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Regulatory T cells modulate monocyte functions in immunocompetent antiretroviral therapy naive HIV-1 infected people

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Abstract

We previously demonstrated that the overall number of regulatory T (Treg) cells decrease proportionately with helper $CD4^+$ T cells and their frequencies increase in antiretroviral therapy (ART)-naive human immunodeficiency virus type-1 (HIV-1) infected individuals. The question now is whether the discrepancies in Treg cell numbers and frequencies are synonymous to an impairment of their functions. To address this, we purified Treg cells and assessed their ability to modulate autologous monocytes functions. We observed that Treg cells were able to down modulate autologous monocytes activation as well as interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) production during stimulation with polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (poly-ICLC). This activity of Treg cells has been shown to be influenced by immunocompetence including but not limited to helper $CD4^+$ T cell counts, in individuals with HIV-1 infection. Compared to immunosuppressed participants (CD4 < 500 cells/μL), immunocompetent participants (CD4 ≥ 500 cells/μL) showed significantly higher levels of transforming growth factor beta (TGF-β) and IL-10 ($p < 0.001$ and $p < 0.05$, respectively), key cytokines used by Treg cells to exert their immunosuppressive functions. Our findings suggest the contribution of both TGF-β and IL-10 in the suppressive activity of Treg cells.

Keywords Antiretroviral therapy-naive HIV-1 infection, Monocyte function, Regulatory T cells, Sustained activation/Inflammation

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Introduction

Chronic infection with HIV-1 maintains the immune system in a perpetual state of activation ultimately culminating into severe $CD4^+$ T cells depletion and loss of immune functions [1, 2]. A gradual decline in immune function during HIV-1 infection is consistently linked to the deterioration of mucosal barrier integrity. This deterioration can lead to ongoing exposure to microbial products found in the gut, such as bacterial endotoxin lipopolysaccharide (LPS), which is a potent stimulator of the immune system. LPS, a Toll-like receptor 4 (TLR4) ligand through binding to CD14 on monocytes activates and induces the production of large amounts of pro-inflammatory cytokines including IL- 1, IL-6, TNF-α and type 1 interferons (IFN-1) [3–8]. In addition, a wide range of chemokines are also produced by activated monocytes further facilitating the recruitment of additional leukocytes to the inflammation site [9]. As a result, this process increases inflammation, triggers activation of naive and central memory $CD4^+$ T cells, which in turn become new targets for HIV-1 infection. Consequently, this accelerates the depletion of helper $CD4^+$ T cells [10]. Sustained depletion of helper $CD4^+$ T cells during ART naive HIV-1 infection may impair critical functions of forkhead box P3 (FoxP3) -expressing $CD4^+$ Treg cells. These functions involve maintaining peripheral tolerance and immune homeostasis [11, 12]. Dysfunction of Treg cells has been linked to a range of conditions, including allergies, autoimmune diseases, cancers, and early rejection of transplanted organs [13, 14]. Therefore, regulating the severity of inflammatory responses could help to slow down the disease progress by reducing the number of target cells available for HIV replication, minimizing tissue injury, preventing the apoptosis of uninfected $CD4^+$ T cells and allowing sufficient immune pressure against HIV.

In steady state, Treg cells have been shown to play an important role in maintaining immune homeostasis by suppressing excessive activation, proliferation and effector functions of adaptive and innate immune cells. To accomplish this, Treg cells can use a variety of mechanisms including direct cell-cell contact, secretion of inhibitory cytokines, or competing for growth factors. However, in the context of ART naive HIV-1 infection, it is not known how the Treg cells function. Previous studies including report from our group [15] have demonstrated that the total number of Treg cells decreases in proportion to helper $CD4^+$ T cell counts [15–19]. Nevertheless, it is not known if a reduction in Treg cell number is synonymous with an impairment of their functions since their frequencies have been shown to be comparatively higher in HIV-1 infected individuals [15].

This study aimed to investigate if autologous Treg cells could modulate monocyte activation as well as their production of pro-inflammatory cytokines when stimulated with poly-ICLC.

Material and methods

Study population

Thirty (30) ART-naive HIV-1-infected participants (21 female and 9 male) were recruited from the Chantal Biya international reference center (CIRCB) African HIV-1 dendritic cell targeted vaccine (AFRODEC) cohort established in Cameroon since 2012. Ten (10) HIV-negative participants (6 female and 4 male) were also recruited as controls. In addition to people who did not provide consent, participants who had been diagnosed with hepatitis B virus, hepatitis C virus, Dengue virus, *Mycobacrerium tuberculosis*, or malaria were excluded from the study. Helper $CD4^+$ T cell counts for both HIV^+ and HIV^- participants were determined in fresh whole blood by BD multitest CD3/CD8/CD45/CD4 and TruCount tubes (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Plasmatic HIV-1 viral load was determined on the *m2000rt* machine using the Abbott Real-Time HIV-1 Assay protocol.

Cell preparation

Isolation of peripheral blood mononuclear cells

Twenty milliliters (20 mL) of peripheral blood were collected from each consenting participant in Ethylene Diamine Tetra Acetic acid (EDTA) tubes by an experienced nurse. The Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood within 2–4 h of sampling by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden). Briefly, venous blood was diluted with an equal volume of 1x phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Mediatech, corning, NY), then carefully overlaid on Ficoll-Paque Plus and centrifuged at 300 g at 21°C for 20 min. The Mononuclear-cell-rich interface was harvested, washed twice in 1x PBS without Ca^{2+} and Mg^{2+} and counted on a bright line hemocytometer (improved Neubauer, 0.100 mm deep; Hausser Scientific, Horsham, PA). The cells were finally re-suspended at a final concentration of $1*10^7$ cells/mL in Magnetic Activated Cell Sorting buffer (MACS BSA stock solution 1:20 autoMACS rinsing solution; Miltenyi Biotec, Bergish Gladbach, Germany) for Treg cell and monocyte purification.

Purification of treg cells

Treg cells were isolated from PBMCs using the $CD4^+CD25^+$ $CD127^{dim/-}$ Treg cell isolation kit II supplied by Miltenyi Biotec, following the manufacturer's protocol (Miltenyi Biotech). Firstly, $CD4^+$ T cells were negatively isolated from PBMCs with $CD4^+CD25^+CD127^{dim/-}$ T cell biotin- antibody cocktail II and anti-biotin microbeads.

Isolated cells were then washed and depleted of $CD4^-$ and $CD127^{high}$ cells using Miltenyi LD columns. Next, Treg cells ($CD4^+CD25^+$ $CD127^{dim/-}$ Treg cells) were purified from $CD4^+$ T cells by positive selection using Miltenyi CD25 microbeads II. The purity of Treg cells was assessed by flow cytometry using a Becton Dickinson (BD) Fortessa X-20 (BD Biosciences).

Purification of monocytes

Monocytes were isolated from PBMCs using the CD14 MicroBeads provided by Miltenyi Biotec, following the manufacturer's protocol (Miltenyi Biotech). Briefly, PBMCs were incubated with CD14 MicroBeads for positive selection of $CD14^+$ cells using Miltenyi LS columns. The purity of $CD14^+$ cells was assessed by flow cytometry using a BD Fortessa X-20 (BD Biosciences).

Note: CD14 microbeads are known to activate monocytes during the purification process. However, this non-specific activation is not expected to affect the comparison between the HIV^- and HIV^+ groups.

Monocyte-T reg cell co-culture

Cells were cultured in a complete medium, R_{10} [RPMI 1640 medium supplemented with glutamine (MACS Media, Miltenyi Biotec), 10% heat inactivated fetal bovine serum (FBS; Mediatech, corning), and 1% penicillin/streptomycin (Gibco)] at 37°C in an atmosphere of 5% CO_2. Monocytes (2×10^5 cells/mL) were either cultured alone, treated with Poly-ICLC (10 µg/mL; Hiltonol, Oncovir, Washington, D.C.) or co-cultured with autologous Treg cells in 96-well U-bottom plates (Costar, Corming incorporated, USA) at a 1:1 ratio in a final volume of 200µL. One hour (1 h) after the addition of Poly-ICLC, brefeldin A (1 µg/mL; BD Biosciences) was added for the last 5 hours.

Evaluation of functional activity of regulatory T cells

Antibodies

The monoclonal antibodies (mAbs) used for surface staining in this study were sourced from various suppliers. They included Brilliant Violet (BV)- 421 conjugated anti- CD127 (clone 9HIL-7R-M21), BV605 conjugated anti-CD25 (clone 2A3), Phycoerythrin (PE)-CF594-labeled anti-CD16 (clone 3G8), Peridin-chlorophyll protein cyanine five-five (PerCP-CY5-5)-conjugated anti- CD14 (clone MØP9), and Fixable Viability stain 510, all purchased from Becton Dickinson (BD Biosciences). Additionally, Allophycocyanin (APC) Vio770-conjugated anti-CD3 (clone BW 264/56) and PE- Texas Red-conjugated anti-CD4 were obtained from Miltenyi Biotec and Beckman coulter, respectively. PE-conjugated anti-CD127 (clone HIL-7R-M21) and APC-labeled anti-CD38 were provided by BD pharmigen. Alexa-Fluor 700-labeled anti-human leukocyte antigen-DR (HLA-DR) (clone LN3) was obtained from eBiosciences.

