Chronically HIV-1 Infected Patients Exhibit Low Frequencies of CD25+ Regulatory T Cells

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Abstract: The characterization of regulatory T cells (Treg) during HIV infection has become of particular interest considering their potential role in the pathogenesis of the acquired immunodeficiency syndrome. Different reports on Tregs in HIV-infected patients vary greatly, depending on the state of disease progression, anatomical compartment, and the phenotypic markers used to define this cell subpopulation. To determine the frequency of Tregs we included paired samples from peripheral blood and rectal biopsies from controls and chronic HIV patients with or without detectable viral load. Tregs were determined by flow cytometry using three different protocols: $CD4^+Foxp3^+$; $CD4^+Foxp3^+CD127^{Low/-}$, and $CD4^+CD25^+CD127^{Low/-}$. In addition, and with the purpose to compare the different protocols we also characterized Tregs in peripheral blood of HIV negative individuals with influenza like symptoms. Here, we report that Treg characterization in HIV-infected patients as $CD4^+Foxp3^+$ and $CD4^+Foxp3^+CD127^{Low/-}$ cells was similar, indicating that both protocols represent a suitable method to determine the frequency of Tregs in peripheral blood mononuclear cells (PBMC) and gut associated lymphoid tissue (GALT). In contrast, in HIV but not in flu-like patients, detection of Tregs as $CD4^+CD25^+CD127^{Low/-}$ cells resulted in a significantly lower percentage of these cells. In both, HIV patients and controls the frequency of Treg was significantly higher in GALT compared to PBMC. The frequency of Tregs in PBMC and GALT using $CD4^+Foxp3^+$ and $CD4^+Foxp3^+CD127^{Low/-}$ was higher in HIV patients than in controls. Similarly, the frequency of Treg using any protocol was higher in flu-like patients compared to controls. The results suggest that relying on the expression of CD25 could be unsuitable to characterize Tregs in PBMC and GALT samples from a chronic infection such as HIV.

Keywords: Regulatory T cell, human immunodeficiency virus, CD25, phenotype.

INTRODUCTION

Regulatory T cells (Treg) play a major role in maintaining self-tolerance and limiting antiviral responses during chronic infections. Treg is a subpopulation of $CD4^+$ T cells characterized by their potent capacity to reduce the activation and expansion of conventional T cells (Tcon) [1, 2]. Considering that immune activation is a hallmark of human immunodeficiency virus type-1 (HIV) infection, a considerable number of studies have explored the role of Treg in different cohorts of patients, reporting contradictory data. In peripheral blood, several studies have reported a reduction in the percentage of Treg in HIV patients, independent of viral load, compared to controls [3,4]; in contrast, other reports indicate either an increase in this T cell subpopulation [5-8] or no change [9,10]. Although several factors, including the stage of infection and the heterogeneity of the HIV populations studied can account for these differences, technical considerations in the phenotypic characterization of these cells cannot be ruled out. The first concern is that most of the original reports indicating frequency and absolute counts of Treg in HIV patients were based on the assumption that a high level of CD25 expression is a reliable marker for human Treg [11, 12]. There is no doubt that the $CD4^+CD25^{hi}$ T cell subset has the

greatest regulatory potential [13]; however, there is also evidence that activated conventional cells have upregulated the CD25 molecule [14].

A better characterization was achieved by determining the expression of Foxp3, the best marker so far identified to characterize this cell subpopulation; however, this molecule is also transiently expressed in a small percentage of activated $CD4^+$ Tcon, and it is not convenient to purify the cells because its detection implies permeabilization [15]. Later, in 2006 the absence or low expression of the alpha chain of the IL-7 receptor, the CD127 molecule, along with the expression of the transcription factor Foxp3, were considered the most specific markers of this cell population [13, 16].

Although flow cytometry is an efficient tool to determine the phenotype and functional activity of Treg [13, 16, 17], there is still some controversy regarding the molecules used for their characterization, particularly in the context of chronic infections [18]. In addition, most of the immune alterations occurring during HIV infection have been well documented in peripheral blood. However this virus has a greater impact in lymphoid organs, particularly in GALT, the main site of HIV replication [19], where little is known regarding Treg phenotype. In this study we compared three different protocols to characterize Tregs. We included samples from peripheral blood and gut associated lymphoid tissue (GALT) of healthy controls and two groups of chronically HIV infected patients: patients with viral load and patients with undetectable viral load. Here, we explore if

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type of tissue sample or the virus influence the Treg phenotypic alteration. In addition, and with the purpose of determining whether changes in Treg are specific of HIV infection or associated to any inflammatory/activation process due to any infection, we also characterized Tregs in peripheral blood of HIV negative individuals with influenza like symptoms. The results suggest that relying on the expression of CD25 is unsuitable to characterize Tregs in peripheral blood mononuclear cells (PBMC) and GALT samples from HIV patients.

MATERIALS AND METHODS

Subjects

Four groups of subjects, from Medellin, Colombia, were included in the study: HIV negative healthy controls (HC, n=10); HIV negative individuals with acute flu-like symptoms (Flu, n=9); chronic HIV-1-infected patients with undetectable viral load (HIV-U, n=13) and HIV-1-infected patients with viral load (HIV-VL, n=19). Flu-like patients had an average of five days with symptoms such as fever, malaise, headache, sore throat, nasal congestion and weakness; all patients had rapid spontaneous resolution of the disease in absence of treatment. To rule out bacterial infection, para-clinical examinations were carried-out. Healthy controls and flu-like patients were screened to rule out HIV infection by electrochemiluminescence (HIV combi PT, Roche Diagnostics; Mannheim, Germany).

HIV patients, with at least two years of confirmed infection, classified in the B clinical category, according to the CDC criteria were included. The demographic and clinical characteristics of these individuals are shown in Table **1**. Absolute number and percentage of CD4^{+} T-cell were calculated in whole blood samples. Plasma HIV-1 RNA levels were assessed by RT-PCRq (COBAS AmpliPrep/ COBAS TaqMan, Roche Molecular).

