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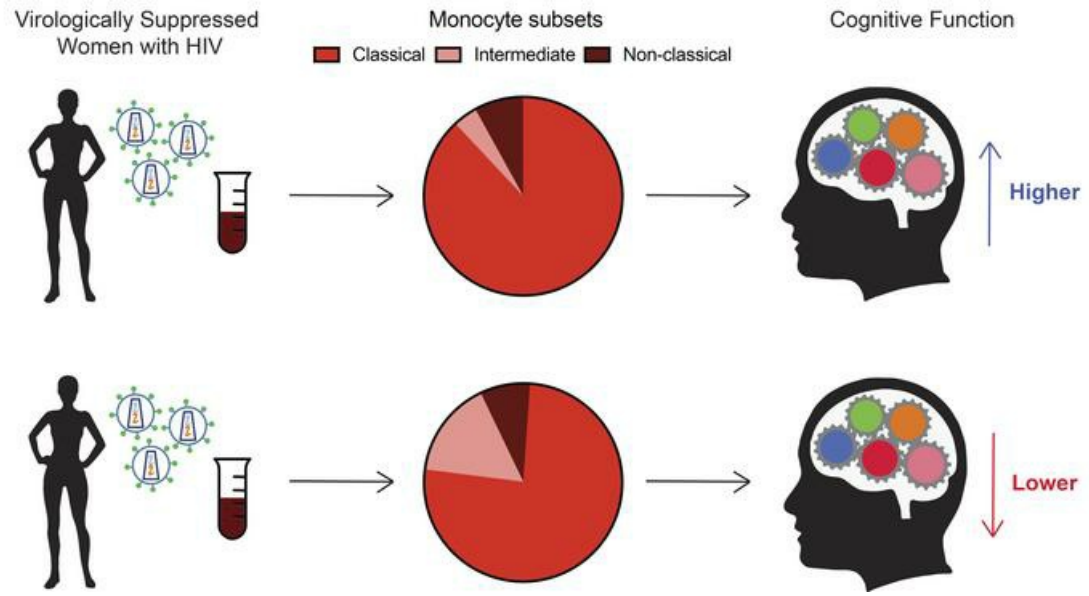
Graphical abstract



Higher circulating intermediate monocytes are associated with cognitive function in women with HIV

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Running Title: Intermediate Monocytes: A Cognitive Biomarker in HIV Infected Women

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Abbreviations

Human Immunodeficiency Virus (HIV); people with HIV (PWH); central nervous system (CNS); women with HIV (WWH); Mononuclear cells (MNC); Toll-like receptor 2 (TLR2); neuropsychological (NP); HIV-associated neurocognitive disorder (HAND);

Hopkins Verbal Learning Test-Revised (HVLT-R); Letter-Number Sequencing (LNS); Trail Making Test- (TMT); Symbol Digit Modalities Test (SDMT); Controlled Oral Word Associations Test (COWAT); Grooved Pegboard (GPEG); Center for Epidemiological Studies Depression scale (CES-D); Perceived Stress Scale (PSS); Post-Traumatic Stress Disorder (PTSD); Checklist-Civilian Scale (PCL-C); phosphate-buffered saline (PBS); peripheral blood mononuclear cells (PBMC); Spearman's Rho correlations (rs); Women's Interagency HIV Study (WIHS)

Abstract

Background: Identifying a quantitative biomarker of neuropsychiatric dysfunction in people with HIV (PWH) remains a significant challenge in the neuroHIV field. The strongest evidence to date implicates the role of monocytes in central nervous system (CNS) dysfunction in HIV, yet no study has examined monocyte subsets in blood as a correlate and/or predictor of neuropsychiatric function in virally suppressed PWH.

Methods: In two independent cohorts of virologically suppressed women with HIV (vsWWH; n=25 and n=18), whole blood samples were obtained either in conjunction with neuropsychiatric assessments (neuropsychological [NP] test battery, self-report depression and stress-related symptom questionnaires) or one year prior to assessments. Immune cell subsets were assessed by flow cytometry.

Results: A higher proportion of intermediate monocytes ($CD14^{+}CD16^{+}$) was associated with lower global NP function when assessing monocytes concurrently and approximately one year before (predictive) NP testing. The same pattern was seen for executive function (mental flexibility) and processing speed. Conversely, there were no associations with monocyte subsets and depression or stress-related symptoms. Additionally, we found that a higher proportion of classical monocytes was associated with better cognition.

Conclusion: Although it is widely accepted that lentiviral infection of the CNS targets cells of monocyte-macrophage-microglial lineage, is associated with an increase in intermediate monocytes in the blood and monocyte migration into brain, the percentage of intermediate monocytes in blood of vsWWH has not been associated with neuropsychiatric outcomes. Our

findings provide evidence for a new, easily measured blood-based cognitive biomarker in vsWWH.