For intracellular cytokine analysis, we used BV-650-labeled anti-IL-10 (clone JES3-9D7), BV-421-labeled anti-IL-6 (clone MQ2-13A5), PE-conjugated anti-TGF-β1 (clone TW4-9E7), phycoerythrin cyanine seven (PECY7)-labeled anti-TNFα (clone MAb11) and APC-labeled anti-IL4 (clone 8D4-8), all purchased from BD. PerCP-CY5-5-labeled anti- IL-17 A (clone N49-653) and PE-Cy7-labeled anti-FoxP3 (clone PCH101) were obtained from eBiosciences.

In order to determine the optimal concentration for each antibody, serial dilutions were performed starting from the concentration recommended by the manufacturers.

Analysis of cytokine profile of monocytes and regulatory T cells using multiparametric flow cytometry

After 6 h of incubation, cells were washed twice with Fluorescence Activated Cell Sorting (FACS) buffer [1x PBS with Ca^{2+} and Mg^{2+} + 2% heat inactivated FBS] and then surface stained with a cocktail of fluorochrome-labeled antibodies either to CD14 and live-dead for monocytes or to CD3, CD4, CD25, CD127 and live-dead for Treg cells. The cells were subsequently fixed and permeabilized with FoxP3 fixation/permeabilization buffer (e-Bioscience) at 4 °C for 45 min in the dark, followed by intracellular staining with a cytokine cocktail consisting of either IL-6 and TNF-α for monocytes or Foxp3, IL-4, IL-10, IL-17 A and TGF-β for Treg cells. Two rounds of cell washes with FoxP3 permeabilization buffer (e-Bioscience) were employed before and after intracellular labeling. Finally, the cells were washed, re-suspended in FACS buffer, and acquired on BD LSRFortessa X-20 cytometer using BDFACS Diva Software.

Determination of the stimulation and suppression indexes

To evaluate the suppressive activity of Treg cells, we measured the responsiveness of monocytes to poly-ICLC stimulation. We calculated the stimulation index by dividing the Mean Fluorescence Intensity (MFI) of antibody (Ab) expression after poly-ICLC treated monocytes by the MFI of Ab expression in unstimulated monocytes. The Treg cell suppression index was determined using the following formula: 1- (MFI of Ab expression of poly-ICLC-treated monocytes in the presence of Treg cells / MFI of Ab expression of poly-ICLC-treated monocytes in the absence of Treg cells).

Statistical analysis

All flow cytometry data were analyzed using FLOWJO software, version 9.8.5, from Treestar (Ashland, OR), and statistical analyses were performed using GRAPHPAD PRISM, version 5.0 (Graphpad, San Diego, CA). The data

were presented as median (25-75th centile), and non-parametric tests were used. To compare medians among two groups, the U-Mann- Whitney test was utilized, while the Kruskal-Wallis test with Dunn's multiple comparisons post-test was used to assess differences between more than two groups. Correlations were determined using the Spearman test, and P-values < 0.05 were considered statistically significant.

Results

Study population

Table 1 summarizes the characteristics of the study population. Overall, 40 participants were enrolled in this study. ART-naive HIV-1 infected participants (30) consisted of 21 (70%) female and 9 (30%) male aged of 37 (33–40) and 40 (37.5–45) years old, respectively. The median ages of their sex-matched seronegative counterparts were 34.50 (31.50-38.75) and 32 (28.50–47.50) years old for female (60%) and male (40%), respectively. As expected, HIV-1^+ participants showed lower helper $CD4^+$ T cell counts than healthy controls: 498 (298.5–538) cells/μL Vs. 938 (765.3–1142) cells/μL in female ($p<0.0001$) and 503 (277.5-526.5) cells/μL Vs. 752 (637.8-833.5) cells/μL in male ($p<0,001$). Whereas a majority of HIV-1^+ participants (51%) showed no significant immune suppression (CD4 ≥ 500 cells/μL), 16% had mild immunosuppression (CD4 between 350 and 499 cells/μL), 20% had advanced immunosuppression (200–349 cells/μL) and 13% had severe immunosuppression (CD4 < 200 cells/μL). Comparatively, male showed a 0.9 Log higher HIV-1 plasmatic viral load than female. There was no significant difference ($P=0.12$) in the duration of HIV-1 infection in both male [4 (2–6) years] and female [5 (4–6) years].

Purity of Treg cells with respect to HIV-1 disease progression

In our recent study [15], we observed that chronic HIV-1 infection leads to a depletion of T reg cell number proportionally to helper $CD4^+$ T cells. Since such an HIV-1 mediated reduction in Treg cell numbers could create an artificial variation during suppression studies, we magnetically sorted Treg cells as described in the methods section, and utilized the same numbers from all participants during the immunosuppression analysis. Dot plots showing the gating strategy for identifying Treg cells are shown in Fig. 1a. The $CD3^+$ $CD4^+$ $CD127^{Low}$ $CD25^+$ T cells obtained were more than 95% pure and highly expressed FoxP3 marker (>70%). The median numbers of $FoxP3^+$ Treg cells after purification were consistent across all CD4-related categorization: severe immunosuppression [539 cells/μL (380.3-585.3)], advanced immunosuppression [502 cells/μL (402.8-730.5)], mild immunosuppression [514 cells/μL (439.5-642.5)], and no significant immune suppression [558 cells/μL (499–615)]. Similar Treg cell numbers were also observed regarding the plasmatic HIV-1 viral loads (VL) after purification: 579 cells/μL (419–660) for VL < 3, 514 cells/μL (490–673) for VL between 3 and 4, and 619 cells/μL (517.5-717.8) for VL > 4 Log_{10} copies/mL, respectively.

Cytokine profile of purified Treg cells from HIV-1 infected participants

To exert immunosuppressive functions, several strategies including direct cell-cell contact, the secretion of inhibitory cytokines or competition for growth factors are employed by Treg cells [20–24]. Here to understand the nature of Treg cells in the context of ART-naive HIV-1 infection, we have assessed key cytokines associated with their functions. Magnetically sorted Treg cells as described in the methods section were stained

Table 1 Study population

Variable	HIV negative participants ($n=10$)		HIV-1-infected participants ($n=30$)	
Sex	Male	Female	Male	Female
Participants (%)	4 (40)	6 (60)	9 (30)	21 (70)
Median age (IQR)	32 (28.50–47.50)	34 (31.50-38.75)	40 (37.50–45)	37 (33–40)
Median CD4 count (cells/μL)	752 (637.8-833.5)	938 (765.3–1142)	503 (277.5–526,5) **	498 (298.5–538) ***
CD4 > 500 cells / μL (n/%)			5 (17)	10 (34)
350–499 cells / μL (n/%)			1 (3)	4 (13)
200–349 cells / μL (n/%)			2(7)	4 (13)
CD4 < 200 cells/ μL			1(3)	3(10)
Median viral load (Log_{10}copies/ml)	NA	NA	4.31 (3.27–5.17)	3.41 (2.96–3.96)

** $P<0.001$; *** $P<0.0001$ in the U Mann Whitney test; NA, Not Applicable; IQR, interquartile range

n = number of participants; %= percentage

A total of 30 antiretroviral naive HIV-1 infected participants were enrolled in this study. The greater number (70%) consisted of females aged 37 (33–40) years and the rest (30%) males aged 40 (37, 5–45) years. As shown in Table 1 above, 10 participants were recruited as HIV negative controls. As expected, helper $CD4^+$ T cell counts were lower in HIV-1 infected males ($p=0.002$) and females ($p=0.0003$) compared to their sex-matched controls. Whereas a majority of participants (51%) showed no significant immune suppression (CD4 > 500 cells/ μL), 16% had mild immunosuppression (CD4 between 350 and 499 cells/ μL), 20% had advanced immunosuppression (200–349 cells/ μL) and 13% had severe immunosuppression (CD4 < 200 cells/ μL). Comparatively, male participants showed a 0.9 Log higher HIV-1 plasmatic viral load than female participants