Isolation of Cells from Peripheral Blood and GALT

PBMC were obtained from 10 ml of heparin venous blood samples by centrifugation on Histopaque (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 400 x g. Mucosal samples were processed as described by the Shacklett protocol [20] with minor modifications. Rectal tissue was obtained by rectosigmoidoscopy from the rectum at 10 cm from the anal verge. A flexible sigmoidoscope with a single endoscopy biopsy forcep FB-24K-1 (Olimpus America Corp, Melville, NY, USA) was used. Samples were

processed by digestion using collagenase type II from *Clostridium histolyticum* (Sigma) at 0.5 mg/ml diluted in RPMI 1640 and 7.5% FCS (fetal calf serum) (penicillin 100 U/ml, streptomycin 100 g/ml, amphotericine B 0.25 g/ml) (Gibco-BRL; Grand Island, NY, USA), during 30 min at 37ºC while shaking. After collagenase digestion, biopsy fragments were further disrupted by repeated passage through a 30 ml syringe with a blunt ended 16 gauge needle (Stem Cell Technologies, Vancouver, BC). Rectal cells (RC) were isolated by passage through a nylon strainer of 70 µM (Falcon, Lincoln Park, NJ, USA). PBMC and RC were washed with Dulbeco´s phosphate buffered saline (DPBS) (Sigma-Aldrich; St Louis, MO, USA) to remove excess histopaque and collagenase, respectively.

Flow Cytometry Analysis of PBMC and RC

PBMC and RC were treated with 20 ug/ml of human IgG (Sigma-Aldrich; St Louis, MO, USA) to block Fc receptors, and stained on the surface with anti-CD4-ECD (clone SFCI12T4D11) (Beckman Coulter Fullerton, CA, USA) anti-CD3-PE-CY7 (clone UCHT1), anti-CD25-PE-CY5 (clone BC96) and anti-CD127-PE (IL-7 Rα, clone RDR5) (e-Bioscience, San Diego, CA, USA) for 30 min at 4°C. The cells were washed with DPBS, then fixed/permeabilized (Foxp3 staining buffer kit, e-Bioscience) and stained intracellularly using monoclonal antibodies against anti-Foxp3-FITC (clone PCH101), for 30 min at 4°C. At least 100,000 events were acquired for each sample in the lymphocyte region, using the flow cytometry FC500 (Beckman Coulter Fullerton, CA), and analyzed using the Kaluza Software (Beckman Coulter Fullerton, CA). T cells were gated first based on forward- and side-scatter properties, then as CD3^{+}CD4^{+} cells (Fig. **1A**). The strategy followed to detect Treg was previously reported by Presicce *et al.*, 2010 [21] and Seddiki *et al.*, 2006a [16] protocols. Briefly, to define the limit of the positive gate, a negative biological population (CD3^{-}CD4^{-} or CD3^{+}CD4^{-}), previously known for not expressing the marker of interest, was used [21]; the Mean fluorescence intensity (MFI) was analyzed within the positive population.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA). The data are shown as median (25th percentile-75th percentile). Comparisons of medians among groups were performed by the U-Mann-Whitney test or the Kruskal Wallis test with Dunn's multiple comparisons post-test. Intragroup comparisons were made

Table 1. Characteristics of Healthy Controls, Flu-Like, HIV with and Undetectable Viral Load

	Healthy Controls (n=10)	Flu-Like (n=9)	HIV Undetectable Viral Load (n=13)	HIV With Viral Load (n=19)
Age (years)[1]	46 (13)	30(7)	42 (9)	38 (12)
% Male/Female	50/50	45/55	85/15	95/5
% CD4^{+} T cell[2]	39 (32-46)	34 (32-41)	16**φ (12-31)	18***φ (10-24)
% CD8^{+} T cell[2]	20 (16-23)	32¥ (22-34)	45***φ (34-56)	46***φ (42-57)
HIV Viral load (copies/ml)[2]	N/A	N/A	<40 (40-40)	12500^ (230-50190)

