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Cerebrospinal fluid CD14^{++}CD16^{+} monocytes in HIV-1 subtype C compared with subtype B

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Abstract

CD14^{++}CD16^{+} monocytes are susceptible to HIV-1 infection, and cross the blood–brain barrier. HIV-1 subtype C (HIV-1C) shows reduced Tat protein chemoattractant activity compared to HIV-1B, which might influence monocyte trafficking into the CNS. We hypothesized that the proportion of monocytes in CSF in HIV-1C is lower than HIV-1B group. We sought to assess differences in monocyte proportions in cerebrospinal fluid (CSF) and peripheral blood (PB) between people with HIV (PWH) and without HIV (PWoH), and by HIV-1B and -C subtypes. Immunophenotyping was performed by flow cytometry, monocytes were analyzed within CD45 + and CD64 + gated regions and classified in classical (CD14^{++}CD16^{-}), intermediate (CD14^{++}CD16^{+}), and non-classical (CD14^{low}CD16^{+}). Among PWH, the median [IQR] CD4 nadir was 219 [32–531] cell/mm^3; plasma HIV RNA (log_{10}) was 1.60 [1.60–3.21], and 68% were on antiretroviral therapy (ART). Participants with HIV-1C and -B were comparable in terms of age, duration of infection, CD4 nadir, plasma HIV RNA, and ART. The proportion of CSF CD14^{++}CD16^{+} monocytes was higher in participants with HIV-1C than those with HIV-1B [2.00(0.00–2.80) vs. 0.00(0.00–0.60) respectively, p = 0.03 after BH correction p = 0.10]. Despite viral suppression, the proportion of total monocytes in PB increased in PWH, due to the increase in CD14^{++}CD16^{+} and CD14^{low}CD16^{+} monocytes. The HIV-1C Tat substitution (C30S31) did not interfere with the migration of CD14^{++}CD16^{+} monocytes to the CNS. This is the first study to

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Authors' contributions SM de Almeida participated in the conception and design of the study, patient recruitment, acquisition, statistical analysis and interpretation of clinical and laboratory data, as well as drafting, revision, and finalization of the manuscript. MP Beltrame participated in the conception and design of the study, patient recruitment, acquisition, statistical analysis and interpretation of clinical and laboratory data, as well as drafting, revision, and finalization of the manuscript. B Tang participated in the statistical analysis, as well as the revision and finalization of the manuscript. I Rotta participated with laboratorial support. I Abramson participated in the statistical analysis. F Vaida participated in the statistical analysis. R Schrier participated in the conception and design of the study, as well as revised the finalized the manuscript. RJ Ellis participated in the conception and design of the study, as well as revised the finalized the manuscript.

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evaluate these monocytes in the CSF and PB and compare their proportions according to HIV subtype.

Keywords

Cerebrospinal fluids (CSF); HIV subtypes; Flow cytometry; Monocytes; Intermediate; Non-classical

Introduction

Monocytes are critical components of the primary innate immune response (Sampath et al. 2018). They are multifunctional cells that can act as either pro- or anti-inflammatory mediators that guide the development of innate and adaptive immune responses to pathogens (Guilliams et al. 2014; Ziegler-Heitbrock et al. 2010). HIV enters the CNS within 4–8 days of a peripheral infection. Monocytes/macrophages play a key role in the neuropathogenesis of HIV because of their ability to enter and activate resident cells of the CNS, as well as their ability to establish and maintain the CNS viral reservoir (Fischer-Smith et al. 2001; Burdo et al. 2010; Valcour et al. 2012). Within the CNS, monocytes/macrophages and microglia are the major cell types that harbor replication-competent viruses even after long-term viral suppression by antiretroviral therapy (ART) (Avalos et al. 2016; Gama et al. 2017; Williams et al. 2001).

In humans, monocytes can be further divided into three circulating subsets based on expression levels of the surface proteins CD14 (co-receptor for TLR4) and CD16 (Fcγ receptor IIIa) as follows: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{low}CD16^{+}$) (Ziegler-Heitbrock et al. 2010). These three subsets of monocytes are phenotypically and functionally distinct (Sampath et al. 2018).

$CD14^{++}CD16^{-}$ monocytes comprise approximately 80–95% of circulating monocytes in the peripheral blood (PB), and are important scavenger cells; few differentiate into macrophages, whereas most differentiate into dendritic cells (Wong et al. 2011; Chimen et al. 2017; Villani et al. 2017; Boyette et al. 2017; Sampath et al. 2018). These cells are effective phagocytes with multiple functions, including coordination of innate immune responses, production of pro-and anti-inflammatory cytokines, and migration into tissues in response to inflammatory signals (Kapellos et al. 2019; Auffray et al. 2009).

$CD14^{++}CD16^{+}$ monocytes account for 2–11% (Sampath et al. 2018) of PB circulating monocytes; they are a non-homogenous population that have the potential to differentiate into macrophages and less frequently into dendritic cells (Villani et al. 2017; Boyette et al. 2017). Their functions include the production of reactive oxygen species (ROS), antigen presentation (Lee et al. 2017), proliferation and stimulation of T lymphocytes, inflammatory responses, and regulation of apoptosis. They are referred to as pro-inflammatory monocytes because they secrete high levels of pro-inflammatory cytokines (Belge et al. 2002; Chimen et al. 2017; Gren et al. 2015; Wong et al. 2011; Kapellos et al. 2019). This subset expresses chemokine receptors that drive their migration into tissues (Ziegler-Heitbrock 2007) and expands in the circulatory system under several conditions, including HIV

infection (Ozanska et al. 2020; Patel et al. 2017). $CD14^{++}CD16^{+}$ monocytes express C–C chemokine receptor (CCR) type 2 (CCR2) and selectively express CCR5, which reacts with macrophage inflammatory protein-1α (MIP-1α), a chemokine that mediates macrophage chemotaxis, and chemokine (C–C motif) ligand 5 (CCL5, also referred to as regulated on activation, normal T cell expressed and secreted, RANTES). $CD14^{++}CD16^{+}$ monocytes express CCR5, which is a co-receptor used by HIV to gain entry into macrophages, at higher levels than classical monocytes, which likely accounts for their higher susceptibility to HIV-1 infection (Zawada et al. 2011; Weber et al. 2000; Ellery et al. 2007; Campbell et al. 2014; Hijdra et al. 2013), and the fact that they preferentially transmigrate across the blood–brain barrier (BBB) (Williams et al. 2013, 2012; Pulliam et al. 1997; Ziegler-Heitbrock 2007; Ellery et al. 2007; Jaworowski et al. 2007; Buckner et al. 2011). The circulation of $CD14^{++}CD16^{+}$ monocytes infected with HIV contributes to chronic immune activation, and these monocytes are thought to transport HIV into the brain (Rao et al 2014). The mature $CD14^{++}CD16^{+}$ monocyte subset enters the CNS in response to chemokines, including chemokine (C–C motif) ligand 2 (CCL2), also referred to as monocyte chemoattractant protein-1, MCP-1). After HIV-infected $CD14^{++}CD16^{+}$ monocytes enter the brain they can differentiate into macrophages. The entry of infected $CD14^{++}CD16^{+}$ monocytes may lead to the infection of other CNS cells, including macrophages, microglia, and astrocytes (Churchill et al. 2006; Thompson et al. 2011). Infected CNS cells produce additional inflammatory mediators such as cytokines and, despite ART, produce early viral proteins, such as Tat and gp120 (Conant et al. 1998; Weiss et al. 1999; Saylor et al. 2016; Yeung et al. 1995; Bansal et al. 2000; Buscemi et al. 2007). This contributes to neuroinflammation and neuronal damage that leads to HIV-associated neurocognitive disorders (HAND) despite ART (Veenstra et al. 2017).

