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Loss of CCR2 Expressing Non-Classical Monocytes are Associated with Cognitive Impairment in Antiretroviral Therapy-Naïve HIV-Infected Thais

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Abstract

HIV DNA in monocytes has been linked to HIV-associated neurocognitive disorders (HAND), however, characterization of monocyte subsets associated with HAND remains unclear. We completed a prospective study of antiretroviral therapy-naïve, HIV-infected Thais, with varying degrees of cognitive impairment, compared to HIV-uninfected controls. Monocyte subsets' CCR2, CCR5 and CD163 expression were profiled and inflammatory markers in plasma and cerebrospinal fluid (CSF), measured. Lower numbers of CCR2⁺non-classical monocytes were associated with worse neuropsychological test performance ($r=0.43, p=0.024$). CCR2⁺non-classical monocyte count inversely correlated with CSF neopterin ($r=-0.43, p=0.035$) and plasma TNF- α levels ($r=-0.40, p=0.041$). These data benchmark CCR2⁺non-classical monocytes as an independent index of cognitive impairment.

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Conflict of Interest Disclosure

The authors declare no conflict of interest

Graphical Abstract

Lower numbers of CCR2 expressing non-classical monocytes (CCR2⁺Mono3^{lo}) correlated with worse neuropsychological test performance. These data highlight a new promising cellular biomarker of neurocognitive disease.

Keywords

HIV-associated neurocognitive disorders (HAND); monocytes; C-C chemokine receptor (CCR) CCR2; CD163; neopterin

1. INTRODUCTION

The prevalence of HIV-associated neurocognitive disorder (HAND) remains high (estimated 30-50%) and persists despite plasma HIV RNA suppression with potent combination antiretroviral therapy (cART) (Tozzi et al. , 2009). These cognitive dysfunctions range in severity, spanning from the milder deficits of asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) to more severe deficits in HIV-associated dementia (HAD). These cognitive deficits are not only widespread but impact everyday functioning, and increase morbidity and mortality with critical public health effects (Ellis et al. , 1997, Heaton et al. , 2004, Ickovics et al. , 2001). To date, there are no clinically proven therapies for HAND for individuals already on stable, virologically suppressive anti-HIV regimens (Shapshak et al. , 2011).

Our studies and those of others have highlighted the association between monocytes and HIV neuropathogenesis. We have shown a direct association between cognitive impairment and a greater number of cells harboring HIV DNA selected from cellular populations enriched with monocytes (CD14⁺) (Kusao et al. , 2012). Recent studies from others further document that *in vitro*, cytosolic HIV RNA in monocytes is mandatory for subsequent microglial activation and neurotoxicity (Faissner et al. , 2014). Studies in an SIV non-human primate model of AIDS, underlined the monocyte/macrophage trafficking into the CNS as a driver neuronal injury (Campbell et al. , 2014).

Monocyte nomenclature can be categorized by CD14 and CD16 expression into distinct populations (Gordon and Taylor, 2005, Zawada et al. , 2011): classical monocytes (Mono1; CD14⁺⁺CD16⁻), intermediate monocytes (Mono2; CD14⁺⁺CD16⁺), non-classical

monocytes (Mono3; CD14⁺CD16⁺) (Ziegler-Heitbrock et al. , 2010) and a recently described 4th ‘transitional’ monocyte subset (Mono4; CD14⁺CD16⁻) (Jalbert et al. , 2013a). These monocyte subsets have different inflammatory profiles (Jalbert, Crawford, 2013a) as well as migratory capacities (D’Antoni *et al*; unpublished data) and can be further subdivided according to additional cell surface receptor expression patterns (Wong et al. , 2012).

During the pre-cART era, high levels of several soluble factors were detected in circulation among subjects with HIV encephalitis and HIV dementia. These include monocyte chemoattractant protein-1 (MCP-1) (Bernasconi et al. , 1996, Conant et al. , 1998, Kelder et al. , 1998), neopterin (Brew et al. , 1990, Fuchs et al. , 1989, Sonnerborg et al. , 1989), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) (Sippy et al. , 1995, Tyor et al. , 1992) and interleukin-6 (IL-6) (Perrella et al. , 1992, Rusconi et al. , 2013). In patients on virally suppressive cART regimens, the levels of neopterin, TNF- α , MCP-1 and IL-6 have been documented to remain elevated and persist when compared to HIV uninfected subjects or those not on cART (Abdulle et al. , 2002, Cassol et al. , 2013, Probasco et al. , 2010, Sevigny et al. , 2004). Most of these soluble mediators, which can be secreted by monocytes and macrophages, have been associated with neurocognitive impairment in HIV-infected individuals on suppressive cART (Cassol, Misra, 2013, Sevigny, Albert, 2004).

The identification of new biomarkers for HIV-infected individuals with milder forms of HAND and among subjects who are suppressed on cART would afford an opportunity for mechanistic insight into the pathogenesis of HAND. Chemokine receptors have been suggested to play a role in HAND. We have shown in a single arm trial that intensification with a C-C chemokine receptor type 5 (CCR5) inhibitor improved cognition in cART suppressed cognitively impaired HIV-infected subjects (Ndhlovu et al. , 2014). Recent studies identified CCR2 on monocytes as a correlate to cognitive impairment in HIV-infected individuals (Williams et al. , 2014). CD163, a scavenger receptor, is emerging as a potential marker of activation and inflammation in HIV and a correlate of neurological injury. The frequency of CD163⁺ CD14⁺ CD16⁺ monocytes are increased in viremic HIV-infected individuals compared to HIV-uninfected individuals or ART suppressed aviremic HIV subjects (Fischer-Smith et al. , 2008).