[1]Results are expressed as mean (SE); [2]Results are expressed as median (25th-75th percentiles). Groups were compared by the Kruskal-Wallis test and the Dunn´s multiple comparison post-test. Significant differences are indicated by **p<0.01, ***p<0.001compared to helthy control group and φp<0.01 with the flu-like group. Comparation by U Mann Whitney test between controls and flu groups are indicated by ¥p<0.01 and between both groups of HIV-1 positive patients by ^ p<0.001. N/A: not applicable.
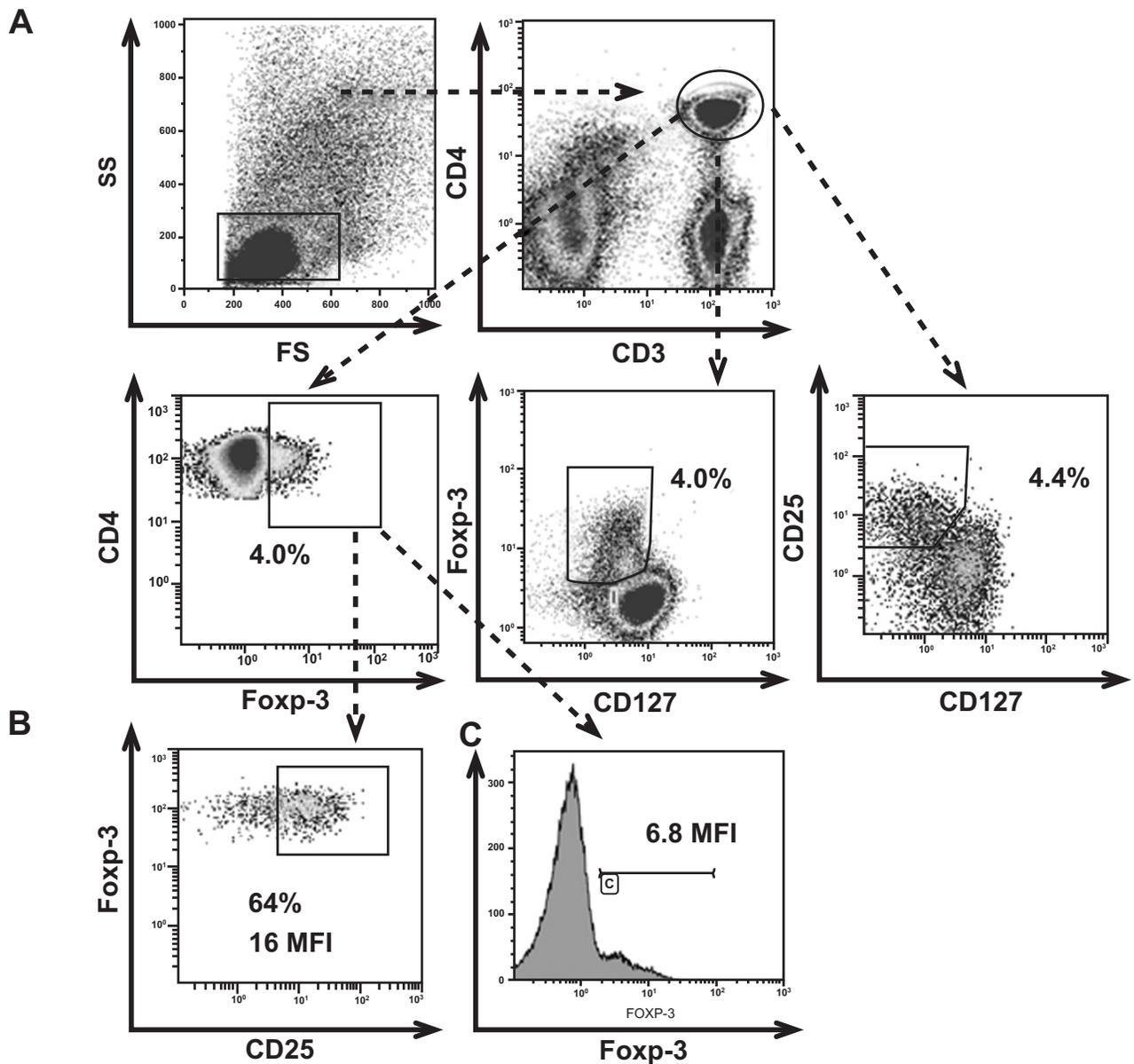
Fig. (1). Chacterization of Treg phenotype. Staining of Treg was made with conjugated monoclonal anti-CD3-PE-CY7, anti-CD4-ECD, anti-CD25-PE-CY5, anti-CD127-PE and anti-Foxp3-FITC antibodies. **A**) Scatter blot from one representative figure of flow cytometry data shows the frequency of Treg in PBMC in an individual from a healthy control group. Three different ways of defyning Treg were analyzed: CD4$^+$Foxp3$^+$ (right panel), CD4$^+$Foxp3$^+$CD127$^{Low/-}$ (middle panel) and CD4$^+$CD25$^+$CD127$^{Low/-}$(left panel). In the Foxp3$^+$ gate the following analysis were done. **B**) percentage and MFI of CD25 molecule; and **C**) MFI of Foxp3.

with Friedmann test. Correlations were made with spearman test and *p* values less than 0.05 were considered to be statistically significant.

RESULTS

Patient Characteristics

As shown in Table **1**, the percentage of CD4$^+$ T cells were reduced in both groups of HIV patients (with detectable and undetectable viral load) compared to controls (HC *vs* HIV-U; p<0.01) (HC *vs* HIV-VL; p<0.001) and flu-like (both p<0.01). Controls, flu-like, HIV-U and HIV-VL

exhibit CD4$^+$ T cell counts of 905 cells/mm^3 (692-1093 cells/mm^3), 815 cells/mm^3 (645-923 cells/mm^3), 439 cells/mm^3(301-986 cells/mm^3) and 426 cells/mm^3 (287-707 cells/mm^3), respectively. The CD4$^+$ T cell counts were strikingly low in the HIV-VL group compared with controls (p<0.05). As expected, the percentages of CD8$^+$ T-cells were higher in all individuals with viral infection (HIV and Flu) compared to controls, with the highest values found in both HIV groups (Table **1**). All the patients with undetectable viral load had been on highly active antiretroviral therapy (HAART) for a median of 8 (3-12) years.

Low Frequency of CD4$^+$CD25$^+$CD127$^{Low/-}$ Tregs Detected During Chronic HIV Infection in Peripheral Blood and Gastrointestinal Tissue

To characterize the Treg subset during HIV infection, we analyzed the frequency of Treg using the following protocols: i) CD4$^+$Foxp3$^+$; ii) CD4$^+$Foxp3$^+$CD127$^{Low/-}$; and iii) CD4$^+$CD25$^+$CD127$^{Low/-}$ (Fig. **1A**) as reported by Seddiki *et al.,* [16]. As shown in Fig. (**2A**), the percentage of Treg in PBMC from healthy donors (upper panel) and flu-like patients was similar intra-group, regardless of the protocol used. In contrast, the percentage of Treg from HIV samples varied depending of the protocol. In the HIV-U group the frequency was similar, 7% (6-10%) and 7% (5-8%) when the Treg were defined as CD4$^+$Foxp3$^+$, and CD4$^+$Foxp3$^+$CD127$^{Low/-}$respectively; however, the frequency was significantly lower, only 4% (4-6%), when these cells were defined as CD4$^+$CD25$^+$CD127$^{Low/-}$ (Fig. **2A**; p<0.05 compared to CD4$^+$Foxp3$^+$). Similarly, in the HIV-VL group the frequency of Treg was 7% (6-10%) and 6% (5-8%) when the Treg were defined as CD4$^+$Foxp3$^+$, and CD4$^+$Foxp3$^+$CD127$^{Low/-}$ respectively; however, the frequency was lower with the CD4$^+$CD25$^+$CD127$^{Low/-}$ protocol compared to CD4$^+$Foxp3$^+$ (5% (4-8%) *vs* 7% (6-10%); p<0.001) (Fig. **2A**).