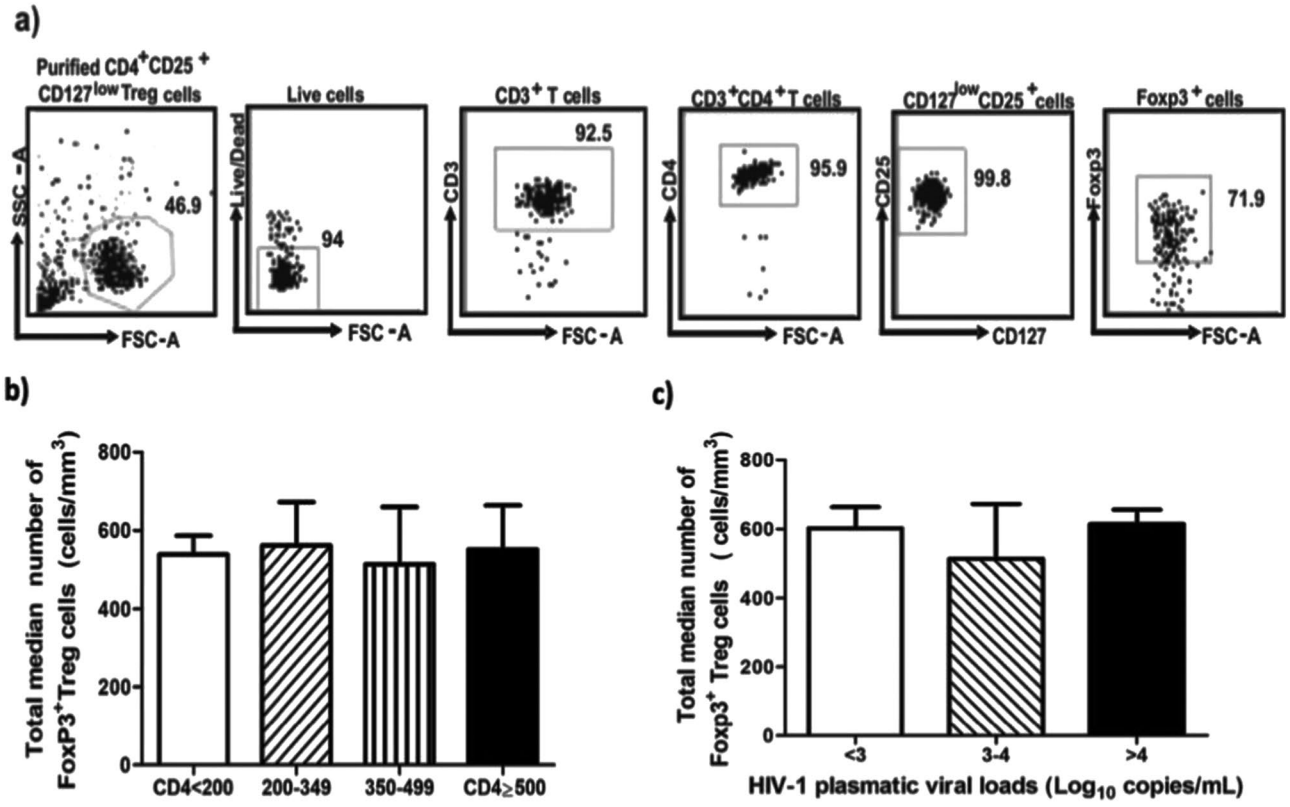

Fig. 1 Purity of Treg cells in relation to the disease categorization. Dot plots of the gating strategy for magnetically purified Treg cells are shown in **a**. Purified $CD4^+CD25^+$ $CD127^{dim/-}$ Treg cells were gated based on forward and side scatter. $CD3^+$ $CD4^+$ T cells were selected from live cells and then analyzed for co-expression of $CD127^{Low}$ $CD25^+$. $CD3^+$ $CD4^+$ $CD127^{Low}$ $CD25^+$ T cells were more than 95% pure and highly expressed FoxP3 marker (> 70%). The number of FoxP3+ Treg cells was stratified by the participants' immunological (**b**) and virological (**c**) statuses. No significant differences ($p > 0.05$) were found in the number of $FoxP3^+$ Treg cells across all the subgroups after purification. Horizontal bars represent the median and statistical difference was calculated using Kruskal-Wallis test

intracellularly for IL-4, IL-10, IL-17 A and TGF-β and then analyzed by multiparametric flow cytometry. Dot plots showing expression of each cytokine by $CD4^+CD25^+CD127^{Low}$ $FoxP3^+$Treg cells are shown in Fig. 2a. The differences between groups were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison post-test. ART-naive HIV-1 infected participants with helper $CD4^+$ T cell counts ≥ 500 cells/ μL showed significantly higher levels of TGF-β and IL-10 MFI (Fig. 2b and c) compared to people with helper $CD4^+$ T cell counts < 200 cells/ μL ($p < 0.001$ for TGF-β_1 and $p < 0.05$ for IL-10) and those with helper $CD4^+$ T cell counts between 200 and 349 cells/μL ($p < 0.05$ for TGF-β). This elevated cytokine expression was similar to that seen in uninfected individuals [826 (749–973) Vs. 824 (689–1056) for TGF-β and 490 (467–510) Vs. 510 (413–524) for IL-10]. Compared to uninfected participants, a significant reduction in IL-4 expression (Fig. 2d) was observed in ART naive HIV-1 infected participants with helper $CD4^+$T cell counts < 200 cells/ μL ($p < 0.001$), between 200 and 349 cells/ μL ($p < 0.001$) and between 350 and 499 cells/ μL ($p < 0.05$). Regarding IL-17 expression (Fig. 2e), no difference was observed in both HIV-1 infected and uninfected participants.

CD38 surface marker is more reliable than HLA-DR for monocyte activation

Chronic immune activation is considered as the major driving force of $CD4^+$ T-cell depletion and the inability of immune system to control a wide range of potential pathogens [25]. In this study, we assessed the activation of purified monocytes by analyzing the surface expression of HLA-DR and CD38 in MFI, as shown by the gating strategy in Fig. 3a. Our findings revealed that a high proportion of monocytes from HIV-1 infected participants expressed increased levels of HLA-DR [92% (88–97%)], while a smaller percentage [61% (50–76%)] expressed CD38. Moreover, nearly all CD38 expressing monocytes [97%; (94–99%)] also expressed HLA-DR, whereas only 64% (57–74%) of HLA-DR expressing monocytes were positive for CD38. As expected, we observed a significant increase in monocyte activation in ART-naive HIV-1 infected participants compared to uninfected individuals ($P < 0.0001$), as determined by the Kruskal-Wallis test