$CD14^{low}CD16^{+}$ monocytes comprise approximately 2–11% of the PB circulating monocytes, and mainly differentiate into macrophages (Villani et al. 2017; Boyette et al. 2017; Sampath et al. 2018). They are mobile in nature and patrol the endothelium in search of injury. They exhibit pro-inflammatory behavior and secrete inflammatory cytokines in response to infection. These cells are also involved in antigen presentation and T-cell stimulation (Wong et al. 2011; Chimen et al. 2017). This subset can survey vasculature, patrol the endothelium (Kapellos et al. 2019), and promote homeostasis (Buscher et al. 2017; Hernandez and Iruela-Arispe 2020). These cells rarely respond to the inflammatory signals that drive $CD14^{++}CD16^{-}$ and some $CD14^{++}CD16^{+}$ monocytes to transmigrate into tissues (Chimen et al. 2017).

Monocytes that express surface $CD16^{+}$ possess a more mature phenotype and are associated with gene ontology terms such as cell-to-cell adhesion, cell trafficking, proliferation, and differentiation (Ancuta et al. 2009). In addition, they express higher levels of CX_3CR1, which explains why they more readily migrate and adhere to the fractalkine-secreting endothelium than $CD16^{-}$ monocytes (Ancuta et al. 2003; Zawada et al. 2011). However, $CD14^{++}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes are transcriptionally and functionally distinct (Gren et al. 2015).

The Tat protein plays a pivotal role in inducing chemokine secretion, mainly β-chemokines (such as CCL2) (Kutsch et al. 2000). Tat also upregulates the expression level of several

cytokines, including TNF-α (Chen et al. 1997; Bennasser and Bahraoui 2002), which is attributed to its C30C31 dicysteine motif (Albini et al. 1998; Beall et al. 1996). In vitro studies have suggested that HIV-1C is less neuropathogenic than subtype B based on a defective Tat chemokine dimotif in the C30C31 position, which might influence cellular trafficking and CNS inflammation (Ranga et al. 2004; Williams et al. 2020). HIV-1C Tat (C30S31) interferes with monocyte and lymphocyte migration into the CNS. An in vitro study found that HIV-1C Tat was less effective at upregulating markers such as TNF-α in monocytes (Gandhi et al. 2009). Furthermore, HIV-1C Tat does not induce calcium influx, resulting in lower levels of IL-10 in monocytes (Wong et al. 2011). Our group reported that the frequency of the C30S31 substitution in HIV-1C Tat in Brazil was 82% vs. 10% in HIV-1B ($p < 0.0001$; de Almeida et al. 2021a). However, we did not find that HIV-1C Tat was associated with less neurocognitive impairment than subtype B (de Almeida et al. 2013).

In the present study, we hypothesized that a lower proportion of monocyte subsets would be present in the cerebrospinal fluid (CSF) of participants with HIV-1C than in those with HIV-1B. This is expected because of the reduced transmigration of these cells, mainly $CD14^{++}CD16^{+}$ monocytes, through the BBB due to the Tat C30S31 substitution that results in a defective HIV-1C Tat chemoattractant property. To investigate this hypothesis, we conducted a cross-sectional survey to analyze monocyte subsets in fresh CSF and PB samples. The aims of this study were to assess the proportions of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{low}CD16^{+}$ monocyte subsets to: (a) compare the proportion of each monocyte subset in CSF and PB between people with HIV (PWH) and HIV-negative controls (people without HIV; PWoH) and (b) compare the proportions in participants with HIV-1B and -C. In addition, (c) secondary exploratory comparisons were performed to assess the relationship between monocyte subsets and HIV RNA in CSF and PB samples categorized by the distribution of HIV RNA in both compartments.

Methods

Ethics statement

This study was conducted in accordance with the principles of the Declaration of Helsinki. The protocols were approved by the Institutional Review Board of the Hospital de Clínicas, Universidade Federal do Paraná (Curitiba, Paraná, Brazil). Written informed consent was obtained from all participants before enrollment in the study.

Study design

This cross-sectional study explored monocyte phenotypes in the CSF of a subset of participants enrolled in the Hospital de Clínicas, Universidade Federal do Paraná (Brazil).

CSF and peripheral blood samples

CSF was collected by lumbar puncture (LP) without the addition of anticoagulant. PB samples were collected with the addition of ethylenediaminetetraacetic acid (K3 EDTA 7.5%) at the time of the LP, and processed in parallel with the CSF.

Participants characteristics

In this study, 36 PB and 34 paired CSF samples were collected from 36 neuroasymptomatic subjects, purely for research purposes, recruited from Curitiba, Southern Brazil. Participants were selected by convenience sampling (PWH, n = 22; PWoH, n = 14).

PWH recruited were all without opportunistic CNS infections. HIV-1 subtypes were genotyped using pol or env sequences. Genotyping revealed that eight individuals were infected with HIV-1B, while 14 were infected with non-B HIV-1 subtypes (C, n = 9; BF, n = 3; CF, n = 1; and F, n = 1). In 9 participants the subtype HIV-1B or C was confirmed by the Tat sequencing (de Almeida et al. 2021a). All HIV-1B (N = 4) have Tat sequence C30C31 and all HIV-1C (N = 5) showed C30S31.

For the PWoH group, 14 age-matched subjects who underwent surgery with spinal anesthesia were recruited. The indications for surgery were hernia (n = 3), lower-limb varicose veins (n = 3), column surgery (n = 2), hysterectomy (n = 2), knee surgery (n = 2), hemorrhoidectomy (n = 1), and perineoplasty (n = 1). These participants had no neurological comorbidities or cognitive complaints, and had negative serological tests for HIV, hepatitis C virus, and syphilis. The CSF inclusion criteria for this group were a white blood cell (WBC) count ≤ 5 cells/mm^3, total protein ≤ 45 mg/dL, and glucose ≥ 55 mg/dL. All volunteers underwent serological testing to confirm their HIV status prior to enrollment (Brasil 2018). Demographic data, clinical and HIV infection characteristics, comorbidities, and biochemical, cytological, and virological characteristics of CSF samples are shown in Table 1.

Laboratory methods

Immunophenotyping—Multiparameter flow cytometry was used to identify and quantify cellular phenotypes in the CSF and PB. Immunophenotyping was performed on fresh CSF samples processed within 20 min of LP. Preservative medium was not added. CSF samples (4–6 mL) were centrifuged at 500 × *g* at room temperature for 5 min. The supernatant was aspirated (it was not decanted to prevent excessive cell loss), and the cell pellets were resuspended in 300 μL of 0.5% bovine serum albumin (BSA) and gently mixed. The goal was to obtain at least 5,000 events per tube. The cells were mixed well by inversion and WBCs were counted using a hemocytometer (Sysmex, Roche, USA).

Sample source and processing—The relative frequency of each monocyte population was determined using paired CSF and PB samples from both groups.

For the conventional technique, fresh PB samples containing 10^6 cells and 100 μL blood were incubated for 15 min at room temperature in the dark with pre-titrated saturating amounts of four-color combinations of fluorochrome-conjugated and monoclonal antibody (MoAb): fluorescein isothi-ocyanate (FITC), peridinin-chlorophyll-protein (PerCP), or peridinin-chlorophyll-protein cyanine 5.5 (PerCPCy5.5) and allophycocyanin (APC). An additional unstained sample was processed in parallel as a negative control. Non-nucleated red blood cells were lysed using FACS lysing solution (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The remaining cells were sequentially

centrifuged at 500 × g at room temperature for 5 min, washed twice in phosphate-buffered saline (PBS pH 7.4) or PBS plus 0.5% BSA (pH 7.4), and resuspended in 300 μL of PBS for FC acquisition and analysis (Craig et al. 2011). For each PB sample aliquot, a minimum of 50,000 events were acquired using the CellQUEST software (BD Biosciences).

Flow cytometry analysis—CSF and PB cells were analyzed for surface marker expression using flow cytometry. Cells were stained with optimal concen-trations of the following monoclonal antibodies: control mouse isotypes and anti-human CD45 conjugated with peridinin chlorophyllprotein/cyanin 5 (PerCP-Cy5.5), anti-human CD14 conjugated with allophycocyanin (APC; BD Biosciences, San Jose, CA, USA), anti-human CD16 conjugated with FITC (BD Biosciences), and anti-human CD64 conjugated with phycoerythrin (PE; Beckman-Coulter, Brea, CA, USA).