Interestingly, and probably due to an inflammatory process in both, chronic (HIV: using CD4$^+$Foxp3$^+$, CD4$^+$Foxp3$^+$CD127$^{Low/-}$ protocol) and acute (flu-like: using any protocol) viral infections, the percentage of Treg on PBMC was increased compared with controls (p<0.05) (Fig. **2A**). However, when the Treg were defined as CD4$^+$CD25$^+$CD127$^{Low/-}$ in HIV, but not in flu-like infected patients, the percentage was similar to that healthy controls, indicating that this protocol is not suitable to define Treg in HIV patients.

Similarly, and related to these differences, absolute counts of Treg were not significantly different in PBMC from healthy controls and flu-like patients (intragroup comparisons) in any of the protocols used (data not shown), but varied in HIV-infected patients; in the HIV-U group the Treg counts were 202 cells/μl (142-243 cells/μl) and 163 cells/μl (116-235 cells/μl) when the Treg were defined as CD4$^+$Foxp3$^+$, and CD4$^+$Foxp3$^+$CD127$^{Low/-}$ respectively; however, the counts were significantly lower, only 117 cells/μl (96-178 cells/μl), when these cells were defined as CD4$^+$CD25$^+$CD127$^{Low/-}$ (Fig. **2B**; p<0.01 compared to CD4$^+$Foxp3$^+$). Similarly, in the HIV-VL group the count of Treg defined as CD4$^+$CD25$^+$CD127$^{Low/-}$ was significantly lower than CD4$^+$Foxp3$^+$ (107 cells/μl (58-187 cells/μl) *vs* (173 cells/μl (108-360 cells/μl); p<0.001) and CD4$^+$Foxp3$^+$CD127$^{Low/-}$ (107 cells/μl (58-187 cells/μl) *vs* (179 cells/μl (104-269 cells/μl); p<0.01) (Fig. **2B**). In PBMC, HIV patients exhibited the highest Treg counts (Foxp3$^+$), compared to controls (HIV-U: 202 cells/μl *vs* HC: 101 cells/μl; and HIV-VL: 173 cells/μl *vs* HC: 101 cells/μl; both p<0.05).

GALT is the primary site of damage during HIV infection, where the virus induces many quantitative and functional changes in the Tcon subset [22]. Therefore, we also analyzed biopsy samples of this tissue. When the frequency of Treg was evaluated in GALT samples from HIV patients, the differences, depending on the protocol used, were more evident. In both groups of HIV-infected individuals the Treg percentage in GALT were similar when the Treg were defined as CD4$^+$Foxp3$^+$, and CD4$^+$Foxp3$^+$CD127$^{Low/-}$; however, when these cells were defined as CD4$^+$CD25$^+$CD127$^{Low/-}$, a significant decrease in the frequency were observed compared to CD4$^+$Foxp3$^+$ (HIV-U: 8% (5-15%) *vs* 14% (12-16%) or HIV-VL: 12% (7-14%) *vs* 18% (16-27%); p<0.001) (Fig. **2C**). Again, in healthy controls the percentage of Treg in GALT was similar independent of the protocol used (Fig. **2C**, upper panel), and the Treg frequency was significantly lower compared to HIV patients, using CD4$^+$Foxp3$^+$CD127$^{Low/-}$ or CD4$^+$Foxp3$^+$ protocols (both, p<0.001). No differences were observed using the CD4$^+$CD25$^+$CD127$^{Low/-}$ protocol. The Treg frequency in GALT was not measured in flu-like patients.

The comparison between tissues showed that the Treg frequency increases in GALT compared to peripheral blood in all 3 groups tested: controls, HIV-U and HIV-VL; the increase was ~1.7, ~2, ~2.4 times, respectively, when the protocols CD4$^+$Foxp3$^+$ (p=0.001) and CD4$^+$Foxp3$^+$CD127$^{Low/-}$ (p=0.003) were used. Treg identification with the protocols CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^+$CD127$^{Low/-}$ in PBMC (r=0.80, p=0.0001) and GALT (r=0.82, p=0.0001) from HIV patients exhibit the highest positive correlation (Fig. **3A**, **B**), indicating that both protocols are suitable to determine Treg frequency in these samples.

HIV-1 Infected Patients Exhibit Alteration in the Expression of CD25 Molecule in CD4$^+$Foxp3$^+$ T Cells in Peripheral Blood and Rectal Tissue

To evaluate potential variations in the Treg phenotype, the expression of CD25 was analyzed in the Foxp3 CD4$^+$ gate. We measured the percentage of cells expressing this molecule and the mean MFI of its expression (Fig. **1B**). As shown in Fig. (**4A**), the percentages of CD25$^+$ Treg cells are lower in HIV patients compared with healthy controls and flu-like patients. In the HIV-U, the percentages of CD25$^+$ cells were 42% (35-58%) and 33% (27-47%) in PBMC and GALT respectively; in HIV-VL these percentages correspond to 39% (25-40%) in PBMC and 35% (26-49%) in GALT, compared to 56% (51-64%) and 47% (35-53%) in PBMC and GALT, respectively, in cells from controls. In fact, the percentage of CD25$^+$ Treg from HIV patients (with or undetectable VL) was about ~1.4-fold lower than from controls in both PBMC and GALT (p<0.05) (Fig. **4A**, lower panel). The percentage of CD25$^+$ Treg in PBMC from flu-like patients was 58% (51-60%), similar to controls (Fig. **4A**, middle panel). Analyzes of CD25 in Tcon (CD4$^+$Foxp3$^-$) showed that the percentage of CD25$^+$ in PBMC and GALT increases in cells from HIV patients (data not shown), suggesting that the low expression of CD25$^+$ occurs only in the Treg subset.