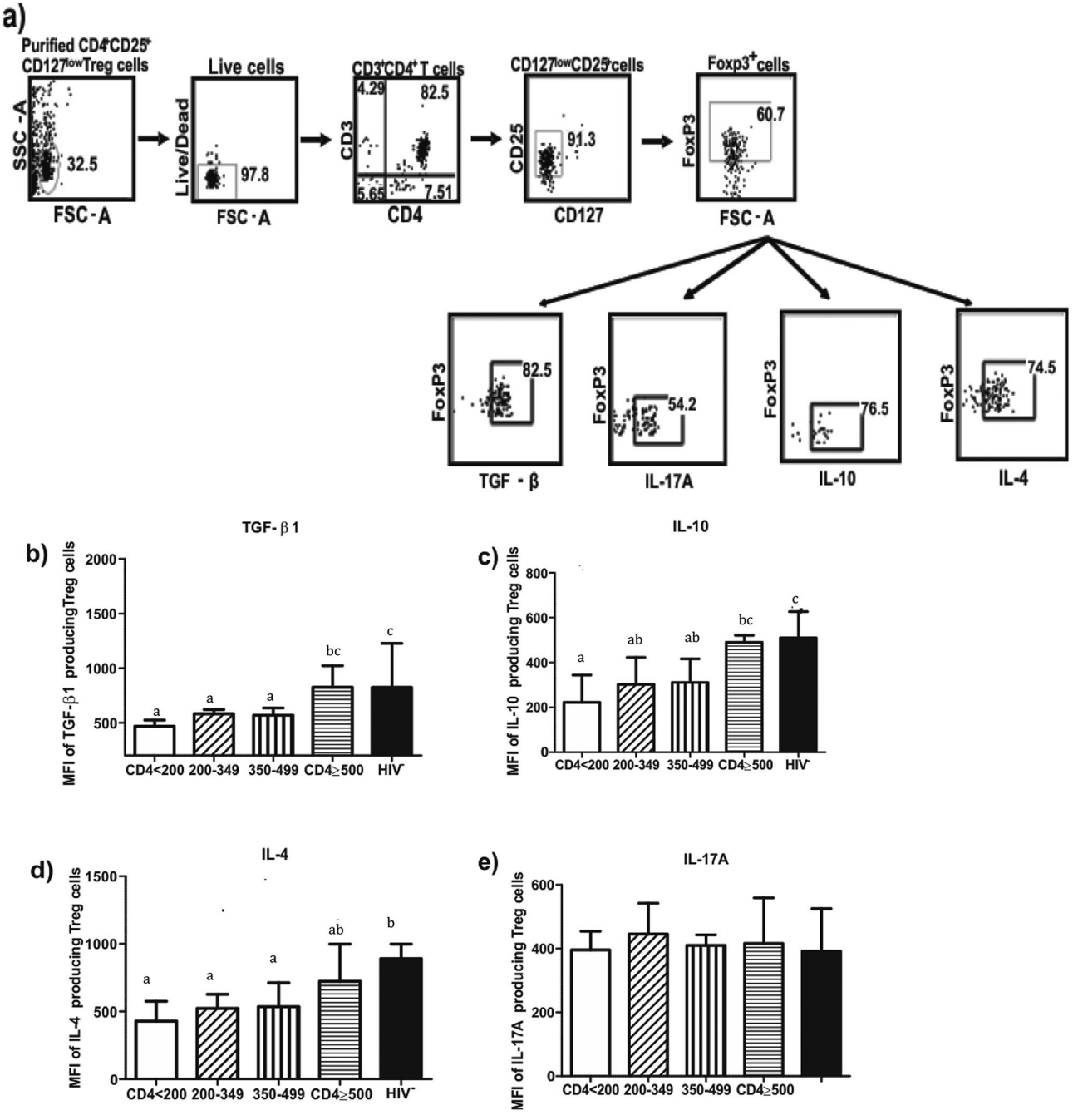

Fig. 2 Cytokine profile of Treg cells following stimulation with poly-ICLC. Figure **a** shows representative dot plots of the gating strategy of cytokines secreting Treg cells. Purified Treg cells obtained from PBMCs of HIV^+ and HIV^- participants were stained intracellularly using mAbs specific for IL-4, IL-10, IL-17 A and TGF-β and then analyzed by multiparametric flow cytometry. Dot plots showing expression of each cytokine on $CD4^+CD25^+CD127^{Low}$ $FoxP3^+$Tregs are shown (**b**, **c**, **d**, **e**). A minimum of 100 events of each intracellular cytokine were acquired. The differences between groups were calculated using Kruskal-Wallis test followed by Dunn's multiple comparison post-test. Treg cells from ART naive HIV-1 infected participants with helper $CD4^+$ T cell counts ≥ 500 cells/mm^3 showed a significant increase in TGF-β and IL-10 MFI compared to those with helper $CD4^+$ T cell counts < 200 cells/ μL ($p < 0.001$ and $p < 0.05$ for TGF-β and IL-10, respectively) and those with CD4 between 200–349 cells/ μL ($p < 0.05$ for TGF-β). Relative to uninfected participants, there was a significant reduction of IL-4 expression in HIV-1 infected participants with helper $CD4^+$ T cell counts < 200 cells/ μL ($p < 0.001$), between 200–349 cells/ μL ($p < 0.001$) and between 350–499 cells/μL ($p < 0.05$). Horizontal bars represent the median values. The letters on horizontal bars identify which groups are significantly different from each other. The same letter implies there is no significant difference between the compared groups, while different letters indicate a significant difference between them

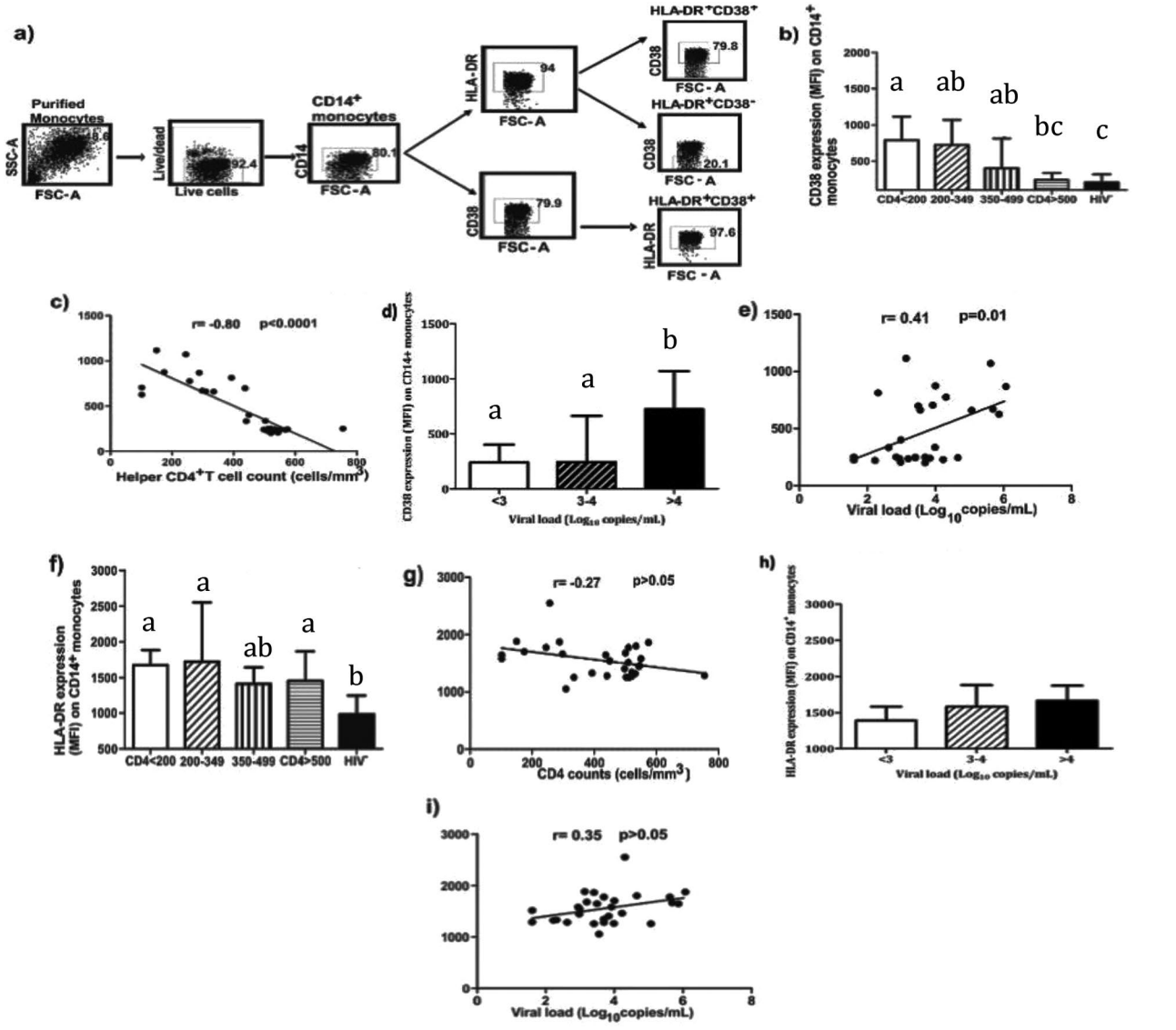

Fig. 3 CD38 surface marker is more reliable than HLA-DR for monocyte activation. (**a**) Gating strategy for HLA-DR and CD38 expression on monocytes: following forward and side scatter selection, magnetically sorted monocytes were selected from live cells and then analyzed for CD14 expression. $CD14^+$ monocytes were more than 80% pure. Next, HLA-DR and CD38 expression was monitored on the purified Monocytes. Whereas all CD38 expressing monocytes were also $HLA\text{-}DR^+$, only 80% of HLA-DR expressing monocytes were $CD38^+$. Relative to seronegative controls, HIV infected individuals display increased levels of immune activation as evidenced by elevated expression of HLA-DR and CD38, using the Kruskal-Wallis test followed by Dunn's multiple comparison test (**b**, **f**). Moreover, in individuals living with HIV, higher monocyte activation levels are associated with elevated viral loads (>4 log copies/ml). Interestingly, this link is notably significant when using the CD38 marker (**d**), whereas the association is not statistically significant when assessed through the HLA-DR marker (**h**). Notably, whereas HLA-DR expression did not correlate with viro-immunological parameters ($p>0.05$; **i**, **g**). On the other hand, CD38 expression correlated negatively with helper $CD4^+$ T cells ($r= -0.80$, $P < 0.0001$; **c**) and positively with plasmatic HIV-1 viral loads ($r= 0.41$, $p=0.01$; **e**). Thus, making CD38 a more reliable marker for tracking monocyte activation during this study. Horizontal bars represent the median values. The letters on horizontal bars identify which groups are significantly different from each other. The same letter implies there is no significant difference between the compared groups, while different letters indicate a significant difference between them

followed by Dunn's multiple comparison test (Fig. 3b, f). Within the ART-naive HIV-1 infected group, we further analyzed the relationship between HLA-DR or CD38 expression in monocytes and virological/immunological parameters using the Spearman test. CD38 expression correlated negatively with helper $CD4^+$ T cells ($r=-0.80$, $P<0.0001$; Fig. 3b, c) and positively with plasmatic HIV-1 viral loads ($r=0.41$, $P=0.01$; Fig. 3d, e). The same trend was observed with respect to the expression of HLA-DR, although no significant difference was detected ($P>0.05$; Fig. 3f, g, h, i). Therefore, we decided to track changes in monocytes activation using CD38 because it was reliable in predicting changes in monocytes activation relative to different clinical stages of HIV infection.

