ± 3 log₁₀ copies/ml). Moreover, the long term follow up of untreated PHI patients up to 24 months showed that they did not develop any AIDS-defining events nor indication to start c-Art according to the current guidelines. This led us to investigate whether the frequency and the suppressive capacity of Treg at PHI diagnosis could be correlated to the level of CD4 T cell counts, plasma viral load or CD4 T cells activation. We found a correlation between the percentage of CD4⁺CD25⁺ cells and CD4 T cell counts, but not plasma viral load, at diagnosis of PHI (r=0.6; P<0.01) (Figure 5A). Interestingly, we found a negative association between the frequency of Treg and of HLA-DR expressing CD4 T cells (r=-0.66; P=0.01) (figure 5B). Moreover, a correlation between the frequency of CD4⁺CD25⁺ at diagnosis and the percentage of
inhibition of CD4+CD25+ proliferation in the presence of p24, but not tuberculin, was found 
(r= 0.8, P<0.05) (figure 5C). These results show that a higher frequency of Treg inhibiting 
HIV-specific immune responses is associated with a higher CD4 T cell counts in PHI patients 
at diagnosis.
Discussion

The purpose of this study was to determine whether HIV-specific Treg are present at early stages of HIV infection and to characterize their suppressive activity in regard to CD4 HIV specific activity. We present data supporting the induction of HIV-specific Treg mediated suppression of HIV-specific CD4 T cells in PHI patients. Treg exhibiting a CD4^CD25^{high}CD127^{low} phenotype and expressing high levels of the transcription factor FoxP3 are detectable in the blood at early stages of HIV infection. These cells displayed suppressive activity by inhibiting the proliferation of CD4^CD25^ stimulated with HIV or recall antigens and secreted IL-10. Interestingly, in vitro stimulation of Treg from PHI patients with HIV antigens led to an increase of IL-10 synthesis demonstrating the presence of a proportion of HIV specific T cells among Treg. Altogether, these data extended those previously reported in chronic patients [23] demonstrating that HIV drives an expansion of Treg since the PHI stage.

Human CD4^CD25^ T cells have been shown to be a heterogeneous population that includes suppressive Treg cells, anergic but not suppressive T cells and normal activated T cells [29]. Interestingly, all these populations were detectable in PHI patients studied here. Although no specific markers have been described for characterization of human Treg, the expression of high levels of CD25 and low density of CD127 molecules has been correlated with immunosuppressive Treg activity in humans [30]. Using this combination of markers and high expression of FoxP3 we attempted to quantify Treg in PHI patients. Although FoxP3 is now widely used for the characterization of human Treg cells in healthy individuals [31] contrasting findings have been reported on the kinetics and functional effects of transient FoxP3 expression in activated human CD4^CD25^ T cells [29, 32-34]. These cells did not exert suppression on CD4 T cell proliferation a characteristic that was limited to CD4^CD25^{high}CD127^{low} T cells expressing higher levels of FoxP3. Thus, our results demonstrate that a true population of Treg may be identified at the early stages of PHI.

The most effective method for assessing suppressive activity of Treg is to determine their capacity to suppress the proliferation of stimulated CD4^CD25^ T cells. We show that Treg from PHI patients suppress HIV specific and non-specific proliferation of CD4 T cells. Interestingly, we found that the percentages of Treg in PHI patients were significantly lower than that observed in chronic patients. However, it is possible that peripheral blood may not be the most appropriate compartment to accurately assess HIV-specific Treg cell activity, particularly at the time of primary infection. Several studies in chronically HIV-infected patients and non-human primate models of SIV infection have shown a lower expression of FoxP3 and suppressor potential of Treg in the periphery compared to lymphoid tissue [25, 27, 28, 35-37], the primary site of virus replication [38].
We found a decrease in frequency of HIV, but not PPD, suppressive activity of Treg throughout a 24-months follow up of PHI patients who remained untreated. This also suggests a preferential homing of these cells to secondary lymphoid. Moreover, since Treg need to be activated through their TCR for exhibiting a suppressive activity [39] these data reinforce the demonstration of the presence of HIV-specific Treg in PHI patients. Alternatively, a decrease of HIV-suppressive activity could result from chronic immune activation or from a deleterious interaction of Treg with viral protein [40, 41] and antigen presenting cells [42]. Finally, although a recent report has shown that these cells are not preferentially infected by HIV [41], one hypothesis could be that Treg are infected and depleted in non-treated PHI patients.

Persistent antigens such as HIV are believed to promote the expansion and activation of antigen-specific Treg. In addition, we may speculate that, as recently reported, antigen presentation of HIV antigens by dendritic cells with tolerogenic properties and upregulation of inhibitory molecules might also participate to expansion of Treg in the context of PHI [43]. The high levels of viral replication and immune activation during PHI increase interactions between HIV or envelope proteins and CD4 or HIV-co-receptors, and may favor the peripheral conversion of memory activated CD4 T cells into Treg [44]. Likely, this is suggested by the persistent capacity of Treg from PHI patients to produce IL-2. However, the major characteristic of Treg populations in PHI patients was to contain a high proportion of cells producing IL-10. The level of IL-10 transcripts and the frequency of those cells increased significantly following in vitro stimulation with p24 antigens.

The definitive role of HIV-suppressive Treg in HIV infection is difficult to assess. In one hand Treg may have potentially beneficial effects by limiting the infection and deletion of HIV-specific effectors. In other hand our study provides new data showing that HIV-specific Treg expanded early after PHI may hamper the establishment of HIV-specific CD4 T cells responses. HIV infection in the humanized rag2−/− γC−/− mouse model support this evidence showing that depletion of Treg before HIV infection decrease viral load [45]. We found a correlation between the percentages of Treg and higher CD4 cell counts, but not plasma viral loads, at diagnosis of PHI. Although, longitudinal studies are needed to confirm the predictive role of Treg in PHI, our data support a beneficial effect of Treg during the acute phase of the infection. This observation is corroborated by recent studies showing a similar effect of Treg in non-pathogenic SIV models of acute infection [46] or more recently, in a mice model of acute mucosal HSV infection [47].