CD25$^+$ Treg from HIV-1 Patients with Detectable Viral Load and Flu-Like Infected Individuals Exhibit Higher Density of the CD25 Molecule

We then examined whether the reduction of the CD25 molecule in Foxp3$^+$CD4$^+$ cells from HIV patients affects not only the percentage of cells expressing this molecule but also the density of its expression per cell. As shown in Fig. (**4B**), the CD25 MFI in Treg from control samples was 17 (13-20) and 11 (8-16), in PBMC and GALT, respectively; in HIV-U

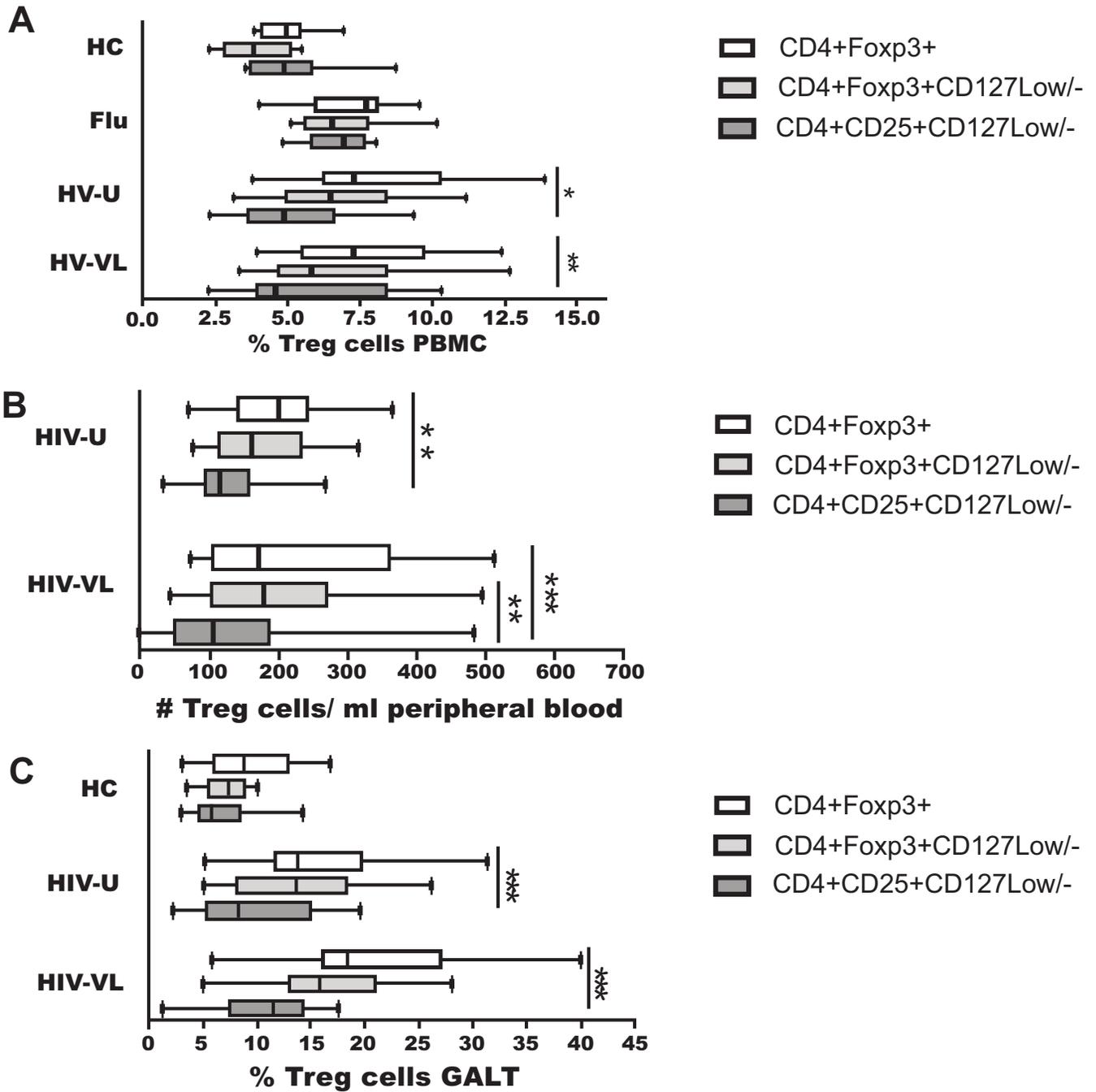**Fig. (2). Frequency of Treg CD4⁺Foxp3⁺, CD4⁺Foxp3⁺CD127^Low/- and CD4⁺CD25⁺CD127^Low/- in samples from healthy controls, individuals with acute flu-like symtoms, chronically HIV-infected with or undetectable viral load.** Whisker box figures show phenotypic characterization of Treg. **A)** Percentage of Treg from PBMC of healthy controls (n=13) (upper panel), flu-like symptoms patients (n=9) (middle panel), HIV-U (n=13) (middle panel) and HIV-VL (n=19) (lower panel). **B)** Treg absolute count in PBMC samples from HIV-U and HIV-VL patients. **C)** Percentage of Treg from GALT of healthy controls (upper panel), HIV-U (middle panel) and HIV-VL (n=10) (lower panel). Median and 25-75 interquartiles ranges are represented. Intra groups were compared by the Friedmann test and Dunn's multiple post-test. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0001.

the CD25 MFI was 35 (18-60) and 15 (9-57). Although in these patients the range was very wide, no significant differences were found in HIV-U *vs* HC.