Here we seek to discover new correlates to cognitive impairment in participants with HAND by utilizing multi-flow cytometry to evaluate distinct monocyte subsets, using CCR2, CCR5 and CD163 expression profiling among treatment naïve chronically HIV-infected subjects from Bangkok.

2. MATERIAL AND METHODS

2.1 Participant Characteristics

Twenty-nine participants were selected from a larger study designed to investigate HIV DNA as a marker of HAND among individuals who were cART-naïve and met Thai Ministry of Health guidelines to initiate therapy (SEARCH 011, [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00782808): NCT00782808), as previously described (Valcour et al. , 2013). For this study, we randomly selected individuals with both high (≥ 1000 copies/ 10^6 cells) and low (< 1000 copies/ 10^6

cells) HIV DNA. The high HIV DNA included 14 participants (median=2137 copies/10⁶ cells; interquartile range (1255, 2658)) and the low HIV DNA, 15 participants (median=615 copies/10⁶ cells; interquartile range (294, 668)). *HAND diagnosis*: Participants were diagnosed by consensus conference as (1) cognitively normal (HIV NL), (2) ANI, (3) MND, or (4) HAD using established guidelines (Antinori et al. , 2007). Neuropsychological testing was performed on all participants. Age-matched HIV-uninfected Thai individuals were evaluated as controls.

NPZ Global Score: Subjects completed a 1-hour neuropsychological (NP) testing battery. All raw scores were transformed to standard z-scores using Thai normative data as previously described. A measure of global performance (NPZ-global) was calculated as the arithmetic mean of all tests with domain scores similarly calculated from domain-specific components of the battery. The neuropsychological battery included tests of attention and concentration, working memory and executive function, verbal and visual learning and memory, psychomotor and manual dexterity, motor, language, and visuospatial domains, as previously described (Schifitto et al. , 2007).

2.2 Monocyte Phenotyping

Blood was separated within eight hours of the blood draw and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation (Pfizer, Inc., New York, NY). Separated PBMCs and plasma were cryopreserved for future phenotypic analyses of cells and plasma cytokines. Cryopreserved PBMCs were thawed using RPMI 1640 (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum, and placed in 96 well polypropylene round bottom plates. Cells were stained with Live/Dead® Fixable Aqua Dead Cell Stain for 15 min at room temperature followed by two, ten minute sequential incubations with anti-CCR2 (AlexaFluor647) and CCR5 (Phycoerythrin (PE)) at 37 °C (Jalbert et al 2013a, b). Cells were then stained at room temperature for 30 minutes with anti-CD3 (V500), CD14 (Qdot605), CD16 (AlexaFluor700), CD56 (PE-Cy7), CD19 (PE-Cy7), CD20 (PE-Cy7), human leukocyte antigen-D related (HLA-DR) (Allophycocyanin (APC)-H7) and CD163 (AlexaFluor488). Cells were fixed with 1% PFA and data were acquired on a custom 4-laser BD LSRFortessa (BD Biosciences, San Jose, CA). Compensation and gating analyses were performed using FlowJo (Treestar Inc, Ashland, OR). All antibodies were purchased from BD Bioscience (San Jose, CA), except for CD14-Qdot605 and yellow Live/Dead® Fixable Aqua Dead Cell Stain (Life Technologies, Grand Island, NY) and CD163-AlexaFluor488 (R&D Systems, Minneapolis, MN).

The gating strategy for identification of monocytes, their subsets, and receptor expression is shown in Figure 1. Monocytes were identified from the PBMC population by the exclusion of dead cells, lymphocytes (CD3 positive cells), natural killer (NK; CD56 positive) and B cells (CD19 or CD20 positive cells). Monocytes, positive for HLA-DR, were subset by CD14 and CD16 expression: Classical monocytes (Mono1; CD14⁺⁺CD16⁻), intermediate monocytes (Mono2; CD14⁺⁺CD16⁺), non-classical monocytes (Mono3^{hi}; CD14⁺CD16⁺ and Mono3^{lo}; CD14^{lo}CD16⁺) and transitional monocytes (Mono4; CD14⁺CD16⁻). Monocyte populations were further assessed for CCR2, CCR5 and CD163 expression (Supplemental

Figure 1). Monocyte numbers were calculated by multiplying monocyte number (Mono/ μ l) from the white blood cell count obtained from the clinical laboratory values and the percent frequency generated by flow cytometry.

2.3 Plasma Soluble Biomarkers

We evaluated the following circulating inflammatory biomarkers: neopterin, TNF- α , IL-6, IL-1 β and MCP-1 in the plasma and CSF in this cohort. TNF- α , IL-6, IL-1 β and MCP-1 were quantified in triplicate as part of a custom multiplex ELISA array according to the manufacturer's protocol (Quansys Biosciences, Logan, UT). Data were captured on the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) and analyzed using Quansys Q-view Plus software (Quansys Biosciences). Single-analyte ELISA was performed in duplicate to detect levels of neopterin (GenWay Biotech, San Diego, CA) and analyzed using SoftMax Pro (Molecular Devices, Sunnyvale, CA).