Suppression of poly-ICLC mediated monocyte activation by autologous Treg cells

A consequence of HIV-1 infection is persistent chronic immune activation driven mainly by HIV-induced and TLR- mediated signaling. Given that, HIV-1 triggers monocyte activation and promotes inflammation through the production of pro-inflammatory cytokines and chemokines, we hypothesized that, Treg cells can target monocytes to counter excessive inflammation. So, in this study, we assessed whether Treg cells can modulate monocytes activation in the context of ART-naive HIV-1 infection following co-culture with autologous monocytes. To achieve this, monocytes were purified as described in the methods section and equilibrated to minimize variations among their numbers between participants. In order to mimic the conditions of chronic HIV-1 infection, we used poly-ICLC, a synthetic double stranded RNA, as a stimulant and then analyzed CD38 expression in monocytes. As shown in Fig. 4a, a significant increase in CD38 expression was observed in poly-ICLC-treated monocytes compared to monocytes cultured alone. The stimulation index (SI) correlated positively with the helper $CD4^+$ T cell counts ($r=0.75$, $P<0.000$; Fig. 4b, c) and negatively with HIV-1 plasmatic viral loads ($r=-0.43$, $P=0.01$; Fig. 4d, e). The addition of autologous Treg cells into the culture medium resulted in a significant suppression of CD38 expression upon the monocytes. Like the stimulation index, the index of Treg cell suppression equally correlated positively with the helper $CD4^+$ T cell counts ($r=0.68$, $P<0.0001$; Fig. 4f, g) and negatively with HIV-1 plasmatic viral loads ($r=-0.45$, $p=0.01$; Fig. 4h, i). These findings indicate a strong correlation between the stimulation index and the index of Treg cell suppression ($r=0.83$, $P<0.0001$; Fig. 4j). This highlights the potential of Treg cells to down modulate monocyte activation and at the same time indicates

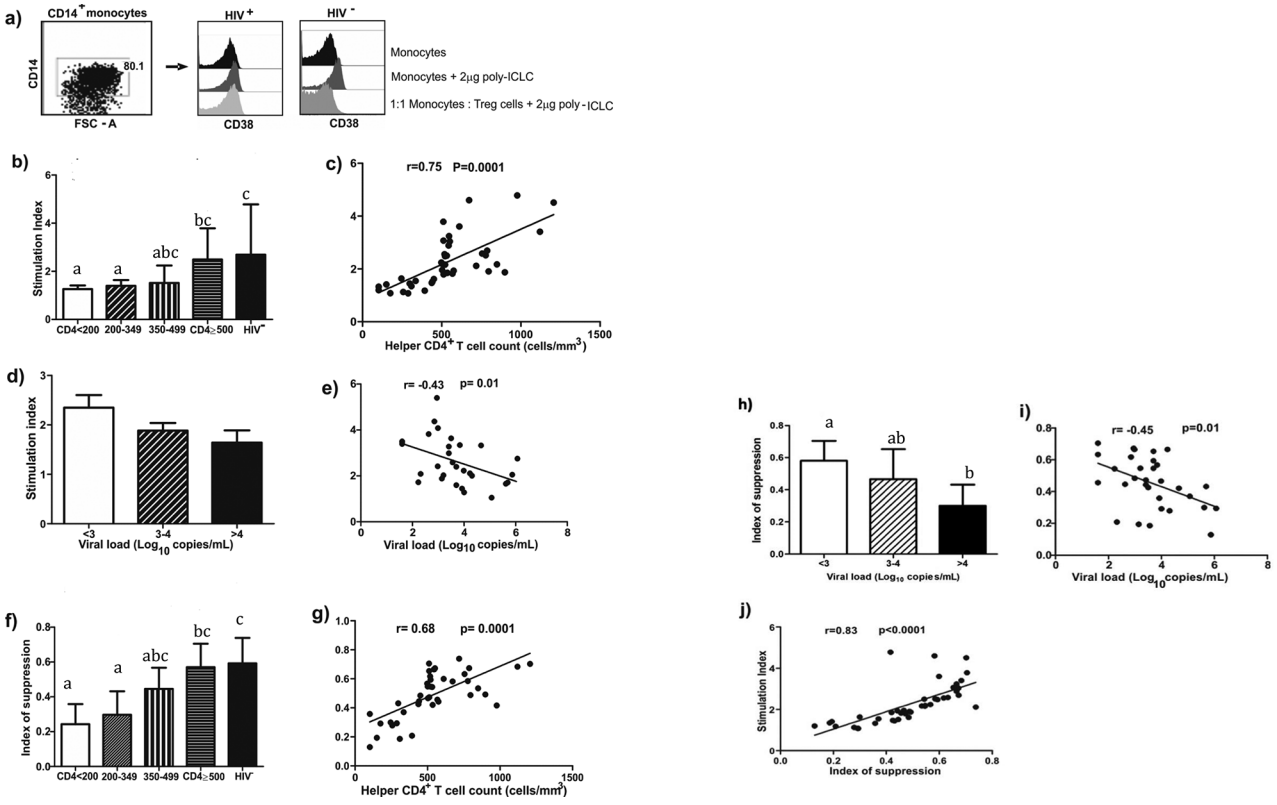

Fig. 4 In coculture with autologous monocytes, Treg cells suppress their activation by poly- ICLC. As shown in **a**, a significant increase in CD38 expression was observed in poly-ICLC treated monocytes compared to monocytes cultured alone. However, the addition of autologous Treg cells resulted into a significant suppression of CD38 expression. The stimulation index (SI) was calculated by dividing the MFI of CD38 expression of poly ICLC treated monocytes by the MFI of CD38 expression in unstimulated monocytes. The suppressive activity of Treg cells defined as index of suppression (IS) was assessed based on the following formula: 1-(MFI of CD38 expression of poly ICLC treated monocytes in the presence of Treg cells / MFI of CD38 expression of poly ICLC treated monocytes in the absence of Treg cells). The SI and IS correlated positively with the helper $CD4^+$ T cell count ($r=0.75$, $P=0.0001$ and $r=0.68$, $p=0.0001$, respectively for the SI and IS; **b**, **c**, **d**, **e**) and negatively with HIV-1 plasmatic viral loads ($r=-0.43$, $P=0.01$ and $r=-0.45$, $p=0.01$, respectively for the SI and IS; **f**, **g**, **h**, **i**), demonstrating a strong correlation between SI and IS ($r=0.83$, $P<0.000$; **j**). Horizontal bars represent the median values that were first compared using the Kruskal Wallis test, then followed by Dunn's multiple comparison post-test. Correlation coefficients and their significance were calculated by Spearman test. The letters on horizontal bars identify which groups are significantly different from each other. The same letter implies there is no significant difference between the compared groups, while different letters indicate a significant difference between them

that this ability is associated with immunocompetence as measured by helper $CD4^+$ T cell counts.

Suppression of poly-ICLC mediated monocyte IL-6 production following invitro coculture with autologous Treg cells

HIV-related chronic inflammation is characterized by elevated levels of cytokines including IL-1β, IL-6 and TNF-α which have been linked to adverse disease outcomes [3, 26, 27]. Here, we explored the potential of autologous Treg cells to suppress the production of pro-inflammatory cytokines by monocytes upon activation with poly-ICLC. Notably, monocytes were first stimulated with poly-ICLC and subsequently analyzed for IL-6 production using multi-parametric flow cytometry. As illustrated in Fig. 5a, poly-ICLC-treated monocytes produced significant elevated amounts of IL-6 relative to their unstimulated counterparts. This was revealed in stimulation index values, all above one. Moreover, the stimulation index correlated positively with helper $CD4^+$ T cell counts ($r=0.40$, $P=0.008$; Fig. 5b, c) and negatively with HIV-1 plasmatic viral loads ($r= -0.52$, $P=0.031$; Fig. 5f, g). However, upon co-culture with autologous Treg cells, there was a significant reduction in IL-6 production levels indicating the inhibitory effect of Treg cells upon poly-ICLC-stimulated monocytes. Similar to the stimulation index, the suppressive activity of Treg cells correlated positively with helper $CD4^+$ T cell counts ($r=0.6$, $P=0.0001$; Fig. 5d, e) and negatively with HIV-1 plasmatic viral loads ($r=-0.46$, $P=0.0089$; Fig. 5h, i). There was a positive correlation between stimulation and suppression indexes ($r=0.40$, $P=0.0093$; Fig. 5j). Thus, Treg cells were able to dampen IL-6 production by poly-ICLC activated monocytes and this ability was dependent upon immune competence.