In contrast, patients with acute flu-like or HIV chronic infection with detectable viral load exhibits the highest levels of CD25 MFI, compared with controls. In flu-like samples,

the CD25 MFI was 75 (64-88) in PBMC; it was 87 (51-97) and 62 (16-76) in HIV-VL samples from PBMC and GALT, respectively (Fig. **4B**). These results suggest that viral replication could modify the density of the expression of CD25 molecule on Treg. To evaluate the strength of the association between the Treg phenotype and viral load,
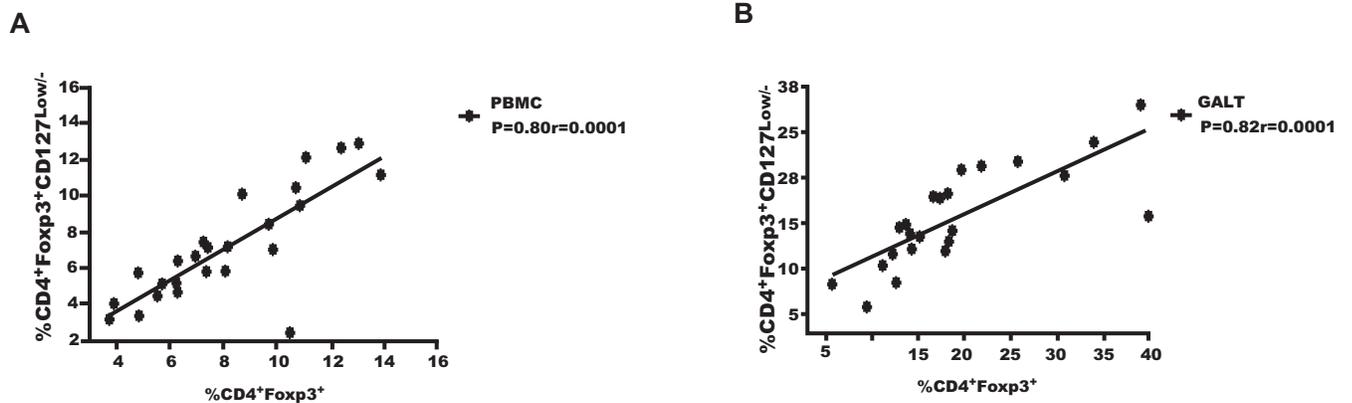
Fig. (3). Correlations between the different protocols to characterize the Treg cells in samples from PBMC and GALT. Spearman correlations between Treg frequency charactized by CD4^{+}Foxp3^{+} and CD4^{+}Foxp3^{+}CD127[Low/-] in **A**) PBMC. **B**) and GALT.
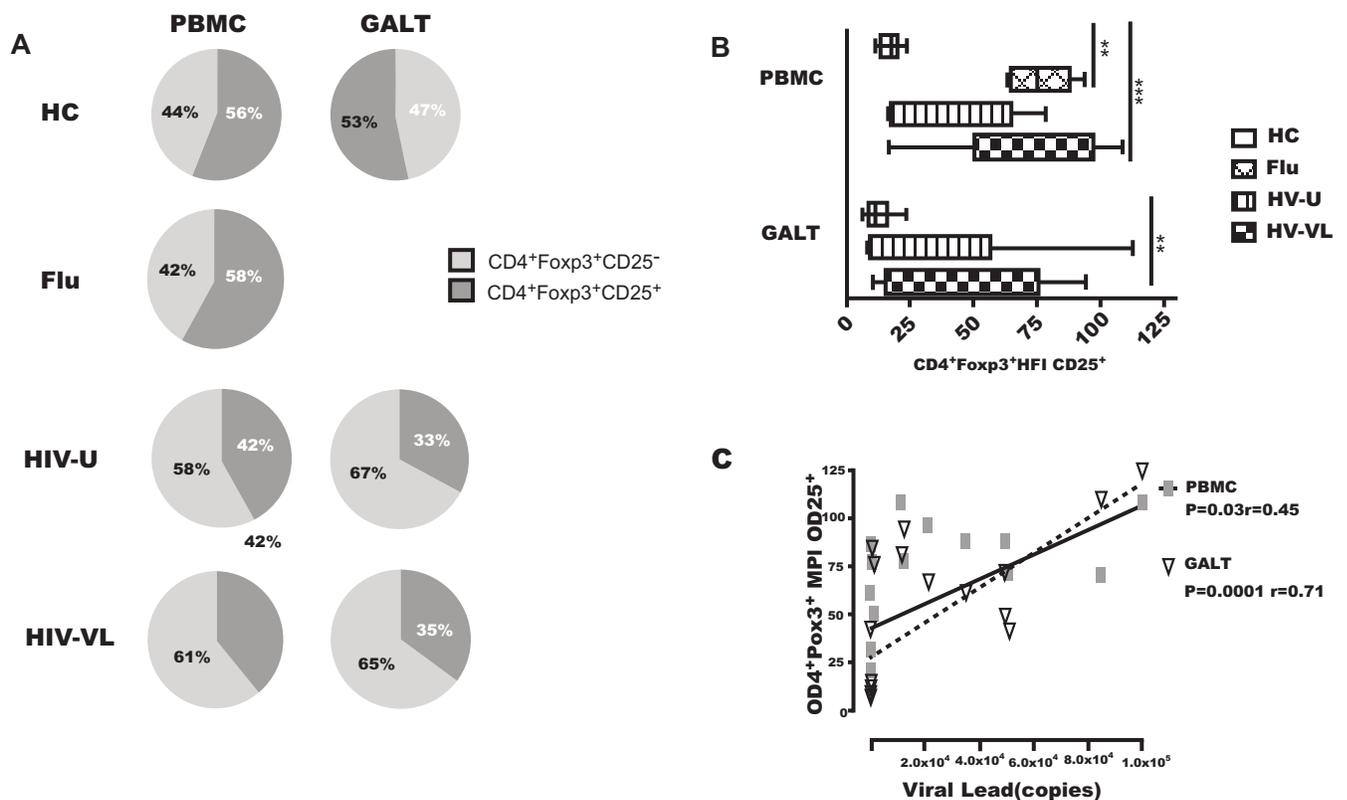

Fig. (4). Percentage and MFI of CD25 molecule of Treg in samples from healthy controls, chronically HIV-infected and individuals with acute flu-like symptoms. A) Pie charts show the percentage of Treg (CD4^{+}Foxp3^{+}) expressing the CD25 (CD4^{+}Foxp3^{+}CD25^{+}) or negative for the CD25 molecule (CD4^{+}Foxp3^{+}CD25^{-}) in PBMC and GALT samples from controls (n=10), HIV-U (n=13); HIV-VL (n=19), and in PBMC from flu-like patients (n=9). **B**) Whisker box figures show MFI of CD25 in Treg (in the gate of CD4^{+}Foxp3^{+}CD25^{+}) in PBMC and GALT samples; median and interquartile ranges are represented. **C**) MFI of CD25 of Treg in PBMC and GALT were positively correlated with HIV viral load. Groups were compared by the Kruskal Wallis test and Dunn's multiple comparisons post-test. Significant differences are indicated by **p < 0.01, ***p < 0001.

correlations were examined in HIV+ patients. Interestingly, the CD25 MFI in Treg from PBMC and GALT was positively correlated with viral replication (PBMC r=0.45, p= 0.03; GALT r=0.71, p=<0.0001) (Fig. **4**C).