2.4 CD14⁺ monocyte isolation and HIV DNA quantification

Cryopreserved PBMCs were thawed in warm media (RPMI 1640 supplemented with 20 % fetal bovine serum), washed once, and resuspended in RoboSep buffer (StemCell Technologies, Vancouver, BC, Canada). Samples were placed in a RoboSep automated cell separator (StemCell Technologies) and CD14⁺ cells were purified through magnetic separation using the EasySep human monocyte enrichment kit without CD16 depletion (StemCell Technologies). DNA was extracted from CD14⁺ monocytes or total PBMCs using the QIAamp DNA Micro Extraction kit (Qiagen, Valencia, CA) and quantified using the ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) as previously described (Shiramizu et al. , 2012). Determination of HIV DNA content was assessed using multiplex real-time PCR with HIV gag and β -globin primer pairs to amplify respective regions that were detected with FAMTM-labeled HIV gag and VIC[®]-labeled β -globin probes. Using standard reference plasmids with one copy of the β -globin housekeeping gene and one copy of the HIV gag gene and appropriate positive/negative controls, samples were run in triplicate on StepOnePlus Real-Time PCR System and analyzed using the StepOne software (Applied Biosystems, Foster City, CA). The copy numbers of each sample gene were analyzed against the standard curves and used to calculate HIV DNA copy number per 1×10^6 cells.

2.5 Statistical Analyses

The demographic and clinical information of participants were summarized by percentage for gender and by median and interquartile range (IQR) for continuous variables, e.g. CD4 count. The gender comparisons were examined by Fisher's exact tests. For continuous variables, Kruskal-Wallis tests were performed to compare multiple neuropsychological diagnosis groups. Wilcoxon-Mann-Whitney tests were conducted to compare two different groups, e.g., monocyte levels between HIV-uninfected and infected subjects, or between HIV NL subjects and HIV HAND subjects, without multiple comparison adjustments. The association analyses between two continuous variables were evaluated by Spearman correlation coefficients, e.g. between NPZ global score and a monocyte subset. The association between NPZ global score and CCR2⁺ Mono3^{lo} subpopulation was further explored by multiple linear regression, adjusting for Mono3^{lo}, HIV DNA from CD14⁺ cells,

and plasma viral load. Model statistical assumptions were checked. Statistical analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC). A two-sided p -value < 0.05 was regarded as statistically significant.

3. RESULTS

3.1 Clinical Cohort

We evaluated 29 HIV-infected Thai participants who were classified by Frascati HAND criteria to have normal cognition (NL; $n=17$), Asymptomatic Neurocognitive Impairment (ANI; $n=6$), Mild Neurocognitive Disorder (MND; $n=1$) and HIV-associated Dementia (HAD; $n=5$) (Table 1). Within the HIV-infected participants, there were no differences between the HAND and the non-HAND groups in terms of education, plasma viral load, CD4 count and CD8 T cell activation (Table 1). NPZ global score and HIV DNA were significantly different between non-HAND and HAND (ANI and MND+HAD) groups ($p=0.0002$ and $p=0.016$, respectively; Table 1). Both the HIV-infected and uninfected individuals were gender and aged matched (Table 1). The frequency of activated CD8⁺ T cells (HLA-DR⁺ CD38⁺) was significantly greater in the HIV-infected individuals compared to the HIV-uninfected group ($p<0.0001$; Table 1).

3.2 Monocyte subsets and HAND diagnosis

Monocytes were categorized into five different subsets based on CD14 and CD16 expression (Figure 1). Cell surface expression for the chemokine receptors, CCR2 and CCR5 and, the scavenger receptor, CD163 were measured on all monocyte subsets (Supplemental Figure 1). By monocyte subset, the HIV-infected HAND group had lower Mono1 and CCR2 expressing Mono1 (CCR2⁺ Mono1) numbers compared to the HIV-uninfected group ($p = 0.0013$ and $p = 0.0040$, respectively; Figure 2A, B). The group of HIV-infected participants with normal cognition had the lowest number of Mono1 and CCR2⁺ Mono1, which differed from that of the HIV-uninfected group ($p < 0.001$ and $p < 0.001$, respectively) and the HIV-infected HAND group ($p = 0.034$ and $p = 0.048$, respectively) (Figure 2A, B).

We observed a significantly lower number of Mono2s in the HAND group compared to the HIV-uninfected participants ($p = 0.0018$; Figure 2C). The numbers of Mono2 and CCR2⁺ Mono2 cells in the HIV-infected normal group were significantly lower than the HIV-uninfected group ($p < 0.001$ and $p = 0.020$, respectively; Figure 2C, D). No differences were seen between Mono2 and CCR2⁺ Mono2 numbers between the HIV-infected normal and HIV-infected HAND groups (Figure 2C, D).