Altogether previous studies in HIV chronic infection and data in PHI patients reported here help to figure out a global picture of Treg in HIV infection. HIV-specific Treg may control potentially pathogenic immune activation during the early phases of infection by limiting excessive activation and depletion of HIV-specific CD4 T cells. In contrast to other microbial
infection, HIV is a persistent antigen that might trigger continuously long-life HIV-specific memory Treg cells resulting in an immune tolerance to HIV in vivo.
Figures legends

**Figure 1:** Characteristics of CD4+CD25high T cells in PHI patients. A) Phenotype of CD4 T lymphocytes in a representative PHI patient. B) Expression of Foxp3 and CD127 markers on gated CD4+CD25-, CD4+CD25low and CD4+CD25high gated T Cells. C) The median percentages of CD4+CD25high in PHI patients at diagnosis (n=17) and chronic c-ART treated patients (n=19). The frequency of CD4+CD25high T cells was evaluated on freshly blood samples after combining a gating on lymphocytes and on CD4+CD3+ T cells. D) Phenotype of CD4+CD25+ and CD4+CD25high T cell subsets in PHI patients (n=13). Median percentages of CD4+CD25+ and CD4+CD25high gated T cells expressing the different markers are indicated. Comparison between CD4+CD25+ and CD4+CD25high subsets are indicated (* P<0.05; ** P<0.01; *** P<0.001; Mann Whitney rank sum test).

**Figure 2:** CD4+CD25+ T cells from PHI and chronic HIV-infected patients are hypo-responsive to antigen specific stimulation and suppress CD4+CD25- proliferation
A) 5x10⁴ purified CD4+CD25+ (white bars) and CD4+CD25+ T cells (black bars) were stimulated for 5 days with 5µg/ml of plate bound either with anti-CD3 mAb and soluble anti-CD28 mAb (5µg/ml). 5µg/ml of purified PPD, p24 or CMV antigen (³H) thymidine was added during the last 16 hours of culture. Results are expressed as median of stimulation index from 9 PHI and 8 chronic HIV infected patients. B) Peripheral CD4+CD25+ T cells from PHI (n=8) and chronic HIV-infected patients (n=6) suppress similarly CD4+CD25+ T cell proliferation in response to PPD and HIV p24 protein. Purified CD4+CD25+ (5.10⁴) cells were incubated at different ratio with CD4+CD25+ T cells and autologous monocytes for 5 days with either 5µg/ml PPD or p24, before adding 0.5 µCi of thymidine for the last 16 hours of culture. Results are expressed as median values of the stimulation index (* P<0.05; ** P<0.01, Mann Whitney rank sum test).

**Figure 3:** HIV-specific production of IL-10 and IL-2 by CD4+CD25high T cells
A) Frequency of CD4+CD25+ and CD4+CD25high producing IL-2 (left) or IL-10 (right) following either polyclonal or p24 stimulation. Cells (5.10⁴/well) were incubated for 24 hours alone or in presence of either 5µg/ml plate bound anti-CD3 mAb and soluble anti-CD28 or of p24 antigen (5 µg/ml) and 5.10⁴ monocytes/well, before assessment of intracellular production of cytokines by flow cytometry. Changes from baseline in the mean frequencies of cells producing cytokines following stimulation are indicated. Results from 6 patients studied are presented. B) Quantitative RT-PCR analyses of Foxp3 (left panel). IL-2 (middle) and IL-10 (right) expression of CD4+CD25+ and CD4+CD25high after purification and non-stimulated (NS), or stimulated either with anti-CD3 and anti-CD28 antibodies or p24 antigen. Mean from 5 patients are presented. Ct values were normalized to mRNA for SF3A11 expression. (* P<0.05; ** P<0.01, Mann Whitney rank sum test).
Figure 4: Evolution of the frequency and suppressive activity of Treg in PHI patients.
A) Frequency of CD4^CD25^{high} T cells at diagnosis and month 24 in 6 PHI patients who remained untreated during the follow up. Evaluation of the suppressive activity of purified CD4^CD25^{high} T cells on PPD (B) and p24 (C) stimulated CD4^CD25 T cells (values at a 1/4 ratio of CD4^CD25^{high} / CD4^CD25 T cells are shown). Mean percentages of inhibition of experiments performed in 6 patients are shown. (* P < 0.05, Mann Whitney rank sum test).

Figure 5: Correlation between the frequency and activity of CD4^CD25^{high} T cells and absolute CD4 cell counts in PHI patients.
The correlations at diagnosis between total number of CD4^CD25^{high} T cells and CD4 cell counts or plasma HIV RNA values (A), expression of activation marker HLA-DR on CD4 T cells (B) inhibition of CD4^CD25 proliferation in the presence of p24 and tuberculin (C) were investigated using a Spearman rank correlation coefficient test.

Supplementary table 1 Virological and immunological characteristics of patients at diagnosis. Decision to treat or not patients were based on usual criteria and current guidelines for initiation of c-Art. (* mean CD4 cell count = 482/mm^3, ** mean percentage of CD4^CD25^{high} = 2.2%)
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Authors contribution: H. K and V.D-P performed the experiments and analyzed the data. J.D. L analyzed the data and wrote the paper. M. B performed the experiments. A. A and G. M recruited the patients and collected clinical data. L.W designed the study and analyzed the data. Y. L designed the study, analyzed the data and wrote the paper.

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