Finally, we analyzed whether the changes on the CD25 expression on Treg was due to an alteration in the expression of the transcription factor Foxp3 (Fig. **1**C); the MFI of Foxp3 was similar among Treg from controls, flu and HIV

patients on both tissues (PBMC; *p*=0.635 and GALT; *p*=0.802), ruling out this possibility (Fig. **5**).

In conclusion, these results indicate that Treg from chronic HIV infected patients exhibit an alteration in the expression of the CD25 molecule, resulting in a reduced percentage of CD25^{+} cells. This finding suggests that detection of Treg based only in the expression of the CD25 molecule, could result in an underestimation of the

frequency of this cell subset in HIV infected individuals. On the other hand, the higher intensity of CD25 expression per cell within the positive cell subpopulation could be associated with the chronic state of hyperactivation during HIV infection.
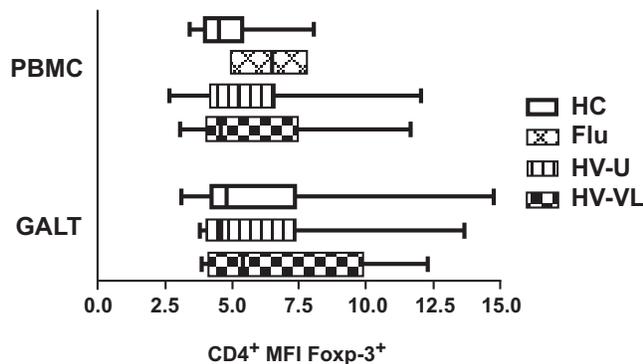

Fig. (5). MFI of the transcription factor Foxp3 on samples from healthy controls, chronically HIV-infected and individuals with acute flu-like symtoms. Whisker box figures show MFI of Foxp3 in Treg in PBMC and GALT samples from controls (n=10) and HIV patients (HIV-U n=13; HIV-VL n=19), and in PBMC from flu-like patients (n=9). Groups were compared by the Kruskal Wallis test and Dunn's multiple comparisons post-test.

DISCUSSION

Since the description of Treg, their phenotypic characterization has been complex; in the beginning, high expression of CD25 in the $CD4^+$ T cell subset was widely used to characterize them. Unfortunately, the ability to accurately gate for CD25 is rather arbitrary and the expression of this molecule is not specific to Treg. Several studies demonstrated that low or negative CD127 expression is an excellent biomarker of Treg, particularly in combination with CD25 or Foxp3. In fact, CD127 and CD25 stain allows human Treg enrichment for *in vitro* functional studies [23, 24]. So far, the combination of CD25, CD127 and Foxp3 expression is considered the best way to distinguish between human Treg and $CD4^+$ Tcon [23]. According to previous studies, similar percentages of Treg were reported when these cells were defined with the combination of these molecules ($CD25^+Foxp3^+$; $CD25^+CD127^{Low/-}$, $Foxp3^+CD127^{Low}$) in basal conditions in PBMC and lymphoid nodes from adults, children and cord blood from neonates, without infection or metabolic diseases [16].

In addition, the characterization of Treg in patients with viral respiratory acute infections is largely unknown. During the first week of influenza H1N1 [25, 26] and influenza A [27] infection an increase in the frequency of Treg in PBMC were reported. In agreement with these results, we also observed high frequency of Treg in patients with flu-like symptoms compared to uninfected controls. No differences were observed in the protocol used to identify these cells. Unfortunately, we could not obtain respiratory tract samples from the flu-like patients to compare with PBMC.

In contrast to controls and patients with flu-like illness, in this study the analysis of Treg in chronic HIV infected individuals shows that the frequency of this cell subpopulation varies depending on the protocol used; in particular, the detection of $CD25^+CD127^{Low/-}$ cells produced a lower frequency of Treg compared to the protocols in which the detection of Foxp3 was included. In this respect, Bi *et al.,* 2009 described that gating $CD4^+CD25^{+/hi}CD127^{Low}$ was difficult to analyze because staining showed a smear of both CD25 and CD127 and proposed that gaiting Foxp3 in CD4 cells gave a clearer staining [28]. In accordance to this previous report, our results also indicate that the identification of Treg cells in samples from HIV patients should rely in the detection of the marked Foxp3 alone or in combination with CD127; in fact, these two protocols showed the highest correlation in both types of samples. Although the CD25 molecule is a key phenotypic and functional marker of Treg, the detection of this cell subset based on its expression might result in underestimation of these cells.