Suppression of poly-ICLC mediated monocyte TNF-α production following *in* vitro coculture with autologous Treg cells

Similarly, the inhibitory effect of Treg cells on TNF-α production by poly-ICLC-activated monocytes was also assessed as shown in Fig. 6a. Upon poly-ICLC stimulation, monocytes displayed increased TNF-α production compared to monocytes cultured alone. However, this increase was not correlated with either helper $CD4^+$ T cell counts ($r=0.28$, $P=0.07$; Fig. 6b, c) or HIV-1 plasmatic viral loads ($r=-0.19$, $P=0.30$ Fig. 6f, g). Following co-culture with autologous Treg cells, a significant reduction of TNF-α production was observed. This suppressive activity of Treg cells correlated positively with helper $CD4^+$ T cell counts ($r=0.67$, $P<0.0001$; Fig. 6d, e) and negatively with HIV-1 plasmatic viral loads ($r= -0.5$, $P=0.0042$; Fig. 6h, i). Unlike IL-6, there was no correlation between the stimulation and the suppression indexes ($r=0.18$, $P=0.24$; Fig. 6j). These findings suggest that Treg cell-mediated suppression of TNF-α production by poly-ICLC-activated monocytes were also dependent upon the immune competence.

Discussion

In steady states, Treg cells are expected to dampen excessive immune activation and inflammation, thereby limiting tissue damage and preventing immune-related disorders. However, in the context of a challenging persistent infection like HIV-1, it is not known how Treg cells function. We have previously demonstrated that the number of Treg cells decreases proportionately with helper $CD4^+$ T cells, while their frequencies within total $CD4^+$ T cells remain higher in ART-naive HIV-1 infected individuals [15]. It is unclear whether the discrepancies in Treg cell numbers and frequencies observed in HIV-1 infected individuals indicate a deficiency in their functionality. To address this challenge, Treg cells were tested for their ability to modulate autologous monocytes activation as well as their function of producing pro-inflammatory cytokines following stimulation with poly-ICLC, a TLR3 and MD5 agonist. We focused on monocytes because they contribute significantly to chronic inflammation during HIV-1 infection, leading to the production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, which are associated with tissue damage [7, 8]. Elevated quantity of these cytokines have been also associated with increased risk of cardiovascular diseases in HIV-1 infected people [28, 29]. Thus, the down-modulation of excessive production of inflammatory cytokines by monocytes could contribute in diminishing chronic immune activation thereby, improving and enhancing the lifespan of HIV-1 infected people.

Similar numbers of Treg cells were used irrespective of the immunological or virological status of the ART-naive HIV-1 infected participants in order to minimize any potential bias.

Treg cells use several immunomodulatory mechanisms to maintain immune homeostasis and prevent autoimmunity. Amongst these, the secretion of suppressive cytokines has been shown in several studies to be necessary for the function of Treg cells [30, 31]. In this light, we have assessed the cytokine profile of Treg cells in the context of ART-naive HIV-1 infection. We found that immunocompetent ART-naive HIV-1 infected participants showed significantly higher levels of TGF-β and IL-10 in MFI compared to people with severe ($p<0.001$ for TGF-β and $p<0.05$ for IL-10) and advanced ($p<0.05$ for TGF-β) immuno-suppression. This elevated level of TGF-β and IL-10 production was similar to that observed in uninfected individuals. Both TGF- β1 and IL-10 are immune suppressive cytokines that are preferentially produced by Treg cells. These cytokines play an

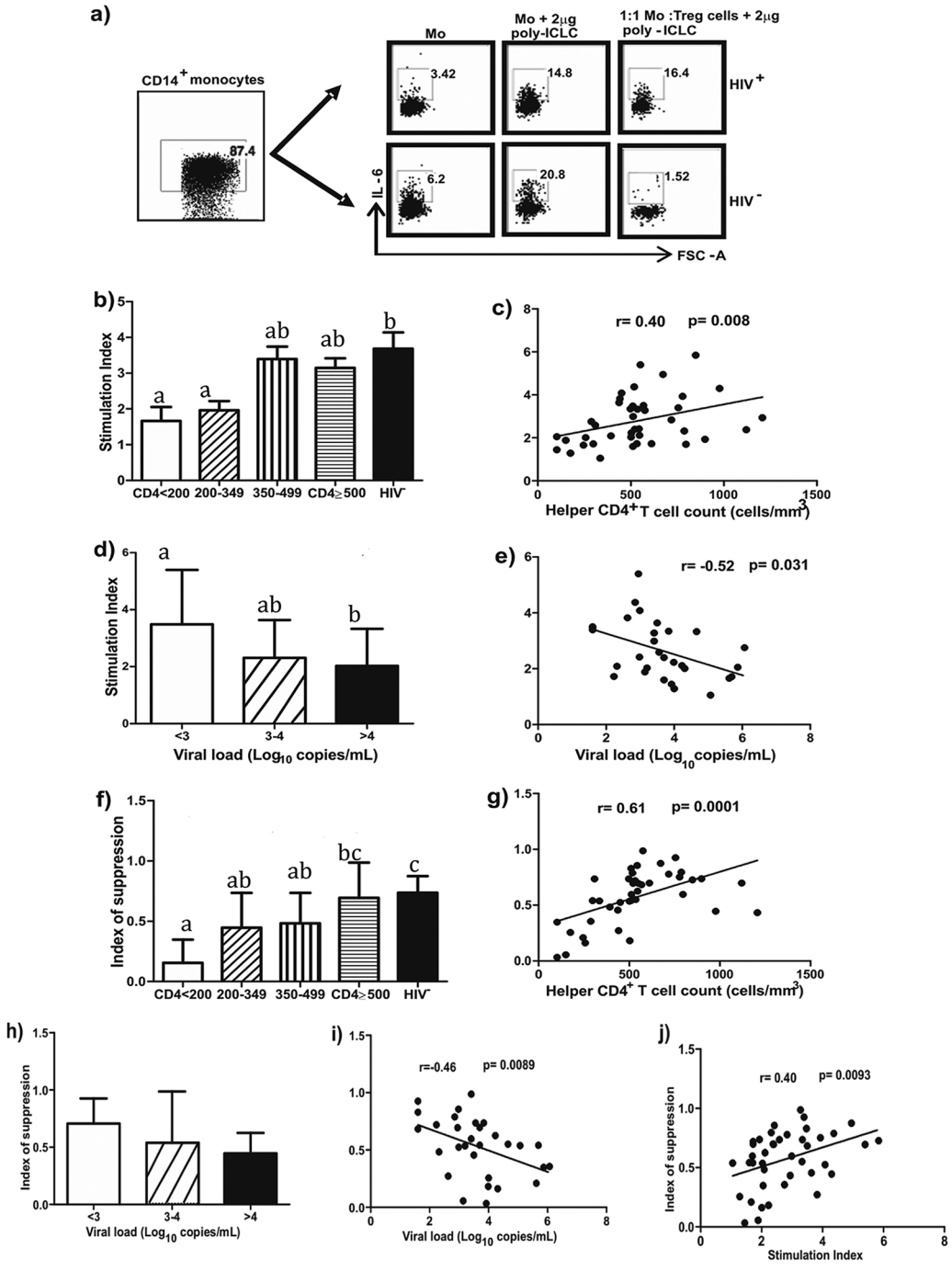

Fig. 5 In coculture with monocytes, Treg cells suppress IL-6 production following stimulation with poly-ICLC. In **a**, representative plots of IL-6 forming monocytes are shown for both HIV^+ and HIV^- participants. Monocytes stimulation with poly-ICLC resulted into increased IL-6 production relative to un-stimulated monocytes. Upon co-culture with autologous Treg cells, there was a significant reduction in IL-6 production indicating the inhibitory effect of Treg cells on poly-ICLC stimulated monocytes. The SI and IS correlated positively with helper $CD4^+$ T cell counts ($r = 0.40$, $P = 0.008$ and $r = 0.61$, $p = 0.0001$, respectively for the SI and IS; **b**, **c**, **g**, **f**) and negatively with HIV-1 plasmatic viral loads ($r = -0.52$, $P = 0.031$ and $r = -0.46$, $p = 0.0089$, respectively for the SI and IS; **d**, **e**, **h**, **i**) demonstrating a strong correlation between SI and IS ($r = 0.40$, $p = 0.0093$; **j**). Horizontal bars represent the median values that were compared using the Kruskal Wallis test followed by Dunn's multiple comparisons post-test. Correlation coefficients and their significance were calculated using the Spearman test. The letters on horizontal bars identify which groups are significantly different from each other. The same letter implies there is no significant difference between the compared groups, while different letters indicate a significant difference between them