CSF cell staining was performed at room temperature in the dark, and washes and incubations were performed in 0.5% PBS/BSA. Whole PB samples were labeled for 15 min at room temperature and then lysed with 2 mL lysis solution (BD Biosciences). The cells were then washed twice. For data acquisition and analysis, a flow cytometer (FACSCalibur ™ Four Color, BD Biosciences) with Infinicyt software 2.0 (Cytognos, Salamanca, Spain) was used. Monocytes were analyzed within the $CD45^+$ and $CD64^+$ gated regions.

The entire cell suspension was acquired for CSF samples, and the median (interquartile range; IQR) number of events was 1328 (885–2648), of which 116 (69.5–478.0) were analyzed. CSF samples with fewer than 10 cells (events) acquired in the phenotype gate were considered to have zero events (Quijano et al. 2009). The percentage of positive cells was measured from a cut-off set using an isotype-matched nonspecific control antibody. The gating strategy used for flow cytometry experiments to define each monocyte population was developed for PB samples and applied to CSF samples (Supplementary Fig. 1).

The total monocytes/$CD3^-CD19^+$ and the $CD3^+$/total monocytes ratios were calculated, in order to evaluate the predominance of adaptive or innate immune response in CSF or PB (Han et al. 2014; Njemini et al. 2014; Bielekova and Pranzatelli 2017).

Clinical laboratory measurements—Nadir $CD3^+CD4^+$ counts were retrieved from the medical records. Total CSF protein, glucose, and WBC counts were quantified using standard laboratory methods. The total CSF WBC count (cells/mm^3) was quantified using fresh, non-centrifuged CSF samples immediately after LP.

Quantification of plasma and CSF HIV RNA levels—HIV RNA levels in PB and CSF were quantified using the *m*24*sp* and *m*2000 Real-Time System (Abbott, Chicago, IL, USA) using 1 mL of CSF or plasma. The assays were performed immediately after sample collection. Samples with HIV RNA levels > 40 copies/mL were within the detection limit. The data are shown as $\log_{10}$ values. HIV groups were categorized based on the quantification of HIV RNA in paired CSF and PB samples, when CSF > PB, the discordance between levels was defined as the CSF HIV RNA level with any value greater than the PB viral load; PB > CSF; and suppressed (aviremic) in both compartments.

Data analyses

The demographic and HIV disease characteristics and biochemical, cytological, and virology measures of the CSF are presented as the median (IQR or range) or number (%), as appropriate, and were compared between individuals infected with HIV-1B and -C using the independent sample *t*-test for continuous variables and Fisher's exact test for binary and categorical variables (i.e., sex, AIDS diagnosis, ART, and HIV RNA in plasma and CSF). Similar methods were used to compare the demographics and biochemical and cytological measures of CSF between PWH (including subtypes B, C, BF, BC, CF, and F) and PWoH.

The proportion was assigned a missing value if the denominator (number of events) was zero. Comparisons of monocyte subsets between CSF vs. peripheral blood (PB), PWH vs. PWoH, and HIV-1B vs. HIV-1C in CSF and PB were performed using weighted linear regression analyses. To stabilize the variance and improve normality, the proportion of monocyte subsets was arcsine square root-transformed prior to statistical analysis. The weight in the regression model was regarded as N (events), and more weight was assigned to larger denominators because the proportions with small denominators (events) were noisy. For some monocyte subsets, the Wilcoxon test was used due to highly skewed data; Welch's two-sample t-test was conducted when two groups had unequal variance (i.e., variance = 0 for one group). Multiple testing corrections for multiple related biomarkers were performed using the Benjamini-Hochberg (BH) procedure. Age and sex were considered as covariates in comparisons of biomarkers between the PWH and PWoH groups, and blood/CSF HIV viral load suppression as covariates in comparisons of biomarkers between HIV-1B and -C.

In the exploratory analysis, PWH were categorized into three groups (CSF > PB, CSF < PB, and aviremic) based on the distribution of HIV RNA in the CSF and PB. The proportion of monocyte subsets in the CSF and PB was compared among groups using the Kruskal–Wallis test and pairwise comparisons were made using the Mann–Whitney test.

Correlation coefficients (ρ) between the proportion of monocyte subsets and the main lymphocyte subpopulations in CSF and PB (described in detail in de Almeida et al. 2022a), CSF characteristics (white blood cell count and total protein), CSF and PB HIV RNA, nadir $CD3^+CD4^+$, PB $CD3^+CD4^+$ recovery, duration of infection, and CNS anti-retroviral Penetration-Effectiveness Rank (CPE, Letendre et al. 2010), were estimated using Spearman's rank-order method.

The R software (version 3.4.1) was used to perform statistical analyses. The significance level of α was set at 5%. The effect size (ES), analogous to Cohen's d (and 95% confidence interval [CI]), was estimated as the coefficient divided by the residual standard deviation.

Results

Subject characteristics

Age and sex were comparable between the PWH and PWoH groups. Among PWH, the median (IQR) CD4 nadir was 219 (33–532) cells/mm^3, the median plasma HIV RNA (log_{10}) was 1.60 (1.60–3.21), and 68% were on ART. Individuals infected with HIV-1C and HIV-1B did not differ in age or HIV infection characteristics, including duration of infection, CSF

and plasma HIV RNA, nadir CD4 counts, CSF WBC count, and frequency of ART use ($p > 0.05$), but differed in sex (33.3% vs. 100% males, respectively, $p = 0.009$; Table 1).

Monocyte subsets in CSF and blood in PWH infected with HIV-1B and HIV-1C

The proportion of $CD14^{++}CD16^{+}$ monocytes in CSF analyzed in the leukocyte gate was higher in participants with HIV-1C than in those with HIV-1B [2.00 (0.00–2.80) vs. 0.00 (0.00–0.60), respectively; Cohen's *d*, 2.12; $p = 0.03$ after BH correction $p = 0.10$] (Table 2 and Fig. 1a). The proportion of total monocytes and monocyte subsets in the PB was comparable between HIV-1C and HIV-1B participants (Table 2).

The proportion of $CD14^{++}$ $CD16^{-}$ monocytes in the CSF was comparable between HIV-1B and HIV-1C participants ($p = 0.63$) (Table 2). There were more CSF samples with total monocytes [7/8 (87.50%) vs. 2/7 (28.57%), $p = 0.041$] and a trend of more CSF samples with $CD14^{++}CD16^{+}$ or $CD14^{++}CD16^{-}$ monocytes in HIV-1C participants than in HIV-1B participants [4/5 (80.00%) vs. 1/7 (17.29%), $p = 0.072$ and 2/5 (40.00%) vs. 1/7 (17.29%), $p = 0.523$, respectively] (Table 2).

A subgroup of PWH was categorized according with the Tat sequencing in C30C31 (HIV-1B, n = 4) and C30S31 (HIV-1C, n = 5), the total monocytes and its subsets in CSF and PB were comparable (Supplementary Table 2). However we must consider the low number of samples in each group.

Monocyte subsets in CSF and blood in PWH and PWoH

HIV-infected and uninfected participants had distinct proportions of monocyte subsets in the CSF and PB (Table 3). The proportion of total monocytes in the CSF did not significantly differ between PWH and PWoH [0.50 (0.00–2.60) vs. 0.00 (0.00–4.35), $p = 0.383$)]. The proportions of $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ monocytes in the CSF were comparable between PWH and PWoH (all $p > 0.05$) (Table 3 and Fig. 1b).

The number of CSF samples with total monocytes was numerically higher in PWH than in PWoH (10/20, 50.00% vs. 3/14, 21.43% respectively, $p = 0.153$), although the difference was not significant (Table 3), as well as the number of CSF samples with $CD14^{++}CD16^{+}$ monocytes [6/17 (35.29%) vs. 3/14 (21.43%), respectively, $p = 0.456$]. $CD14^{low}$ $CD16^{+}$ monocytes were not present in any CSF samples in either group (Table 3). PB $CD14^{++}CD16^{-}$ monocytes were predominant in both PWH and PWoH, with a higher proportion in PWoH ($p < 0.05$). The proportions of $CD14^{++}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes were higher in the PWH group than in the PWoH group (both $p < 0.05$) (Table 3).