In accordance with our results, Ndhlovu *et al.,* 2008 reported a similar frequency of Treg, defined as $CD25^+$ $CD127^{Low/-}$ cells in PBMC in HIV patients, with both primary and chronic infection, and controls; however, when these authors characterized these cells as $Foxp3^+CD127^{Low/-}$, the patients exhibited an increase of this subset compared to controls [10]. In this report, the authors hypothesized that HIV patients contain different sub-subsets of Treg with different phenotypes and functions. Also, Pozo-Balado *et al.,* 2010 reported that the $CD4^+CD25^{+/hi}CD127^{Low}$ phenotype does not accurately identify Treg in PBMC from viremic patients [29]. However, in this report the comparison did not include healthy controls or $Foxp3^+$, $Foxp3^+CD127^{Low/-}$ analysis. In samples from GALT, epple *et al.,* 2006 [5] and Shaw *et al.,* 2011 demonstrated that the frequency of Treg was consistently higher in HIV patients with high viral loads compared to seronegatives [30]. However, in peripheral blood, the detection of Treg with the $CD25^+CD127^{lo}$ protocol indicated a lower frequency of these cells in HIV patients with undetectable viral load (controllers) compared to uninfected individuals [30]. Together, these data are in agreement with the fact that Treg increase during HIV infection and their characterization changes depending on how these cells are analyzed, indicating that classic strategies to detect Treg cells might be suitable, but carefully considered, depending on the type of viral infection.

The results of the present study suggest that there is an expansion/accumulation of Treg during HIV infection in both peripheral blood and GALT tissue; the expanded cells exhibit an altered expression of the CD25 molecule. Indeed, it was previously shown that the percentage of Treg in PBMC expressing the CD25 molecule ($Foxp3^+CD25^+$) is about 45% in controls and 34% in HIV patients [31]. Interestingly, the majority of evidence indicating the low frequency of CD25 in Treg is reported particularly in tissue samples [5, 32, 33]. In fact, using immunohistochemistry and immunofluorescence analyses in samples from lamina propia, lower CD25 expression in $Foxp3^+$ cells has been reported [5]. Confocal micrographs from tonsillar biopsies have shown $Foxp3^+CD25^+$ percentages of 19%, 44%, and 75% in HIV progressors, non progressors, and uninfected donors, respectively [33]. Other reports on tonsillar biopsies detected only a 19% and 16% of $Foxp3^+CD25^+$ cells in HIV-untreated and treated patients, respectively [32]. Analysis of $CD25^+$ and $CD25^-$ $CD4^+$ T cells from in spleens and lymph

nodes of SIV-infected macaques, confirm that the population of Foxp3 expressing cells in lymphoid tissues is not limited to a $CD25^+$ phenotype and that $CD25^-$ $Foxp3^+$ may be present in lymphoid tissues during chronic infection [34]. Unfortunately, comparisons in the frequency of Treg using different phenotypic markers were not performed by these authors. Although previous findings have demonstrated a decrease in the expression of CD25, this article along with the recently published by Shaw *et al.,* [30] are the only ones that include a comparison of different current protocols for identifying Treg cells in peripheral blood and GALT samples in HIV infected patients.

In the present study we report that significant changes in the CD25 expression occur in Treg from HIV patients in both at the percentage of cells expressing this molecule and at the density of expression, measured in terms of MFI. Experimental evidence suggest several possibilities to account for this observation: (i) chronic expansion of Treg *in vivo* results in low levels of CD25 expression, retaining their suppressive activity, as previously reported in a murine model [35]; this constant expansion (high Treg frequency) is observed also in GALT from HIV infected patients [5, 30]; (ii) it might reflect internalization of the CD25 molecule after IL-2 binding [36]; in fact, the expansion of $CD4^+CD25^{lo}CD127^{lo}Foxp3^+$ Treg is one of the long-term effects of IL-2 therapy in HIV patients [37, 38]; (iii) *in vitro* assays have shown that HIV induces down regulation of the CD25 molecule in $CD4^+$ infected cells [39]. In addition, HIV viral proteins are involved in this process, inhibiting IL-2R expression; HIV p29 protein induces a two fold increase in the intracellular cyclic adenosine 3',5'-monophosphate (cAMP) in PBMC which is associated with the decrease of CD25 [40]. HIV Tat protein and the Gp120 also decrease the expression of this molecule in Jurkat cells line [41] and $CD4^+$ T cells [42], respectively.

On the other hand, it is important to note that CD25 expression per cell in Treg from HIV-untreated and flu-like patients increases compared to controls despite similar levels of Foxp3 expression, suggesting a competitive advantage for IL-2, reducing the available levels of this cytokine in the microenvironment [43]. The increase of CD25 MFI can be explained as a consequence of cellular activation; $CD4^+$ T cells exposed to HIV upregulated the expression of CD25, in Tcon ($CD4^+CD25^-$) [44] and in Treg ($CD4^+CD25^+$) cells [33], along with the expression of other activation molecules such as HLA-DR [45, 46]. In fact, there was a positive correlation between the CD25 MFI and the viral load, supporting that HIV induces activation of $CD4^+$ and Treg cells as previously reported [46, 47]. Interestingly, the MFI of CD25 in HIV-U was lower compared to HIV-VL patients, supporting the idea that cellular activation is partially controlled by antiretroviral treatment [48].

In conclusion, here we report an additional phenotypic alteration of Treg from HIV patients. The percentage of Treg expressing CD25 decreases, making the detection of this molecule an unsuitable tool to characterize Treg in HIV patients, particularly in tissue samples. The data presented here also suggest that the use of flow sorting and magnetic columns to deplete Treg based on the expression of CD25 [49, 50] does not ensure complete elimination of this cell population, which may affect functional assays. Although we are aware that the sample size was limited, and the results required further verification, these results point to the urgent requirement to develop additional methods to deplete Treg from PBMC, as a way to measure the role of this cell subpopulation in the context of chronic infections, in particular HIV.

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COMPETING INTERESTS

The authors have no conflicts of interest.

ETHICAL APPROVAL

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Antioquia, Medellin, Colombia.

ABBREVIATIONS

PBMC = Peripheral blood mononuclear cells

GALT = Gut associated lymphoid tissue

RC = Rectal cells

Tregs = Regulatory T cells

Tcon = Conventional T cells

HC = Healthy controls

HIV = Human immunodeficiency virus

FLU = Individuals with acute flu-like symptoms

HAART = Highly active antiretroviral therapy

VL = Viral load

FCS = Fetal calf serum

DPBS = Dulbeco´s phosphate buffered saline

MFI = Mean fluorescence intensity.

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