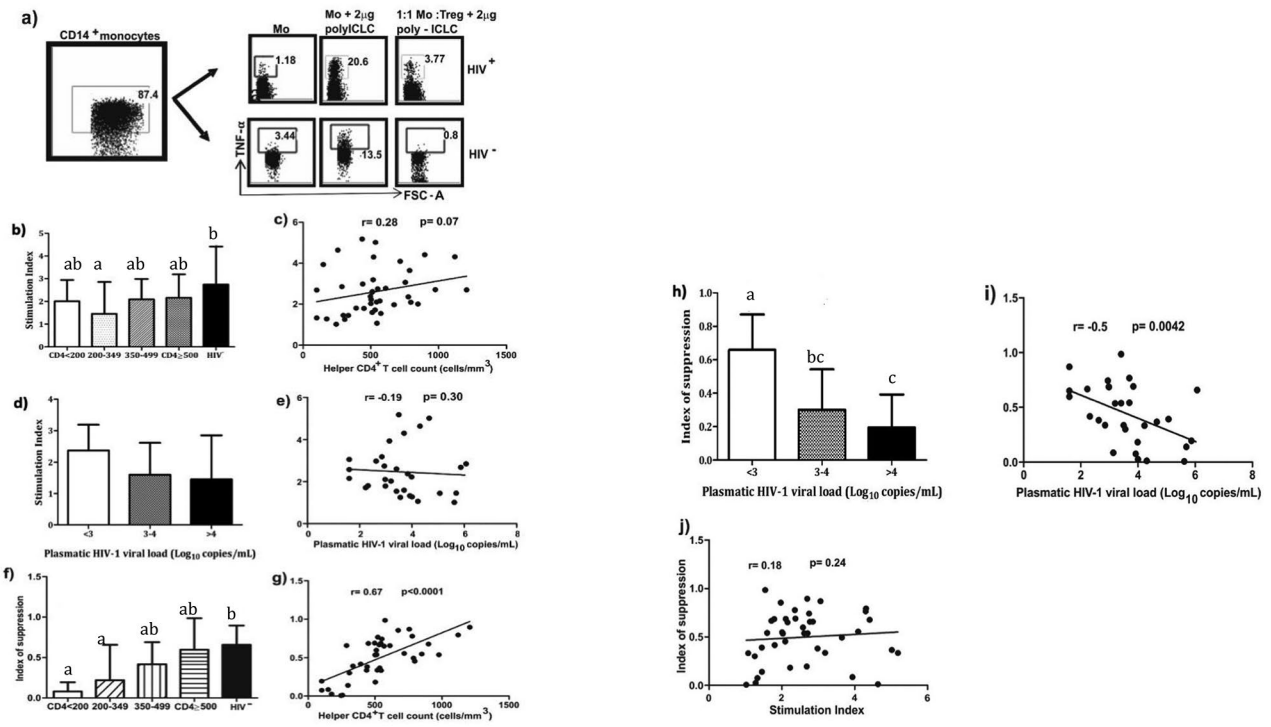

Fig. 6 In coculture with monocytes, Treg cells suppress TNF-α production following stimulation with poly-ICLC. In **a**, representative plots of TNF-α forming monocytes are shown for both ART naive HIV-1 infected and uninfected participants. The addition of poly-ICLC in the culture medium enhanced the production of TNF-α by monocytes compared to monocytes cultured alone, without significant difference ($P > 0.05$; **b**, **c**, **d**, **e**). Upon co-culture with autologous Treg cells, a significant reduction of TNF-α production was observed. This suppressive activity of Treg cells correlated positively with helper $CD4^+$ T cell counts ($r = 0.67$, $P < 0.0001$; **f**, **g**) and negatively with HIV-1 plasmatic viral loads ($r = -0.5$, $p = 0.0042$; **h**, **i**). In contrast to IL-6, no correlation was observed between the stimulation and the suppression index ($P > 0.05$; **j**). Horizontal bars represent the Median values that were compared using the Kruskal Wallis test followed by Dunn's multiple comparisons post-test. Correlation coefficients and their significance were calculated using the Spearman test. The letters on horizontal bars identify which groups are significantly different from each other. The same letter implies there is no significant difference between the compared groups, while different letters indicate a significant difference between them

important role in maintaining mucosal immune homeostasis [32]. IL-10 exerts its immune regulatory effects by inhibiting the maturation and activation of dendritic cells (DCs). This is achieved by downregulating the expression of both costimulatory molecules and major Histocompatibility Complex (MHC) class II on DCs [33]. In addition, IL-10 can limit effector T cell function as well as the recruitment of inflammatory myeloid cells such as monocytes to inflamed tissues by inhibiting the production of IL-2. It also promotes the phagocytic activity, allowing for increased clearance of cellular debris at the inflammation site [26, 34].

Previous studies have demonstrated that the transfer of IL-10 producing $FoxP3^+$ Treg cells to colitic mice can effectively resolve inflammatory responses, leading to the restoration of normal intestinal architecture [35]. This therapeutic approach has demonstrated promising results in managing colitis and promoting intestinal homeostasis by suppressing excessive immune responses and attenuating inflammation in the colon [36]. Similarly, TGF-β contributes to immunomodulatory activity of Treg cells by suppressing both innate and adaptive immune cells [21, 37]. Notably, TGF-β promotes the conversion of conventional $CD4^+$ T cells to $FoxP3^+$ Treg cells, thus inhibiting immune responses [38]. Our data suggests that both IL-10 and TGF-β may play a role in preventing and/or modulating sustained activation and inflammation. Additionally, in ART-naive HIV-1 infected participants, a significant reduction in IL-4 production was observed compared to uninfected participants ($p < 0.001$; Fig. 2d). This finding suggests a potential involvement of IL-4 in the immune regulatory mechanisms of Treg cells. Previous reports have shown that IL-4 produced by $FoxP3^+$Treg cells is capable of inhibiting LPS-induced TNF-α and IL-6 production by monocytes. Importantly, it has been observed that this suppression is completely reversed upon IL-4 blockage [9, 39].

In addition to the aforementioned anti-inflammatory cytokines, our study revealed that $Foxp3^+$Treg cells can also produce IL-17 A, a pro-inflammatory cytokine, which is typically produced by $CD4^+$ T helper 17 cells. IL-17 A is believed to contribute to the expansion of the inflammatory responses by facilitating cell recruitment

and activation at the sites of inflammation [40, 41]. In a related study, Voo et al. [42] demonstrated that IL-17 A producing Treg cells can be generated from conventional Treg cells in the periphery during inflammatory responses. These cells were found to co-express FoxP3, CCR6 and RORγt, the Th17 lineage-specific transcription factor. In another study reported by Walter and colleagues [26], it was demonstrated that besides IL-17 A, Treg cells can also produce IFN-γ and TNF-α upon interactions with activated monocytes. Despite the production of these pro-inflammatory cytokines, Treg cells were shown to maintain their ability to suppress T cell proliferation and inflammation. Indeed, Pandiyan and Zhu (2015) reported that, in in vitro studies, IL-17 A continues to effectively modulate inflammatory Bowel disease when transferred into immunodeficient mice. In addition, INF-γ-producing Treg cells are able to uphold their regulatory functions and suppressed colitis in mice. It should be noted that in vivo, inflammatory conditions may trigger the production of IL-17 A or INF-γ in a subset of FoxP3 Treg cells without impairing their ability to suppress T cell proliferation and inflammation [43]. Indeed, the production of IL-17 A by Treg cells is thought to allow them to contribute to the antimicrobial innate immune defense while controlling inflammation at the same time, particularly at mucosal sites [42, 44]. However, contradictory studies reported that IL-17 A producing Treg cells may rapidly lose their suppressive capacity when strongly activated in the presence of pro-inflammatory mediators such as IL-1β, IL-6, IL-7, IL-23 and TNF-α [41, 45, 46]. In the present study, we did not observe a significant difference in IL-17 A producing Treg cells between HIV-1 infected and uninfected participants. Based on our understanding that Treg cells respond to synthetic dsRNA mimic copolymer by inducing the production of IL-17 A, it was expected that there might be a difference in IL-17 A producing Treg cells between HIV-1 infected and uninfected participants. This suggests that, in the context of HIV-1 infection, the presence of this cytokine does not play a major role in influencing Treg cell function or their immunoregulatory activity.

During chronic HIV infection, sustained immune activation and inflammation are strong predictors of disease progression. Monocytes contribute extensively to the chronic inflammatory process and tissue destruction through the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [3, 26, 47, 48]. Down-regulation of monocyte activation and functions by Treg cells may therefore contribute to slow down disease progression during chronic HIV-1 infection. In this study, to determine the activation state of monocytes, we analyzed the surface expression of HLA-DR and CD38. We observed that 92% of monocytes expressed high levels of HLA-DR, while only 61% expressed CD38. Moreover, 97% of CD38-expressing monocytes were also HLA-DR positive, but only 64% (57–74%) of HLA-DR- expressing monocytes were CD38 positive. This allowed us to select CD38 expression as a surrogate marker to evaluate monocyte activation as HLA-DR was constitutively expressed at high levels by monocytes. As expected, monocytes from ART-naive HIV-1 infected participants displayed significantly high levels ($P<0.0001$) of CD38 compared with their HIV-1 negative counterparts, we noted a negative correlation between the level of monocyte activation, as measured through CD38 expression, and helper $CD4^+$ T cell count. On the other hand, plasma HIV-1 viral load correlated positively with CD38 expression. This is consistent with existing literature describing highly activated monocytes in HIV-1 infected people, especially those in advanced HIV-1 disease progression [49–51].