As seen with the previous subsets and compared to the HIV-uninfected individuals, we noted a lower number of Mono3^{hi} and CCR2⁺ Mono 3^{hi} in the HIV-infected normal group ($p < 0.001$ and $p = 0.036$, respectively; Figure 2E, F) and in the HIV-infected HAND group ($p = 0.0057$ and $p = 0.0065$, respectively; Figure 2E, F). No differences were seen in the numbers of Mono3^{hi} and CCR2⁺ Mono 3^{hi} between the HIV-infected HAND and HIV-infected normal groups (Figure 2E, F). There were no significant differences between the Mono3^{lo} populations across groups, however, the HIV-infected HAND group had a

significantly lower number of CCR2⁺ Mono 3^{lo} subsets compared to the HIV-infected normal group ($p = 0.031$) and the HIV-uninfected group ($p = 0.0056$) (Figure 2G, H). The HIV-infected normal group also had significantly lower CCR2⁺ Mono 3^{lo} numbers than that of the HIV-uninfected group ($p = 0.032$; Figure 2H). There were no significance differences observed with the Mono4 cells however the HIV-infected HAND group had lower CCR2⁺ Mono4 numbers compared to the HIV-uninfected individuals ($p = 0.025$; Figure 2I, J).

3.3 Relationship between neuropsychological function and circulating monocyte subsets

The NPZ global score directly correlated with the number of the non-classical monocyte subset, Mono3^{lo}, ($r = 0.43$, $p = 0.024$), as well as the number of CCR2⁺ Mono3^{lo} ($r = 0.43$, $p = 0.024$) (Figure 3A) among HIV-infected participants. We did not find other correlations between the NPZ global scores and any other monocyte subset expressing the various receptors we measured (data not shown). The number of Mono3^{lo} cells directly correlated with the NPZ-psychomotor score ($r = 0.44$, $p = 0.022$; Figure 3B) and a trend was noted with the NPZ-motor score ($r = 0.37$, $p = 0.060$; data not shown). The number of CCR2 expressing Mono3^{lo} directly correlated with the NPZ-psychomotor score ($r = 0.47$, $p = 0.014$; Figure 3B) and the NPZ-motor score ($r = 0.38$, $p = 0.050$; Figure 3C). On the corollary, CCR2⁻ Mono3^{lo} numbers, correlated with both the NPZ global and NPZ-psychomotor score ($r = 0.43$, $p = 0.025$ and $r = 0.44$, $p = 0.022$, respectively, data not shown).

3.4 Correlations between plasma and cerebrospinal fluid (CSF) inflammatory markers, monocyte HIV DNA content and monocyte subsets

Statistically significant negative correlations between the inflammatory markers, CSF neopterin and plasma TNF- α levels, and the number of CCR2⁺ Mono3^{lo} cells were observed ($r = -0.43$, $p = 0.035$ and $r = -0.40$, $p = 0.041$; Figure 4A, B). No significant correlations were observed between CSF neopterin or plasma TNF- α levels and the number of monocyte subsets, including Mono3^{lo} and CCR2⁻ Mono3^{lo} populations (data not shown). Plasma TNF- α levels also inversely correlated with the number of CD163⁺ Mono3^{lo} cells ($r = -0.41$, $p = 0.035$; Figure 4C). There was also significant correlation between CSF IL-6 levels and Mono3^{hi} numbers ($r = -0.45$, $p = 0.027$) (data not shown). There were no correlations found between any of the monocyte subsets evaluated and CSF and plasma IL-1 β or MCP-1 levels (data not shown). There were no significant correlations however we did observe a positive trend between the total number of classical monocytes (Mono1) and total HIV DNA within the CD14⁺ bead selected monocytes ($r = 0.34$, $p = 0.067$) (data not shown).

3.5 Multiple linear regression analysis

A multiple linear regression analysis was performed to further explore the relationship between neuropsychological testing performance (NPZ global) and the number of monocyte populations and subpopulations, HIV DNA from CD14⁺ enriched peripheral blood mononuclear cells (PBMCs) and plasma viral load. In this model, **the number of CCR2⁺ Mono3^{lo} cells** was the only independent predictor of NPZ global ($p = 0.043$; Table 2), which was consistent with the earlier simple regression analysis shown in Figure 3B.

4. DISCUSSION

Monocytes are thought to contribute to HAND pathogenesis by mediating HIV-neuroinvasion, which ultimately leads to HIV in the brain tissue, neuro-inflammation and neuronal damage. Many studies have focused on soluble factors, such as cytokines and chemokines, which might contribute to pathogenesis. Data regarding receptors are still lacking, as assessing multiple cell surface chemokine receptors can be technically challenging. We have developed a flow cytometry based assay to measure several cell surface receptors simultaneously (including chemokine and scavenger receptors) on cryopreserved PBMCs.

When examining the different receptors found on the monocyte subsets in this cohort, our data show unexpectedly, that lower numbers of a unique subset of non-classical monocytes, with low CD14 and high CD16 expression and more specifically, those expressing CCR2 in the periphery were linked to cognitive impairment among treatment naïve individuals. The loss of this population in circulation may represent an increased transmigration of this subset into tissue sites. Since CCR2⁺ Mono3^{lo} prominently correlated with clinical performance and given the patrolling nature of non-classical monocytes (Wong, Yeap, 2012) one may hypothesize that the CCR2⁺ Mono3^{lo} subset, may be important in mediating neuroinvasion into the CNS compartment.