In CSF and PB of PWH there was predominance of adaptive, mainly $CD3^{+}$ cell response, over innate immune response (Table 3). In the four CSF samples with $CD3^{-}CD19^{+}$ identified, there was predominance of innate immune response in two; all these four samples were HIV-1C (Table 2).

Monocyte subsets in CSF and peripheral blood in PWH categorized according to the distribution of HIV RNA in CSF and blood compartments

The proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF was higher in PWH without viral suppression in the CSF or PB (i.e., greater HIV RNA levels in CSF than PB, or greater in PB than in CSF) than participants with viral suppression (i.e., aviremic in CSF and PB) [66.00 (31.00–100.00); 73.05 (0.0–100.00); 0.00 (0.00–0.00), respectively, p = 0.003] (Table 4 and Fig. 1c). This indicates that there is more transmigration of $CD14^{++}CD16^{+}$ monocytes through the BBB in PWH with uncontrolled HIV infection.

The proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF was comparable between the two non-suppressed groups. In the HIV-suppressed group, there were no $CD14^{++}CD16^{+}$ monocytes in the CSF ($p > 0.05$, Table 4).

A pairwise comparison of the non-suppressed group with the PWoH group is shown in Table 4.

Analyzing the groups on-HAART and non-HAART in CSF total monocytes and its subsets were comparable; in PB $CD14^{low}CD16^{+}$ were higher in the on-HAART than the non-HAART (p = 0.07), the others were comparable (Supplementary Table 3).

Correlations among HIV-1 infection characteristics

$CD14^{++}CD16^{+}$ monocytes in the CSF were positively correlated with CSF and PB HIV RNA [ρ = 0.685 (0.290–0.880), p = 0.002 and ρ = 0.752 (0.412–0.908), p = 0.0005, respectively]; $CD14^{++}CD16^{+}$ monocytes in the PB were negatively correlated with CSF HIV RNA [ρ = −0.558 (−0.812– −0.124), p = 0.013]; and total monocytes and HIV RNA in the PB were negatively correlated [ρ = −0.437 (−0.731– −0.005), p = 0.042] (Fig. 2b, f–h). There was no correlation between total monocytes or their subsets and $CD3^{+}CD4^{+}$ nadir, PB $CD3^{+}CD4^{+}$ recovery, duration of infection, and CNS anti-retroviral Penetration-Effectiveness Rank (CPE) (data not shown).

Correlations between monocytes in the leukocyte gate (gate 45) and the main lymphocyte populations

Total monocytes in the CSF were negatively correlated with CSF $CD3^{+}$ [ρ = −0.528 (−0.792–−0.097), p = 0.017]. CSF $CD14^{++}CD16^{-}$ monocytes were correlated with CSF $CD3^{-}CD19$ + [ρ = 0.508 (0.020–0.800), p = 0.038)]. $CD14^{++}CD16^{+}$ monocytes in the CSF were positively correlated with CSF WBC count [ρ = 0.506 (0.017–0.799), p = 0.038] and CSF $CD3^{-}CD19^{+}$ [ρ = 0.688 (0.295–0.882), p = 0.002], whereas they were negatively correlated with CSF $CD3^{+}$ [ρ = −0.866 (−0.953–−0.652), p < 0.0001] (Fig. 2a, c–e). $CD14^{++}CD16^{-}$ monocytes in the PB were not related to the other PB monocytes (data not shown). There was no correlation between monocyte subsets in the CSF and their corresponding subsets in the PB (data not shown). Data showing the main lymphocyte populations in the studied groups have been published previously (de Almeida et al. 2022a).

Discussion

In this study, we used flow cytometry to analyze the direct impact of HIV subtypes on the migration of monocytes from the PB to CSF. Different from other studies that indirectly investigated this impact by analyzing inflammatory and immunologically soluble biomarkers (de Almeida et al. 2016, 2020, 2021b, 2022b, c). The proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF was higher in participants with HIV-1C than in those with HIV-1B, while the proportion in PB was comparable between the two subtypes. Although these findings do not support the hypothesis of this study, and in fact oppose our hypothesis, they are valuable as they show that HIV-1C Tat and HIV-B Tat are capable of stimulating the in vivo transmigration of $CD14^{++}CD16^{+}$ monocytes through the BBB. This is the first study to demonstrate this in human samples.

In this study, the proportion of total monocytes in the PB was higher in PWH than that in PWoH, mainly because of the higher proportions of $CD14^{++}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes. In the CSF, although there was an increase in the proportion of $CD14^{++}CD16^{+}$ monocytes in PWH, this increase was not significant, probably due to the small sample size. Monocytes were identified in 50% of the CSF samples in the PWH group. In normal CSF, the proportion of total monocytes in the CSF is small, ranging from 0 to 22% (Ho et al. 2013; Kowarik et al. 2014). Lymphocyte T cells are the most abundant cell type in the CSF, with a predominance of $CD3^{+}CD4^{+}$ over $CD3^{+}CD8^{+}$ T cells (de Graaf et al. 2011; de Almeida et al. 2022a).

This study adds to the published literature by analyzing paired CSF and PB samples of HIV-1C compared with HIV-1B subtypes. It is widely accepted that HIV infection of the CNS targets cells of monocyte-macrophage-microglial lineage and is associated with an increase in $CD14^{++}$ $CD16^{+}$ monocytes in the blood and monocyte migration into the brain (Veenhuis et al. 2021), causing immune activation and BBB disruption (Williams et al. 2012; Williams et al. 2013, 2014a, b; Campbell et al. 2014; Fischer-Smith et al. 2001).

Previously, our group found comparable levels of CCL2 in HIV-1C and −1B CSF and serum samples, showing that in vivo, HIV-1C Tat as well as HIV-1B Tat were capable of activating CCL2 and other β-chemokines at comparable levels (de Almeida et al. 2016). These findings are in line with those of the present study, as prior to entering the CNS, monocytes must be directed to their site of entry at the BBB by the chemokine gradient. HIV-infected $CD14^{++}$ $CD16^{+}$ monocytes transmigrated across the BBB in far greater numbers in response to CCL2 than their uninfected counterparts did. This heightened sensitivity to CCL2 is mediated by increased CCR2 expression in HIV-infected $CD14^{++}$ $CD16^{+}$ monocytes (Williams et al. 2013).

The findings of the present study provide additional support for our previously published studies, which found a comparable frequency of HAND between HIV-1B and -C participants (de Almeida et al. 2013). Moreover, CSF inflammation was investigated based on the increase in CSF WBC count, interleukin levels, IgG intrathecal synthesis, and the presence of oligoclonal bands, as well as CSF discordance or CSF viral escape, which were comparable between HIV-1C and −1B participants (de Almeida et al. 2016,

2020, 2021b). Recently, our group investigated differences in the proportions of the main lymphocyte subpopulations between HIV subtype using the same cohort of PWH as in the present study, and found that the proportions of $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$, and $CD3^{-}CD19^{+}$ lymphocytes in the CSF were comparable between HIV-1B and C samples. This shows that the HIV-1C Tat substitution (C30S31) did not interfere with migration of the main lymphocyte subpopulations to the CNS (de Almeida et al. 2022a).

Our group evaluated the soluble form of CD14 (sCD14, a marker of monocyte activation) in CSF and serum in a different cohort of PWH than that included in the present study. During cellular activation, sCD14 is generated by proteolytic shedding of the membrane-associated form (mCD14) (Striz et al. 1995). However, in contrast with the findings of this study, we found an increase in sCD14 that was subtype-dependent, with a greater increase in HIV-1B than in C participants, which was in accordance with our hypothesis (de Almeida et al. 2022b). This was the first time that subtype-dependent differences were described in terms of inflammatory biomarker stimulation. The reduced monocyte stimulation observed in HIV-1C samples when compared to HIV-1B could be attributed to the Tat C30S31 mutation present in HIV-1C Tat, which would be in line with the results of in vitro studies (Gandhi et al. 2009; Ranga et al. 2004; Williams et al. 2020). However, our group evaluated the impact of HIV-1 on other monocyte activation biomarkers in CSF and serum, including suPAR, β2m, and neopterin, and did not find differences by subtype (de Almeida et al. 2022c).