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Brief summary: Higher proportions of intermediate monocytes in blood correlate with impaired cognitive function in virally suppressed women with HIV, and act as a readily accessible blood-based cognitive biomarker.

Introduction

Neuropsychiatric complications persist despite effective antiretroviral therapy (ART) in people with HIV (PWH). Ongoing challenges remain in understanding the underlying pathophysiology and in identifying an easily measurable, quantitative blood-based biomarker for predicting, detecting, and monitoring these complications. The role of monocytes in central nervous system (CNS) dysfunction is well-supported by preclinical and clinical studies in HIV(1-3), yet no monocyte marker has been identified as a biomarker of neuropsychiatric dysfunction in PWH.

Monocytes are the initial defense against invading pathogens and play an essential role in the innate immune response. Monocytes are multi- functional and are either pro- or anti-inflammatory mediators that guide the development of the innate and adaptive immune response to a pathogen(4, 5). In humans, monocytes can be identified using Toll-like receptor 2 (TLR2) as a surface marker(6, 7) and then divided into three circulating subsets, classified based on the expression levels of the surface proteins CD14 (co-receptor for toll-like receptor 4) and CD16 (Fc gamma receptor IIIa). Classical monocytes ($CD14^+/CD16^-$) are the most abundant, comprising approximately 80-95% of all monocytes(8). These cells are effective phagocytes with multiple functions including the coordination of innate immune responses, production of pro- and anti-inflammatory cytokines, and migration into tissues in response to inflammatory signals(9, 10). Intermediate monocytes ($CD14^+/CD16^+$), account for 2-11%(8) of monocytes and are also called pro-inflammatory monocytes. This subset presents antigen(11), secretes high levels of pro-inflammatory cytokines(12), expresses chemokine receptors that drive their migration into tissues(13), and can expand in circulation following cardiac events, cancer, autoimmune disease, stroke, and in a number of bacterial and viral infections, including HIV(14, 15). Intermediate

monocytes are also permissive to HIV infection due to high levels of CCR5 expression on the cell surface(16, 17). Non-classical monocytes ($CD14^{-}/CD16^{+}$) also known as patrolling monocytes, are present between 2-8% (8). This subset can survey the vasculature, patrol the endothelium (9) and promote homeostasis(18, 19). These cells rarely transmigrate into tissues in response to the inflammatory signals that drive classical and some intermediate monocytes(20). Similar to their intermediate precursors, this subset can also be infected with HIV, and are increased during a variety of infections(16).

There is strong evidence that alterations in monocytes may contribute to neuropsychiatric complications in people with HIV (PWH). Increased circulation of intermediate monocytes ($CD14^{+}/CD16^{+}$) infected with HIV are associated with immune activation and cognitive impairment in cross-sectional studies(2, 21-24). These monocytes are thought to transport HIV into the brain(3, 25-28) where HIV proteins, cytokines, and chemokines damage cells and tissue. Additionally, increases in peripheral soluble CD14 and CD163 remain markers commonly associated with CNS injury, specifically with cognitive impairment(1-3, 29). Other CNS outcomes, including mental health factors such as depression and post-traumatic stress, have yet to be linked to monocyte subsets in PWH. There is evidence in HIV-uninfected individuals that higher levels of intermediate monocytes can be used to differentiate between people exhibiting depressive symptoms and those individuals not exhibiting these symptoms(30). Further, the presence of monocyte subsets can be used in conjunction with other markers (e.g., C-reactive protein) to distinguish subtypes of depression(30).

There are well-documented sex differences in monocyte phenotypes in HIV-uninfected populations, however, studies are limited in PWH. Studies report a lower proportion of non-classical ($CD14^{-}CD16^{+}$) monocytes in healthy women compared to men(31). Moreover, after adjusting for age, monocytes from healthy women have different expression patterns of CD38, CD62L and CD115, and plasma levels of CXCL10, and sCD163 are elevated while sCD14 is decreased compared to healthy men(32). In the context of disease, preclinical studies indicate that trafficking of monocytes to the site of inflammation is decreased in female compared to male mice in a model of acute inflammation(33). Additionally, women with systemic lupus erythematosus show increased monocyte activation compared to men with lupus(34). Given these sex differences, we focus here on women with HIV (WWH) because the monocyte-cognition associations may not be the same in women and men with HIV.

Currently there is no clinical biomarker to predict neuropsychiatric function (includes cognition and mental health) in virally suppressed WWH. In the present study, we examined associations between subtypes of monocytes, CD4+ T cells, CD8+ T cells, CD4:CD8 ratio and neuropsychiatric function in WWH. We hypothesized that higher levels of intermediate monocytes would be associated with lower cognitive function (via a comprehensive neuropsychological test battery) and more mental health symptoms (depression, post-traumatic stress via questionnaires).