Others have identified CCR2 as being an important predictor of HAND. In an *in vitro* blood brain barrier (BBB) model, Williams *et al* (2014) demonstrated that CCR2 mean fluorescence intensity (MFI) was significantly higher on the intermediate monocyte subset population from participants with HAND compared to unimpaired HIV-individuals. The functional consequence to this difference was an increase in migration across the BBB toward an MCP-1 gradient. The discrepancies in the specific monocyte subset identified in relation to HAND may reflect differences in the cohort and experimental parameters examined between studies. Interestingly, a recent study by Uleri *et al* document, using an *in vitro* model system, that HIV Tat may promote HIV neuroinvasion *in vivo* through increases in CD16 and CCR2 on monocytes (Uleri *et al.* , 2014). It would be interesting in future studies to determine the HIV content in these unique minor monocytoid subsets as triggers for neuroinvasion and neuroinflammation.

A clear consensus on human monocyte nomenclature has not yet been achieved including agreement on how to assess and quantify this circulating population using single cell flow cytometric techniques (Abeles *et al.* , 2012, Tallone *et al.* , 2011, Wong, Yeap, 2012). A number of studies have documented the frequency of monocytes populations in limited depth in HAND (Ancuta *et al.* , 2008, Pulliam *et al.* , 1997). When calculated by number of monocytes, our data document changes in the proportion of monocyte subsets in HAND, a finding that differs from other studies. This could be due to the distinct populations assessed but also on the design and depth of the immunophenotyping presented (Abeles, McPhail, 2012, Ndhlovu, Umaki, 2014). We have observed that to accurately assess chemokines on monocytes due to their recycling nature sequential and coordinated methods of staining are required (Jalbert *et al.* , 2013b). Our recent studies presented here have assessed monocytes

using a strategy adopted by several groups using HLA-DR gating to obtain better resolution of these populations in the setting of disease (Abeles, McPhail, 2012).

Neopterin is a soluble factor that reflects the intensity of monocyte/macrophage activation and has shown utility as a biomarker of HIV-neuropathology (Hagberg et al. , 2010, Valcour, Ananworanich, 2013). We found that CCR2 expressing monocytes were associated with several measured soluble biomarkers. There was an inverse correlation between the number of CCR2⁺Mono3^{lo} and CSF neopterin and plasma TNF- α levels. That is the fewer CCR2⁺Mono3^{lo} in circulation, the greater the peripheral inflammatory profile. Given these data, one might conclude that the greater presence of CCR2⁺ Mono3^{lo} may serve a protective role in cases where HAND exists. Although their presence is associated with less inflammation for cytokines important to HAND, in this study, they also correlate with disease severity (based on NPZ global score). Indeed CCR2 expressing monocytes have been shown to have greater sensitivity in entry to the CNS (Williams et al. , 2013). On the corollary however, a lower frequency in the circulation of these subsets may suggest increased seeding into the brain through a CCR2-CCL2 driven mechanism resulting in a greater inflammatory environment as revealed by higher levels of neopterin and TNF- α . Recent studies have identified a novel biomarker, 6-Sulfo LacNAc (SLAN) that is linked to the secretion of TNF- α by Mono3 non-classical monocytes (Dutertre et al. , 2012). Given our data, it appears that the entire Mono3^{lo} population may be an important marker of neurocognitive impairment but that the CCR2⁺ Mono3^{lo} cells may be particularly noteworthy as these cells correlate with the inflammation associated with cognitive impairment. Whether this unique subset is responsible for the release of these inflammatory mediators warrants further investigation. Taken together our data suggest that CCR2⁺Mono3^{lo} may serve a novel biomarker for assessing the degree of HAND.

The strength of this study is the ability to correlate multiple monocyte cellular subpopulations and clinical neurocognitive parameters in subjects with HAND. Although the cohort is small, it has highlighted new promising cellular biomarkers of neurocognitive disease. Moreover, it is interesting to note that the HIV-infected individuals analyzed in this study were not on cART and may benefit from additionally from cART and chemokine mediated blockade targeting peripheral monocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CCR2	C-C chemokine receptor type 2
CSF	cerebrospinal fluid
HAND	HIV-associated neurocognitive disorders
TNF-α	tumor necrosis factor- α
A	area
AIDS	acquired immune deficiency syndrome
APC	Allophycocyanin
ANI	asymptomatic neurocognitive impairment
BBB	blood brain barrier
cART	combination antiretroviral therapy
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CNS	central nervous system
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assay
FSC	forward scatter
H	height
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen-D related
IL	interleukin
IQR	interquartile range
MCP-1	monocyte chemoattractant protein-1
MFI	mean fluorescence intensity
MND	mild neurocognitive disorder
Mono	monocyte
NK	natural killer
NL	cognitively normal
NPZ	neuropsychological performance
PBMC	peripheral blood mononuclear cell

PCR	polymerase chain reaction
PE	Phycoerythrin
SIV	Simian immunodeficiency virus
SSC	side scatter
SLAN	Sulfo LacNAc
TNF-α	tumor necrosis factor- α
YARD	Yellow Reactive Amine Dye