There was no correlation between monocyte subsets in the CSF and the corresponding population in PB, in accordance with a previous study that found no correlation between total monocytes in the CSF and PB (Ho et al. 2013).

In this study, the proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF was high and comparable in PWH groups without viral suppression (i.e., HIV RNA level greater in CSF than in PB, or greater in PB than in CSF), but these monocytes were not present in the HIV-suppressed group. $CD14^{low}CD16^{+}$ monocytes were not identified in the CSF of any group. One study showed that 24 weeks after ARV, the percentage of non-classical monocytes decreased in the CSF (Amundson et al. 2020).

In the current study, the proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF increased with increasing CSF and PB HIV RNA levels, indicating a higher proportion of transmigration of $CD14^{++}CD16^{+}$ monocytes in non-suppressed PWH. It has been reported that HIV-infected $CD14^{++}CD16^{+}$ monocytes preferentially transmigrate across the BBB, which is mediated by increased surface expression of junctional adhesion molecule A (JAM-A) and activated leukocyte cellular adhesion molecule (ALCAM) (Williams et al. 2015; Veenstra et al. 2017), and the chemokine receptor CCR2, which is the only known receptor for CCL2 on monocytes (Volpe et al. 2012). CCR2 and the adhesion markers are not the only receptors that may mediate monocytes transmigration, monocytes can express CXCR3 and respond to the CXCL10 gradient among others (Niu et al. 2021). The finding that HIV-infected $CD14^{++}CD16^{+}$ monocytes preferentially transmigrate across the BBB suggests that this contributes to the initial seeding and replenishment of reservoirs within the CNS (Veenstra et al. 2017). Similar to their intermediate precursors, the $CD14^{low}CD16^{+}$ monocyte subset

can also be infected with HIV and this monocytes subset expands during HIV infection (Campbell et al. 2014).

In this study, no $CD14^{++}CD16^{+}$ monocytes were identified in the CSF of participants with HIV suppression in either the CSF and PB, indicating that the intermediate monocytes did not transmigrate to the BBB in these conditions, or did so in a limited number. The proportions of $CD14^{++}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes were higher in the PB of PWH than in PWoH, despite virological control, in accordance with a previous report (Williams et al. 2015). This is important because, although $CD14^{++}CD16^{+}$ monocytes were not present in the CSF in the group with HIV suppression in the CNS and PB (aviremic group) in this study, these monocytes can migrate from the PB to CNS, replenish, and restore the reservoir. The $CD14^{++}CD16^{+}$ monocyte population is the most susceptible to HIV-1 infection (Campbell et al. 2014; Hijdra et al. 2013), and preferentially transmigrates across the BBB (Williams et al. 2013; Pulliam et al. 1997; Ziegler-Heitbrock 2007; Ellery et al. 2007; Jaworowski et al. 2007).

The main strength of this study is the fact that it was the first to examine monocyte subsets in the CSF and serum of participants infected with HIV-1C and compare them with HIV-negative healthy controls. All previous studies limited their analyses to HIV-1B samples. Additionally, participants with HIV-1B and −1C were from the same geographical region in southern Brazil and were similar in age and sex. We analyzed a subgroup of participants categorized according with the Tat sequencing (C30C31 or C30S31), although the number of samples was small in each group, this was the first time it was analyzed.

The PWH and PWoH samples were analyzed concurrently. The healthy HIV-negative control group (PWoH) consisted of neuro asymptomatic HIV- and hepatitis C virus-seronegative participants, rather than subjects with non-inflammatory neurological disease. The use of fresh samples allowed us to account for all monocytes present in the PB and CSF at the time of drawing. Freezing and subsequent thawing of PBMCs can lead to preferential loss of inflammatory cell types (Rundgren et al. 2018).

This study had the following limitations:

The small sample size increased the possibility of a type II error when comparing PWH and PWOH, as well as when comparing HIV-1B and HIV-1C participants, and when comparing groups based on CSF/plasma HIV RNA, limiting the capacity of meaningful conclusions. However, the absolute values of Cohen’s d effect sizes were medium to large, indicating that the sample size was sufficient for power analysis of the comparisons of the PWH and PWoH, as well as HIV-1B and HIV-1C.

Despite monocytes were minor leucocytes population in CSF, other studies, including from PWH, have detected monocytes in CSF (Ho et al. 2013; Amundson et al. 2020), while in this study, many samples did not have monocytes detected at all. We must consider that the CSF monocytes reference range, calculated in symptomatic controls (i.e. headache and paresthesia of unspecific origin) was 0.0 to 21.6% (Kowarik et al. 2014)

The study enrolled both untreated PWH and those using ART, with most of the participants being on ART. This could be a major confounding factor, and the differences in the populations may be based on CSF viral load rather than viral sequences. We attempted to overcome this limitation by controlling for the effect of plasma HIV viral load in the multivariable analysis, and analyzing the groups on-HAART and non-HAART. Nonetheless, HIV-1B and HIV-1C were found to be comparable in the CSF to plasma HIV RNA ratio.

As the sample size was small, mainly when analyzing HIV-1C and HIV-1B, to avoid overfitting and in order to generate more precise statistical results, we included only one covariate. The authors decided to include peripheral blood HIV viral load, to the detriment of CSF HIV RNA, age or sex.

In this study HIV-1 was genotyped based on pol or env sequences, Tat sequencing was not addressed. In a previous study of our group sequencing HIV-1 Tat, with another cohort of participants, HIV-1C was confirmed by the presence of the C30S31 substitution in 82% and HIV-1B was confirmed, by the absence of this substitution, in 90% of the participants (de Almeida et al. 2021a).

The method used to quantify HIV RNA in PB and CSF samples had a limit of detection of 40 copies/mL, which is less sensitive than other comparable assays currently available. Single-molecule assays, which quantify plasma HIV-1 RNA down to a single copy, have enhanced our understanding of the source and dynamics of persistent HIV-1 in the plasma, cells, and reservoirs of PWH. Future CSF studies will benefit from the utilization of these methodologies, as viremia persists in the plasma and CSF even after years of effective therapy (Wang and Palmer 2018).

The study was limited by its cross-sectional design.

In addition, the present study did not evaluate neuropsychological function, and evidence from cross-sectional studies indicates that increased circulation of $CD14^{++}$ $CD16^{+}$ monocytes infected with HIV is associated with neuropsychiatric impairment (Shiramizu et al. 2005; Williams et al. 2013; Williams et al. 2014a, b; Veenstra et al. 2019; Veenhuis et al. 2021).

Conclusion

The results showed that the proportions of PB $CD14^{++}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes were higher in PWH than those in PWoH, despite viral suppression. The impact of HIV-1 on $CD14^{++}CD16^{+}$ monocytes indicated that BBB transmigration was subtype-dependent and higher in HIV-1C than in −1B participants, showing that both HIV-1 subtypes effectively stimulate the transmigration of $CD14^{++}CD16^{+}$ monocytes. Thus, the HIV-1C Tat substitution (C30S31) did not affect the migration of $CD14^{++}CD16^{+}$ monocytes in the CNS. In addition, other HIV proteins such as gp-120 or the auxiliary protein Nef are involved in cytokine and chemokine stimulation and WBC transfer to the CNS. To our knowledge, this is the first study to evaluate monocyte subsets in both the CSF and serum of HIV-1C participants, thereby contributing to the understanding of the pathophysiology of HIV infection in the CNS and the impact of HIV-1 genetic diversity on intermediate

monocyte activation, as well as reservoir establishment and replenishment. This was an exploratory study, its importance is that it is the first study to address monocytes subsets in CSF HIV-1 C compared with HIV-1B, however more studies are necessary for more meaningful conclusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Prof. Sergio Monteiro de Almeida, declare that he has full access to all of the data. Anonymized data from the current study will be made available at the request of qualified investigators if approved by our Research Ethics Board.