To mimic chronic HIV-1 infection, we used poly-ICLC, a synthetic double stranded RNA, to stimulate monocytes in vitro prior to analyzing for CD38 surface expression as described in the material and methods section. HIV-1 negative and immunocompetent people living with HIV-1 showed significantly higher stimulation index compared to immunosuppressed HIV-1 infected participants. This observation was supported by a positive correlation between the stimulation index and the helper $CD4^+$ T cell counts, as well as a negative correlation between the stimulation index and the HIV-1 plasmatic viral loads. When autologous Treg cells were added during poly-ICLC stimulation of the monocytes in culture, a significant reduction of CD38 expression upon the surface of monocytes was observed. Similar to the stimulation index, the index of Treg cell suppression correlated positively with the helper $CD4^+$ T cell counts and negatively with HIV-1 plasmatic viral loads. Of interest, there was a strong correlation between the suppression and stimulation indexes. This demonstrates the potential of Treg cells to down modulate monocyte activation, while also indicating that this ability is associated with immunocompetence, as measured by helper $CD4^+$ T cell counts. Our results are in agreement with a study conducted by Taams et al. [52] in which they reported that Treg cells can modulate monocyte activation by challenging LPS receptor (TLR4/CD14 triggering) and hampering the function of antigen-presenting cells. In accordance to our results involving HIV-1 infected participants, Karlson et al. [53] demonstrated that the in vitro suppressive capability of peripheral Treg cells from chronically infected *Cynomolgus* macaques is associated with preserved helper $CD4^+$ T-cell counts and reduced T-cell activation. Based on these insights, we can speculate that, suppression of immune activation by Treg cells in chronic HIV-1 infection might diminish over the course

of disease progression thereby, contributing to exhaustion of the immune system [53].

Regarding the production of pro-inflammatory cytokines, we investigated whether autologous Treg cells were capable of suppressing IL-6 and TNF-α production by monocytes during activation with poly-ICLC. We noted a noteworthy decrease in the levels of IL-6 and TNF-α production, indicating the inhibitory effect of Treg cells upon poly-ICLC-stimulated monocytes. This suppressive activity of Treg cells correlated positively with helper $CD4^+$ T cell counts and negatively with HIV-1 plasmatic viral loads. There was also a positive correlation between the stimulation and suppression indexes, especially for IL-6. These findings suggest that Treg cell-mediated suppression of IL-6 and TNF-α production by poly-ICLC activated monocytes was also dependent upon immune competence. Similar results were obtained by Tiemessen et al. [39] who worked with healthy individuals. They found that following co-culture with Treg cells, LPS treated monocytes were significantly suppressed in their capacity to produce proinflammatory cytokines/chemokines (TNF- α IL-6, IL-1β, IL-8/CXCL8, and MIP-1 α / CCL3) compared with monocytes cultured alone. This suppressive activity of Treg cells required both cell-cell contact and the presence of soluble factors such as IL-10, IL-4, and IL-13. These cytokines were thought to inhibit NF-kB activation, which is required for proinflammatory cytokine gene expression and which is involved in the regulation of surface markers such as CD40 and CD86. According to a study conducted by Younes and colleagues in 2018, it was found that ART-treated individuals with HIV-1 infection who were immune non-responders (INR) exhibited a pro-inflammatory environment. This was associated with a reduced number of circulating Treg cells and dysfunction in the remaining Treg cells. The study also revealed that the activation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) and mitochondrial transcription factor A (TFAM) by IL-15 led to an increase in mitochondrial mass and oxidative phosphorylation (OXPHOS) activity in Treg cells of HIV-1 infected INR subjects. These findings highlight the connection between metabolic pathways, Treg cell maintenance, and CD4+T cell numbers in INR individuals with HIV-1 infection [54]. In the present study, we speculate that Treg cells may directly interact with autologous monocytes through the cytotoxic T lymphocyte antigen 4 (CTLA-4). This inhibitory molecule which is constitutively expressed by Treg cells [55] has been shown to be capable of binding to costimulatory ligands, CD80 and CD86, which are upregulated on the surface of activated monocytes further resulting in down-regulation of T cell activation [56].

Indeed, the ability of Treg cells to suppress HIV-1 specific effector immune responses has been associated with decreased immune hyperactivation and target cells available for HIV-1 replication leading to a slow disease progression [37, 57–59]. Therefore, in HIV-1 infected individuals where Treg cells are depleted, the active suppression of effector immune cells decreases leading to a generalized immune activation and subsequently to rapid disease progression [60–63]. It has been previously shown that, Treg cells, as a subset of $CD4^+$ T cells express HIV co-receptors CCR5 and CXCR4, making them vulnerable to HIV-1 infection [58, 60, 64, 65]. Consequently, Treg cells may constitute a potent reservoir, thus leading to consider infected Treg cells as an obstacle to efficient control of HIV-1 infection [60, 63, 64, 66, 67]. This impairment on Treg cells function was associated with a down-regulation of FoxP3 and CD25 markers [60, 65]. On the other hand, certain studies have suggested that the suppressive activity of Treg cells could impede the establishment of HIV-specific $CD4^+$ and $CD8^+$ T cell responses resulting in the persistence of HIV-1 infection [63, 68]. This is in keeping with data on an increase in Treg cell frequencies within the $CD4^+$ T cell compartment during advanced HIV-1 infection resulting in an elevated suppressor to helper ratio [16, 68–70]. Importantly, the faster depletion of conventional $CD4^+$T cells compared to the expansion of Treg cells is not solely attributed to the suppressive activity of Treg cells, but also to the significant conversion rate of helper $CD4^+$T cells into Treg cells, resulting from the intensive T cell activation associated with HIV-1 infection [59].

Despite the valuable insights gained from this study, it is important to acknowledge the limitations regarding the small sample size, especially, when divided into subgroups. This limitation may compromise the statistical power of the analysis. Future studies with larger sample size are warranted.

Conclusion

Our findings indicate that following monocyte stimulation with a TLR3 ligand poly-ICLC, autologous Treg cells are capable of down modulating monocyte activation as well as IL-6 and TNF-α production by activated monocytes. Although we did not rule out the contribution of IL-4, IL-10 and TGF-β in Treg cell suppressive function, our data suggest that these cytokines are participating in the suppressive mechanisms of Treg cells, given that their expression levels in HIV-1 infected participants without immunosuppression were comparable to those in uninfected individuals. The suppressive activity of Treg cells correlated positively with the helper $CD4^+$ T cell count and negatively with HIV-1 plasmatic viral load. We speculate that the effectiveness of Treg cells in suppressing excessive immune activation and inflammation during HIV-1 infection depends on their presence in adequate proportions relative to the helper $CD4^+$ T cell count. This

is supported by our previous findings, showing an aberrant increase in Treg cell frequencies in HIV-1-infected participants with lower helper $CD4^+$ T cell count. Our research provides further evidence for the potential use of Treg cells as an immunotherapeutic strategy in the long-term management of HIV-1 infection.

Abbreviations

ART	Antiretroviral therapy
FoxP3	Forkhead box P3
HIV-1	Human immunodeficiency virus type-1
IL	Interleukin
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
PBMCs	Peripheral blood mononuclear cells
Poly-ICLC	Polyinosinic-polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose
TGF-β	Transforming growth factor-beta
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
Treg	$CD4^+CD25^+CD127^{Lo}FoxP3^+$Regulatory T cells

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Author contributions
ANG contributed to the study conception and design, data acquisition, analysis and interpretation, as well as manuscript drafting and revising. NEC was responsible for the manuscript revision and final approval. SNC contributed to data acquisition, analysis and interpretation, NNL contributed to data acquisition. LA contributed to data acquisition, analysis and interpretation. TTF contributed to data acquisition, analysis and interpretation. SM contributed to data acquisition. ABW contributed to the revision of the manuscript. MO contributed to the revision of the manuscript. CE contributed to the revision of the manuscript. CGP contributed to the revision of the manuscript. CV contributed to the revision of the manuscript. EFX contributed to the revision of the manuscript. NWG was involved in the study conception and design, data acquisition, analysis and interpretation, as well as revising the manuscript. All authors read and approved the final manuscript.

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Data availability
All data, table and figures are included in the manuscript.

Declarations

Ethics approval and consent to participate
This study received ethical approval from the Cameroon National Ethics Committee for Human Health Research. (Protocol numbers: CIRCB/14 – 11/DROS631-1112; 2014/10/499/CE/CNERSH/SP; 2015/03/569/CE/CNERSH/SP). All participants provided written informed consent. Data were processed using specific identifiers for privacy and confidentiality purposes. All methods were carried out in accordance with relevant guidelines and regulations. Clinical data generated during the course of this study were provided free of charge to all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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