REFERENCES

- Abdulle S, Hagberg L, Svennerholm B, Fuchs D, Gisslen M. Continuing intrathecal immunoactivation despite two years of effective antiretroviral therapy against HIV-1 infection. *AIDS*. 2002; 16:2145–9. [PubMed: 12409735]
- Abeles RD, McPhail MJ, Sowter D, Antoniadis CG, Vergis N, Vijay GK, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi)/CD16(neg) monocytes: Expansion of CD14(hi)/CD16(pos) and contraction of CD14(lo)/CD16(pos) monocytes in acute liver failure. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2012; 81:823–34. [PubMed: 22837127]
- Ancuta P, Kamat A, Kunstman KJ, Kim EY, Autissier P, Wurcel A, et al. Microbial translocation is associated with increased monocyte activation and dementia in AIDS patients. *PloS one*. 2008; 3:e2516. [PubMed: 18575590]
- Antinori A, Arendt G, Becker JT, Brew BJ, Byrd DA, Cherner M, et al. Updated research nosology for HIV-associated neurocognitive disorders. *Neurology*. 2007; 69:1789–99. [PubMed: 17914061]
- Bernasconi S, Cinque P, Peri G, Sozzani S, Crociati A, Torri W, et al. Selective elevation of monocyte chemotactic protein-1 in the cerebrospinal fluid of AIDS patients with cytomegalovirus encephalitis. *The Journal of infectious diseases*. 1996; 174:1098–101. [PubMed: 8896515]
- Brew BJ, Bhalla RB, Paul M, Gallardo H, McArthur JC, Schwartz MK, et al. Cerebrospinal fluid neopterin in human immunodeficiency virus type 1 infection. *Annals of neurology*. 1990; 28:556–60. [PubMed: 2252366]
- Campbell JH, Ratai EM, Autissier P, Nolan DJ, Tse S, Miller AD, et al. Anti- α 4 antibody treatment blocks virus traffic to the brain and gut early, and stabilizes CNS injury late in infection. *PLoS pathogens*. 2014; 10:e1004533. [PubMed: 25502752]
- Cassol E, Misra V, Morgello S, Gabuzda D. Applications and limitations of inflammatory biomarkers for studies on neurocognitive impairment in HIV infection. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*. 2013; 8:1087–97. [PubMed: 24259252]
- Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, et al. Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:3117–21. [PubMed: 9501225]
- Dutertre CA, Amraoui S, DeRosa A, Jourdain JP, Vimeux L, Goguet M, et al. Pivotal role of MDC8(+) monocytes from viremic HIV-infected patients in TNF α overproduction in response to microbial products. *Blood*. 2012; 120:2259–68. [PubMed: 22802339]
- Ellis RJ, Deutsch R, Heaton RK, Marcotte TD, McCutchan JA, Nelson JA, et al. Neurocognitive impairment is an independent risk factor for death in HIV infection. San Diego HIV Neurobehavioral Research Center Group. *Archives of neurology*. 1997; 54:416–24. [PubMed: 9109743]

- Faissner S, Ambrosius B, Schanzmann K, Grewe B, Potthoff A, Munch J, et al. Cytoplasmic HIV-RNA in monocytes determines microglial activation and neuronal cell death in HIV-associated neurodegeneration. *Experimental neurology*. 2014; 261:685–97. [PubMed: 25150097]
- Fischer-Smith T, Tedaldi EM, Rappaport J. CD163/CD16 coexpression by circulating monocytes/macrophages in HIV: potential biomarkers for HIV infection and AIDS progression. *AIDS research and human retroviruses*. 2008; 24:417–21. [PubMed: 18373432]
- Fuchs D, Chiodi F, Albert J, Asjo B, Hagberg L, Hausen A, et al. Neopterin concentrations in cerebrospinal fluid and serum of individuals infected with HIV-1. *AIDS*. 1989; 3:285–8. [PubMed: 2504232]
- Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nature reviews Immunology*. 2005; 5:953–64.
- Hagberg L, Cinque P, Gisslen M, Brew BJ, Spudich S, Bestetti A, et al. Cerebrospinal fluid neopterin: an informative biomarker of central nervous system immune activation in HIV-1 infection. *AIDS research and therapy*. 2010; 7:15. [PubMed: 20525234]
- Heaton RK, Marcotte TD, Mindt MR, Sadek J, Moore DJ, Bentley H, et al. The impact of HIV-associated neuropsychological impairment on everyday functioning. *Journal of the International Neuropsychological Society : JINS*. 2004; 10:317–31. [PubMed: 15147590]
- Ickovics JR, Hamburger ME, Vlahov D, Schoenbaum EE, Schuman P, Boland RJ, et al. Mortality, CD4 cell count decline, and depressive symptoms among HIV-seropositive women: longitudinal analysis from the HIV Epidemiology Research Study. *Jama*. 2001; 285:1466–74. [PubMed: 11255423]
- Jalbert E, Crawford TQ, D'Antoni ML, Keating SM, Norris PJ, Nakamoto BK, et al. IL-1Beta enriched monocytes mount massive IL-6 responses to common inflammatory triggers among chronically HIV-1 infected adults on stable anti-retroviral therapy at risk for cardiovascular disease. *PloS one*. 2013a; 8:e75500. [PubMed: 24086545]
- Jalbert E, Shikuma CM, Ndhlovu LC, Barbour JD. Sequential staining improves detection of CCR2 and CX3CR1 on monocytes when simultaneously evaluating CCR5 by multicolor flow cytometry. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2013b; 83:280–6. [PubMed: 23426986]
- Kelder W, McArthur JC, Nance-Sproson T, McClernon D, Griffin DE. Beta-chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus-associated dementia. *Annals of neurology*. 1998; 44:831–5. [PubMed: 9818943]
- Kusao I, Shiramizu B, Liang CY, Grove J, Aagsa M, Troelstrup D, et al. Cognitive performance related to HIV-1-infected monocytes. *The Journal of neuropsychiatry and clinical neurosciences*. 2012; 24:71–80. [PubMed: 22450616]
- Ndhlovu LC, Umaki T, Chew GM, Chow DC, Aagsa M, Kallianpur KJ, et al. Treatment intensification with maraviroc (CCR5 antagonist) leads to declines in CD16-expressing monocytes in cART-suppressed chronic HIV-infected subjects and is associated with improvements in neurocognitive test performance: implications for HIV-associated neurocognitive disease (HAND). *Journal of neurovirology*. 2014
- Perrella O, Carrieri PB, Guarnaccia D, Soscia M. Cerebrospinal fluid cytokines in AIDS dementia complex. *Journal of neurology*. 1992; 239:387–8. [PubMed: 1403021]
- Probasco JC, Deeks SG, Lee E, Hoh R, Hunt PW, Liegler T, et al. Cerebrospinal fluid in HIV-1 systemic viral controllers: absence of HIV-1 RNA and intrathecal inflammation. *AIDS*. 2010; 24:1001–5. [PubMed: 20299968]
- Pulliam L, Gascon R, Stubblebine M, McGuire D, McGrath MS. Unique monocyte subset in patients with AIDS dementia. *Lancet*. 1997; 349:692–5. [PubMed: 9078201]
- Rusconi S, Vitiello P, Adorni F, Colella E, Foca E, Capetti A, et al. Maraviroc as Intensification Strategy in HIV-1 Positive Patients with Deficient Immunological Response: an Italian Randomized Clinical Trial. *PloS one*. 2013; 8:e80157. [PubMed: 24244635]
- Schifitto G, Zhang J, Evans SR, Sacktor N, Simpson D, Millar LL, et al. A multicenter trial of selegiline transdermal system for HIV-associated cognitive impairment. *Neurology*. 2007; 69:1314–21. [PubMed: 17652642]