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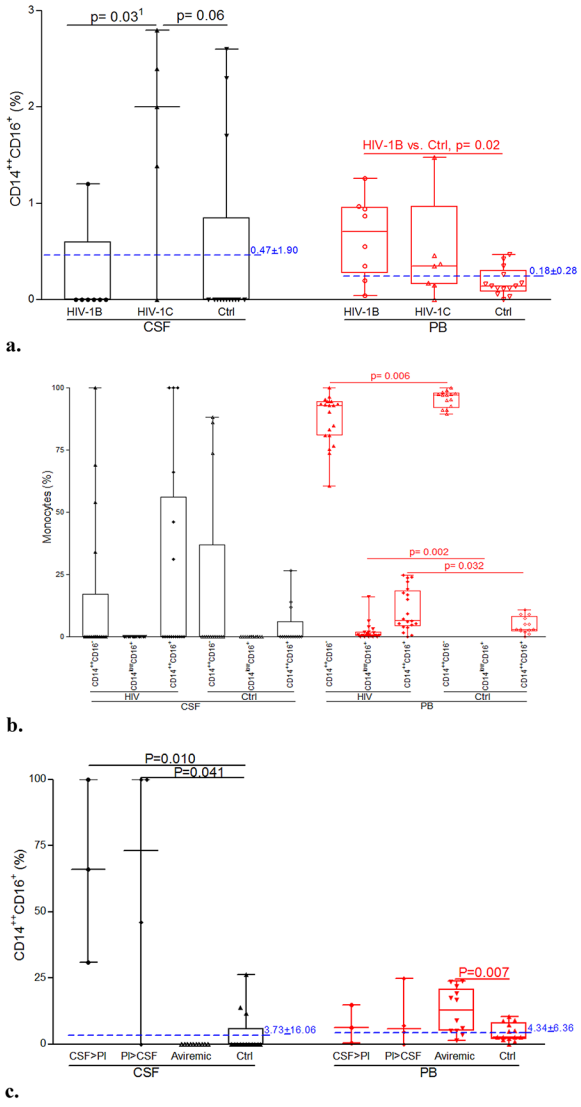

Fig. 1.

Proportion of monocyte subsets in CSF and peripheral blood by HIV serostatus and HIV-1 subtype. Boxes show the medians and interquartile ranges and whiskers indicate the maximum and minimum values. Dots indicate the number of individuals in each group. Monocytes: $CD14^{++}CD16^{-}$ (classical), $CD14^{low}CD16^{+}$ (non-classical), $CD14^{++}CD16^{+}$ (intermediate). **a** Proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF and peripheral blood of individuals with HIV-1B or HIV-1C and PWoH analyzed within the $CD45^{+}$-gated region. Pairwise comparisons were performed using Welchś t-test. [1]In CSF: HIV-1B vs. HIV-1C p = 0.03 and p = 0.10 after multiple testing correction using the BH method. P values were showed if $p < 0.05$. Blue dashed lines indicate mean ±two stand - ard deviations for PWoH (CSF: 0.47 ± 1.90; PB: 0.18 ± 0.28). **b** Proportion of monocyte subsets in the CSF and peripheral blood by HIV serostatus, analyzed within the $CD64^{+}$ gated region. P-values were adjusted for age and sex in the adjusted model and then corrected for multiple testing using the BH method within the CSF and blood. c. Proportion of $CD14^{++}CD16^{+}$ monocytes in the CSF and peripheral blood of the PWH and PWoH groups analyzed within the $CD64^{+}$

gated region. Samples were categorized according to HIV RNA in the CSF and blood compartments. Statistical significance was set at $p < 0.05$. Blue dashed lines indicate mean ± two standard deviations for PWoH (CSF: 3.73 ± 16.06, PB: 4.34 ± 6.36). CSF and PB were compared using the Wilcoxon signed-rank test, and all $p > 0.05$; for the aviremic group not done

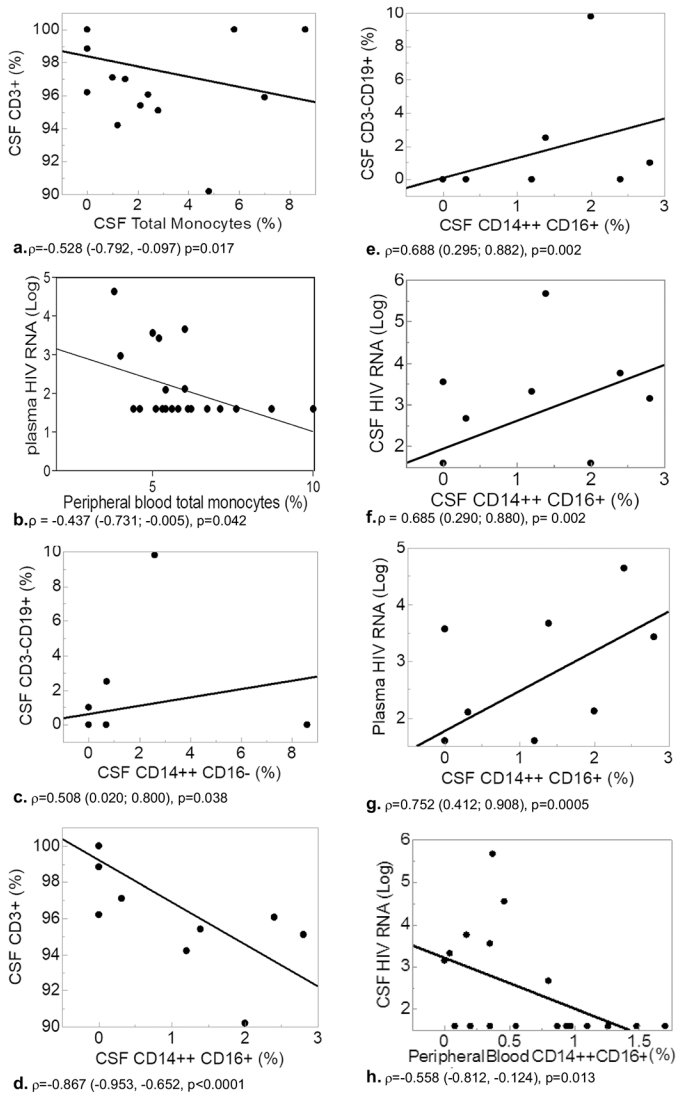

Fig. 2.

Spearman's rank correlation coefficients [ρ (95% CI)] for the proportions of CSF monocyte subsets in PWH, analyzed within the $CD45^{+}$ gated region. Monocytes: $CD14^{++}$ $CD16^{-}$ (classical), $CD14^{++}$ $CD16^{+}$ (intermediate)

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Table 1

Demographics and characteristics of HIV infection and CSF in PWH and PWoH

	all HIV + (n = 22)	**HIV− (n = 14)**	***P***	**HIV-1B (n = 8)**	**HIV-1C (n = 9)**	***P***
Age, years	43.5 (40; 50)	43 (35.5; 52.5)	0.987	43 (41; 48.50)	43 (39.50; 54)	0.743
Male, n (%)	14 (63.64)	5 (37.71)	0.171	8 (100)	3 (33.3)	0.009
AIDS, n (%)	15 (68.18)	-	-	6 (75)	5 (55.56)	0.620
Duration, months	153 (64.97; 182.5)	-	-	156.7 (94.48; 159.70)	150.0 (67.08; 184.50)	0.481
Nadir CD4, cells/mm^3	219 (32.5; 531.5)	-	-	92.00 (26.00; 675.00)	138.0 (26.50; 800.50)	0.805
Current CD4, cells/mm^3	487 (403; 722)	-	-	637.6 (454.2; 797.1)	403.3 (326.7; 438.7)	0.065
Current CD8, cells/mm^3	825 (705; 1571)	-	-	838.5 (716.0; 1259.0)	761.0 (688.0; 1665.0)	0.867
CD4 recovery, cells/mm^3	359 (−34.30; 670)	-	-	326.8 (53.20; 982.60)	359.4 (−27.35; 587.20)	0.535
on CART[b], n (%)	15 (68.18)	-	-	6 (75.00)	5 (55.56)	0.620
CPE[c]	6 (6; 8)	-	-	7 (6; 9)	6	0.537
Log Plasma HIV RNA[a]	1.60 (1.60; 2.55)	-	-	1.6 (1.6; 1.6)	2.12 (1.60; 3.55)	0.281
Plasma HIV RNA < 50 copies/mL, n (%)	14 (63.64)	-	-	7 (87.50)	4 (44.44)	0.131
CSF, n	20	14	-	7	8	-
WBC, cells/mm^3	2.80 (1.20; 6.55)	1.75 (0.6; 2.5)	0.128	1.20 (0.60; 6.85)	4.80 (2.00; 17.80)	0.121
WBC count > 5 cells/mm^3, n (%)	6 (30)	0	0.031	1 (14.3)	4 (50)	0.282
Glucose, mg/dL	57.5 (53; 61.0)	59.5 (54.0; 64.5)	0.473	60.00 (59.00; 69.00)	53.00 (50.00; 56.50)	0.040[d]
Total protein, mg/dL	36.25 (28.90; 43.55)	30.55 (27.35; 31.75)	0.034	42.00 (40.65; 54.55)	28.90 (24.80; 39.90)	0.072
Total protein > 45 mg/dL, n(%)	4 (20)	0	0.126	2 (28.60)	2 (25)	1.000
Lactate, mmol/L	1.4 (1.3; 1.65)	1.4 (1.1; 1.5)	0.432	1.3	1.65 (1.30; 1.70)	-
RBC, cells/mm^3	0.45 (0.3; 3.75)	2.7 (0.3; 12.6)	0.344	0.60 (0.30; 7.50)	1.75 (0.15; 101.70)	0.613
Log CSF HIV RNA	1.6 (1.6; 2.91)	-	-	1.6 (1.6; 2.46)	1.6 (1.6; 3.46)	0.694
CSF HIV RNA < 50 copies/mL, n (%)	13 (65)	-	-	5 (62.5)	4 (44.4)	0.608
HIV RNA CSF > blood, n (%)	3 (15)	-	-	1 (14.3)	1 (12.5)	1.000

[a] Plasma viral load, $\log_{10}$

[b] CART, combination anti-retroviral therapy

[c] CPE, anti-retroviral CNS penetration effectiveness rank (Letendre et al. 2010)

[d] The CSF glucose level was significantly higher in patients with HIV-1 subtype B than those with subtype C, but in both groups CSF glucose levels were within the reference range

Table 2

Proportion of monocyte subsets in the CSF and peripheral blood ofparticipants with HIV subtypes B and C

	A.HIV-1B (n = 7)	B.HIV-1C (n = 8)	Cohen's d (95%CI)	A vs. B P	C.HIV-1B (n = 8)	D.HIV-1C (n = 9)	Cohen's d (95%CI)	C vs. D p	A vs. C p	B vs. D p
	CSF				**Peripheral blood**					
Gate Monocytes										
CD14 $^{++}$ CD16^{-}	0.00 (0.00; 50.00)	0.00 (0.00; 53.90)[1]	0.50 (−0.70; 1.66)	0.63	91.60 (79.85; 93.35)	94.40 (93.20; 98.13)	0.66 (−0.43; 1.71)	0.47	0.03	0.06
CI)I4 ^{low} CD16 $^{+}$	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	1.03 (0.23; 4.61)	0.44 (0.00; 1.25)	−0.91 (−2.00; 0.23)	0.40	-	-
CD14 $^{++}$ CD16 $^{+}$	0.00 (0.00; 50.00)	66.00 (0.00; 100.0)[1]	1.67 (0.16; 3.11)	0.07/0.14[3]	8.02 (4.45; 19.45)	5.160 (4.18; 15.51)	−0.47 (−1.50; 0.59)	0.50	0.30	0.13
Gate Leucocytes										
Total Monocytes	0.00 (0.00; 4.90)	2.60 (1.80; 5.30)	0.68 (−0.36; 1.73)	0.05	5.45 (5.05; 6.60)	6.00 (4.60; 6.45)	−0.26 (−1.22; 0.69)	0.96	0.03	0.06
CD14 $^{++}$ CD16^{-}	0.00 (0.00; 4.30)	0.00 (0.00; 2.60)[1]	0.06 (−1.09; 1.20)	0.92	4.92 (3.80; 5.82)	5.20 (4.13; 6.72)	0.07 (−0.95; 1.08)	0.91	0.05	0.06
CD14 ^{low} CD16 $^{+}$	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	0.06 (0.015; 0.42)	0.03 (0.00; 0.09)	−0.87 (−1.96; 0.26)	0.40	-	-
CD14 $^{++}$ CD16 $^{+}$	0.00 (0.00; 0.60)	2.00 (0.00; 2.80)[1]	2.12 (0.44; 3.72)	0.03/0.10[3]	0.71 (0.28; 0.95)	0.35 (0.16; 0.97)	−0.59 (−1.62; 0.49)	0.47	0.22	0.13
Ratio										
Total monocytes/ CD3^{-}CD19 $^{+}$		1.25 (0.49; 2.80)[1], n = 4			0.75 (0.42; 1.03)	0.51 (0.46; 0.91)	−0.32 (−1.28; 0.64)	0.81		0.15
CD3 $^{+}$ /Total monocytes	45.06 (11.63; 78.50)[1], n = 2	33.96 (33.96; 55.05), n = 7	−0.47 (−2.06; 1.11)	1.00	15.76 (12.18; 16.86)	13.40 (12.18; 17.96)	0.16 (−0.79; 1.12)	0.81	0.71	0.01
N of samples (%) [2]										
Total Monocytes	2/7 (28.57)	7/8 (87.50)		0.04	8/8 (100.00)	9/9 (100.00)		-	0.007	0.47
CD14 $^{++}$ CD16^{-}	1/7 (17.29)	2/5 (40.00)		0.52	8/8 (100.00)	7/7 (100.00)		-	0.001	0.05
CD14 ^{low} CD16 $^{+}$	0/7 (0.00)	0/5 (0.00)		-	7/8 (87.50)	4/7 (57.14)		0.28	0.001	0.08
CD14 $^{++}$ CD16 $^{+}$	1/7 (17.29)	4/5 (80.00)		0.07	8/8 (100.00)	6/7 (85.71)		0.47	0.001	1.00

Values represent the median (IQR); (1) median (min; max); (2) number of samples with the cell type (N, %); p value corrected for multiple testing with the Benjamini-Hochberg (BH) method within CSF and blood and HIV-1 subtype separately; (3) p value before and after corrected for multiple testing with the BH method

Monocytes: **CD14^{++}CD16^{-}** (classical), **CD14^{low}CD16^{+}** (non-classical), **CD14^{++}CD16^{+}** (intermediate)

HIV-1B vs. HIV-1C, p-value adjusted for plasma HIV viral load suppression and nadir CD4 count. Group differences are presented as Cohen's d; CI, confidence interval The Cohen's d (95%CI) effect size for the comparisons of group A vs. C and B vs. D were shown in Supplementary Table 1

Table 3

Proportion of monocyte subsets in CSF and peripheral blood compared between the PWH and PWoH groups