- Sevigny JJ, Albert SM, McDermott MP, McArthur JC, Sacktor N, Conant K, et al. Evaluation of HIV RNA and markers of immune activation as predictors of HIV-associated dementia. *Neurology*. 2004; 63:2084–90. [PubMed: 15596754]
- Shapshak P, Kanguane P, Fujimura RK, Commins D, Chiappelli F, Singer E, et al. Editorial neuroAIDS review. *AIDS*. 2011; 25:123–41. [PubMed: 21076277]
- Shiramizu B, Ananworanich J, Chalermchai T, Siangphoe U, Troelstrup D, Shikuma C, et al. Failure to clear intra-monocyte HIV infection linked to persistent neuropsychological testing impairment after first-line combined antiretroviral therapy. *Journal of neurovirology*. 2012; 18:69–73. [PubMed: 22207583]
- Sippy BD, Hofman FM, Wallach D, Hinton DR. Increased expression of tumor necrosis factor- α receptors in the brains of patients with AIDS. *Journal of acquired immune deficiency syndromes and human retrovirology : official publication of the International Retrovirology Association*. 1995; 10:511–21.
- Sonnerborg AB, von Stedingk LV, Hansson LO, Strannegard OO. Elevated neopterin and beta 2-microglobulin levels in blood and cerebrospinal fluid occur early in HIV-1 infection. *AIDS*. 1989; 3:277–83. [PubMed: 2504231]
- Tallone T, Turconi G, Soldati G, Pedrazzini G, Moccetti T, Vassalli G. Heterogeneity of human monocytes: an optimized four-color flow cytometry protocol for analysis of monocyte subsets. *Journal of cardiovascular translational research*. 2011; 4:211–9. [PubMed: 21308491]
- Tozzi V, Balestra P, Salvatori MF, Vlassi C, Liuzzi G, Giancola ML, et al. Changes in cognition during antiretroviral therapy: comparison of 2 different ranking systems to measure antiretroviral drug efficacy on HIV-associated neurocognitive disorders. *J Acquir Immune Defic Syndr*. 2009; 52:56–63. [PubMed: 19731418]
- Tyor WR, Glass JD, Griffin JW, Becker PS, McArthur JC, Bezman L, et al. Cytokine expression in the brain during the acquired immunodeficiency syndrome. *Annals of neurology*. 1992; 31:349–60. [PubMed: 1586135]
- Uleri E, Mei A, Mameli G, Poddighe L, Serra C, Dolei A. HIV Tat acts on endogenous retroviruses of the W family and this occurs via Toll-like receptor4: inference for neuroAIDS. *AIDS*. 2014
- Valcour VG, Ananworanich J, Agsald M, Sailasuta N, Chalermchai T, Schuetz A, et al. HIV DNA reservoir increases risk for cognitive disorders in cART-naïve patients. *PloS one*. 2013; 8:e70164. [PubMed: 23936155]
- Williams DW, Byrd D, Rubin LH, Anastos K, Morgello S, Berman JW. CCR2 on CD14(+)CD16(+) monocytes is a biomarker of HIV-associated neurocognitive disorders. *Neurology(R) neuroimmunology & neuroinflammation*. 2014; 1:e36. [PubMed: 25340088]
- Williams DW, Calderon TM, Lopez L, Carvallo-Torres L, Gaskill PJ, Eugenin EA, et al. Mechanisms of HIV entry into the CNS: increased sensitivity of HIV infected CD14+CD16+ monocytes to CCL2 and key roles of CCR2, JAM-A, and ALCAM in diapedesis. *PloS one*. 2013; 8:e69270. [PubMed: 23922698]
- Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. *Immunologic research*. 2012; 53:41–57. [PubMed: 22430559]
- Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, et al. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood*. 2011; 118:e50–61. [PubMed: 21803849]
- Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010; 116:e74–80. [PubMed: 20628149]