	A.HIV + (n = 20)	B.HIV− (n = 14)	Cohen's d (95% CI)	A vs. B p	C.HIV + (n = 22)	D.HIV− (n = 14)	Cohen's d (95% CI)	C vs. D p	A vs. C p	B vs. D p
	CSF				**Peripheral blood**					
Gate Monocytes										
CD14 $^{++}$ CD16^{-}	0.00 (0.00; 0.17)	0.00 (0.00; 36.80)	0.010 (−0.70; 0.72)	0.980	92.90 (80.74; 94.40)	97.20 (91.90; 97.95)	−1.06 (−1.74; −0.39)	0.006	<0.0001	<0.0001
CD14 ^{low} CD16 $^{+}$	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	0.58 (0.05; 1.72)	0.00 (0.00; 0.00)	1.28 (0.60; 1.95)	0.002	0.006	-
CD14++CD16+	0.00 (0.00; 56.05)	0.00 (0.00; 5.90)	0.50 (−0.21; 1.21)	0.350	6.63 (4.18; 18.36)	2,80 (2.05; 8.10)	0.77 (0.10; 1.45)	0.032	0.818	0.718
Gate Leucocytes										
Total Monocytes	0.50 (0.00; 2.60)	0.00 (0.00; 4.35)	0.26 (−0.43; 0.94)	0.383	5.60 (5.05; 6.45)	4.30 (3.40; 5.45)	−1.08 (−1.79; −0.36)	0.005	0.0004	0.217
CD14 $^{++}$ CD16^{-}	0.00 (0.00; 0.35)	0.00 (0.00; 3.20)	−0.31 (−1.01; 0.40)	0.660	4.92 (4.04; 5.86)	4.09 (3.25; 5.30)	0.65 (−0.04; 1.33)	0.073	<0.0001	0.135
CD14 ^{low} CD16 $^{+}$	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	0.04 (0.005; 0.11)	0.00 (0.00; 0.00)	0.82 (0.14; 1.50)	0.038	0.079	-
CD14 $^{++}$ CD16 $^{+}$	0.00 (0.00; 1.30)	0.00 (0.00; 0.85)	0.16 (−0.55; 0.87)	0.660	0.51 (0.19; 0.96)	0.14 (0.09; 0.31)	1.18 (0.50; 1.86)	0.006	0.818	0.542
Ratio										
Total monocytes/ CD3^{-}CD19 $^{+}$	1.25 (0.49; 2.80)[1], n = 4	-	-	-	0.60 (0.44; 0.93)	0.55 (0.33; 0.81)	−0.42 (−1.09; 0.26)	0.390	0.095	-
CD3 $^{+}$ /Total monocytes	36.99 (15.47; 71.58), n = 10	7.20 (3.48; 10.49)[1], n = 3	−1.31 (−2.70; 0.07)	0.007	14.54 (12.18; 16.86)	18.22 (14.82; 21.73)	1.01 (0.302; 1.72)	0.013	0.003	0.010
N **of samples (%)**[2]										
Total Monocytes	10/20 (50.00)	3/14 (21.43)		0.153	22/22 (100.00)	14/14 (100.00)		-	0.0001	<0.0001
CD14 $^{++}$ CD16^{-}	4/17 (23.53)	3/14 (21.43)		1.000	20/20 (100.00)	14/14 (100.00)		-	<0.0001	<0.0001
CD14 ^{low} CD16 $^{+}$	0/17 (0.00)	0/14 (0.00)		-	15/20 (75.00)	0/14 (0.00)		<0.0001	<0.0001	-
CD14 $^{++}$ CD16 $^{+}$	6/17 (35.29)	3/14 (21.43)		0.456	19/20 (95.00)	13/14 (92.86)		1.00	0.0002	0.0003

Values represent median (IQR); (1) median (min; max); (2) number of samples with the cell type (N, %)

HIV+vs. HIV−, p value was adjusted for age and gender in the adjusted model, and then corrected for multiple testing with the Benjamini-Hochberg (BH) method within CSF and blood and cell subtype separately; Group differences are presented as Cohen's d; CI: confidence interval;

Monocytes: **CD14^{++}CD16^{-}** (classical), **CD14^{++}CD16^{+}** (intermediate), **CD14^{low}CD16^{+}** (non-classical). The Cohen's d (95%CI) effect sizes for the comparisons between blood and CSF, including group A vs. C and B vs. D, are shown in Supplementary Table 1

Table 4

Proportion of monocyte subsets in CSF and blood by group categorized according to the distribution of plasma and CSF HIV RNA

	A. CSF > Plasma (n = 3)[1]	B. Plasma > CSF (n = 4)[1]	C. Aviremic (n = 13)	p[3]	C vs. Ctrl p	D. CSF > Plasma (n = 3)[1]	E. Plasma > CSF (n = 4)[1]	F. Aviremic (n = 14)	p[3]	F vs. Ctrl p
	CSF					**Peripheral blood**				
Gate Monocytes										
CD14^{++}CD16^{-}	34.00 (0.0; 69.00)	0.0 (0.0; 53.90)	0.00 (0.00; 100.00)[1]	0.216	0.712	92.90 (84.70; 93.30)	94.15 (73.60; 100.00)	86.70 (78.65; 93.95)	0.613	0.001
CD14^{low}CD16^{+}	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	0.85 (0.43; 6.26)	0.05 (0.00; 1.64)	0.72 (0.40; 2.38)	0.250	-
CD14^{++}CD16^{+}	66.00 (31.00; 100.0)	73.05 (0.00; 100.0)a	0.00 (0.00; 0.00)	0.003	–	6.25 (0.44; 14.90)	5.80 (0.00; 24.76)	12.97 (5.20; 20.61)	0.579	0.007
Gate Leucocytes										
Total Monocytes	1.20 (1.00; 2.10)	2.60 (0.00; 4.80)	0.00 (0.00; 3.65)	0.344	0.920	6.00 (5.40; 10.00)	5.10 (3.80; 6.00)	5.70 (5.20; 6.90)	0.173	0.003
CD14^{++}CD16^{-}	0.69 (0.0; 0.71)	0.00 (0.00; 2.60)	0.00 (0.00; 8.60)[1]	0.268	0.623	5.57 (4.57; 9.33)	4.52 (3.63; 5.20)	5.35 (3.82; 6.23)	0.369	0.095
CD14^{low}CD16^{+}	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	-	-	0.06 (0.03; 0.63)	0.005 (0.00; 0.12)	0.06 (0.03; 0.15)	0.279	-
CD14^{++}CD16^{+}	1.20 (0.31; 1.39)	2.20 (0.0; 2.80)	0.00 (0.00; 0.00)	0.003	-	0.37 (0.04; 0.80)	0.26 (0.00; 1.48)	0.91 (0.41; 1.04)	0.256	0.0008
Ratio										
Total monocytes/ CD3^{-}CD19^{+}	0.84	1.65 (0.49; 2.80)	1.67	-	-	0.43 (0.35; 1.07)	0.53 (0.51; 1.72)	0.72 (0.48; 0.93)	0.653	0.223
CD3^{+}/Total monocytes	78.50 (45.43; 97.10)	33.96 (18.79; 40.03)	15.47 (11.63; 64.67)[1]	0.078	0.057	12.99 (5.494; 14.18)	15.76 (13.40; 21.73)	14.52 (11.66; 16.86)	0.208	0.011
N of samples (%)[2]										
Total Monocytes	3/3 (100.00)	3/4 (75.00)	4/13 (30.77)	0.098		3/3 (100.00)	4/4 (100.00)	14/14 (100.00)	-	
CD14^{++}CD16^{-}	2/3 (66.67)	1/4 (25.00)	1/10 (10.00)	0.127		3/3 (100.00)	4/4 (100.00)	12/12 (100.00)	-	
CD14^{low}CD16^{+}	0/3 (0.00)	0/4 (0.00)	0/10 (0.00)	-		3/3 (100.00)	2/4 (50.00)	10/12 (83.33)	0.228	
CD14^{++}CD16^{+}	3/3 (100.00)	3/4 (75.00)	0/10 (0.00)	0.001		3/3 (100.00)	3/4 (75.00)	12/12 (100.00)	0.138	

Values represent the median (IQR); (1) median (min; max); (2) number of samples with the cell type (N, %)

Monocytes: **CD14^{++}CD16^{-}** (classical), **CD14^{low}CD16^{+}** (non-classical), **CD14^{++}CD16^{+}** (intermediate)

CI: confidence interval; Ctrl, HIV negative; (3) p, Krukal-Wallis test; Pairwise comparison using Mann–Whitney test: A vs. B, and D vs. E, all ps > 0.05; A vs. HIV negative control, for CSF **CD14^{++}CD16^{+}** monocytes (gate monocytes) p = 0.010; D vs. HIV negative control, for PB total monocytes p = 0.044, for all other pairwise comparisons ps > 0.05; B vs. HIV negative control, for CSF **CD14^{++}CD16^{+}** monocytes (gate monocytes) p = 0.041, for all other pairwise comparisons ps > 0.05