Highlights

- Monocyte HIV DNA correlates with HAND, which persists despite cART.
- Monocyte subset characterization in the context of HAND remains incomplete.
- Lower CCR2⁺ non-classical monocyte number associated with worse NPZ testing.
- CCR2⁺ non-classical monocyte count inversely correlated with neopterin and TNF- α .
- Loss of CCR2⁺ non-classical monocytes may serve as a biomarker for HAND.

Figure 1. Monocyte Identification and Phenotype Gating Strategy

Top row: Singlets were identified using forward scatter (FSC) height (H) and area (A). Dead cells, staining positive for Yellow Reactive Amine Dye (YARD), were excluded and PBMCs were identified by size and granularity (side scatter (SSC) vs FSC). Middle row: CD3 positive cells (lymphocytes) were excluded, along with CD19-20-56 positive cells (B cells and natural killer cells). Cells positive for HLA-DR were identified as monocytes. Bottom row: HLA-DR positive cells were subset according to CD14 and CD16 expression. Mono1 are characterized as being CD14⁺⁺CD16⁻; Mono2, CD14⁺⁺CD16⁺; Mono3^{hi}, CD14⁺CD16⁺; Mono3^{lo}, CD14^{lo}CD16⁺ and Mono4, CD14⁺CD16⁻.

Figure 2. Monocyte subset numbers and HAND diagnosis

(A-J) Monocyte subset numbers (left column) and the number of CCR2⁺ monocytes sorted by subset (right column), expressed as cell number per μl, were compared in HIV-uninfected individuals with normal cognition, HIV-infected individuals with normal cognition (normal) and HIV-infected individuals with HAND.

Figure 3. Correlations between Mono3^{lo} subsets and neuropsychological testing data
Correlations between Mono3^{lo} and CCR2⁺Mono3^{lo} subsets, expressed as cell number per μl, and NPZ global score (A), NPZ-psychomotor score (B) and NPZ-motor score (C), were assessed.

Figure 4. Correlations between Mono3^{lo} subsets and inflammatory marker levels
 Correlations between the CCR2⁺Mono3^{lo} subset, expressed as cell number per μl, and neopterin CSF levels (pg/μl; A); and TNF-α plasma levels (pg/μl; B) were assessed. Correlations between CD163⁺Mono3^{lo} number and plasma TNF-α (pg/μl; C) were also assessed.

Table 1

Clinical and Demographic Characteristics of Participants (n = 73)

	HIV-Uninfected		HIV-Infected			P-value (HIV- vs. HIV+)	P-value ** (Comparing 3 groups within HIV+)
	n = 44		Normal n = 17	HAND (HIV-associated neurocognitive disorders) n = 6 ANI = 6	MND = 1 HAD = 5		
Gender (% male)	45.5%		64.7%	50.0%	33.3%		p = 0.46
Age (years)	35.1 (32.2, 38.2)	p = 0.48	33.2 (28.9, 36.5)	33.8 (26.4, 39.6)	33.3 (29.3, 37.1)		p = 0.97
Education (years)	-	-	11 (9, 15)	14 (12, 19)	6 (2, 14)		p = 0.11
NIPZ global score	-	-	0.277 (0.086, 0.569)	-0.280 (-0.451, 0.026)	-1.562 (-1.620, -1.466)		p = 0.0002
Log ₁₀ Plasma Viral Load (copies/ml)	-	-	4.998 (4.350, 5.469)	4.914 (4.405, 5.161)	5.565 (4.760, 5.589)		p = 0.33
Log ₁₀ CD4 ⁺ Total HIV DNA	-	-	1.342 (1.255, 1.568)	1.831 (1.663, 2.199)	2.090 (2.025, 2.152)		p = 0.016
CD4 count (cells/ μ L)	-	-	239 (159, 328)	331 (261, 397)	190 (30, 310)		p = 0.14
CD8 T cell activation (%)	9.1 (6.8, 12.2)	p < 0.0001	35.9 (31.5, 42.3)	44.7 (38.9, 48.5)	36.8 (35.2, 53.5)		p = 0.60

Data are shown as median (interquartile range), except for gender.

* P-values calculated by Fisher's exact test or Wilcoxon-Mann-Whitney test

** P-values calculated by Kruskal-Wallis tests

Table 2

Multiple linear regression analysis

R-Square	Coeff Var	Root MSE	NPZglobal Mean
0.328	-708.2	0.661	-0.093343

Parameter	Estimate	Standard Error	t Value	p-value
Intercept	1.166	0.956	1.22	0.2356
CCR2 Mono6 count	27.497	12.809	2.15	0.0431
Mono6 count	-0.010	0.014	-0.73	0.4752
Log ₁₀ CD14 ⁺ HIV DNA	-0.477	0.311	-1.53	0.1399
Log ₁₀ plasma viral load	-0.145	0.194	-0.75	0.4627