Results

Cohort characteristics

The Baltimore cohort included 25 Black, non-Hispanic WWH between 46 and 64 years of age (mean=55, standard deviation [SD]= 5.5, **Supplemental Table 1**). All women were on effective ART, of whom 80% had HIV RNA <20 copies/ml and 100% had HIV RNA <250 copies/ml. The majority of participants were on an integrase inhibitor-based regimen with 16 (64%) on second generation INSTI and 7 (28%) on first generation INSTI: 10 (40%) dolutegravir, 6 (24%) bictegravir, 5 (20% elvitegravir, 2 (8%) raltegravir. The median monocyte subset percentages were 73.3% classical ($CD14^+/CD16^-$), 15.1% non-classical ($CD14^-/CD16^+$), and 8.9% intermediate ($CD14^+/CD16^+$). Cognitively, 40% of participants had global cognitive impairment, with a global neuropsychological (NP) demographically-adjusted Z-score less than one SD from the reference group (Women's Interagency HIV Study [WIHS] HIV-uninfected women(35, 36)). On average, WWH in the Baltimore cohort demonstrated the greatest difficulty in verbal learning, delayed free recall, and recognition (demographically-adjusted Z-scores <1). A second cohort, the Bronx study cohort, was used to validate our findings in the Baltimore cohort. The Bronx study cohort was a predictive cohort as monocyte percentages were measured a 1 year (median) prior to NP assessment. These data were used to determine if monocyte proportions may predict future NP function in WWH. The Bronx cohort included 18 Black, non-Hispanic WWH between 32 and 58 years of age (mean=48.5, SD=8.3, **Supplemental Table 1**). All women were on effective ART, of whom 83% had HIV RNA <20 copies/ml and 100% had HIV RNA <250 copies/ml. The majority of participants were on Tenofovir+Emtricitabine (72%), Ritonovir was used in 7 (39%) and atazanavir 4 (22%). The median monocyte subset percentages were 72% classical ($CD14^+/CD16^-$), 17% non-classical ($CD14^-/CD16^+$), and 11% intermediate ($CD14^+/CD16^+$).

The demographic, behavior, and cellular composition in both cohorts were similar. However, the Baltimore cohort demonstrated greater cognitive difficulties compared to the Bronx cohort. Only two women (11%) in the Bronx cohort had a global NP demographically-adjusted Z-score <1 SD from the reference group (WIHS HIV-uninfected women(35, 36)), meaning 2 out of 18 women were globally impaired whereas 10 out of 25 women in the Baltimore cohort were globally impaired. On average, z-scores for all NP outcomes did not fall below the one standard deviation cutoff. The most difficult test on average for the participants in the Bronx cohort was the LNS working memory (Z=-0.56), whereas for the Baltimore cohort it was delayed free recall and recognition (demographically-adjusted Z-scores <1). In the case of both cohorts, sociodemographic (e.g., age, education), behavioral (e.g., substance use, smoking), and clinical factors (e.g., CD4+ count) were not related to biomarkers (monocytes or T-cells) and NP outcomes and thus were not confounders of the associations of interest. Full characteristics on the participants from both cohorts, including education, mental health, drug use etc, are available in **Supplemental Table 1.**

A larger proportion of MNC monocytes is associated with better cognitive function

To assess whether the proportions of mononuclear cells (MNC) in whole blood are associated with neuropsychiatric outcomes, we obtained whole blood samples in conjunction with NP testing and mental health measures from participants in the Baltimore cohort. Whole blood samples were analyzed by FACS to assess the proportion of MNC, including monocytes, CD4+ T cells, and CD8+ T cells (**Figure 1A-F**). MNC were defined as the sum of TLR2+ monocytes and TLR2- lymphocytes and estimate the total proportion of MNC in a whole blood sample (**Figure 1G**).

194 TLR2 was used as a marker for monocytes because it separates monocytes more definitively than
195 FSC and SSC alone. This marker is ubiquitously expressed on human and macaque monocytes
196 and the percentage of monocytes expressing TLR2 does not change with SIV or HIV infection
197 status ((6) and **Supplemental Figure 1**). However, TLR2 is also expressed on other cell types and
198 there for it is necessary to use both TLR2 and SSC to cleanly gate out this population. Only
199 monocytes expressing TLR2 are included in our analysis, any T cells or granulocytes that express
200 low levels of TLR2 are removed via SSC gating (**Supplemental Figure 2**). TLR2+ monocytes are
201 negative for CD3, CD8, CD159a, and CD20, and show dim expression of CD4 as expected in
202 human monocytes(37). Additionally, it is important to note, TLR2 is being used as a phenotyping
203 marker similar to CD14 and CD16, and not being used to assess immune function, similar to how
204 CD14 (LPS-receptor) and CD16 (FC receptor) are not being used to assess LPS and antibody
205 signaling in these cells. A higher proportion of MNC monocytes (TLR2+) was associated with
206 higher performance on all HVLT-R outcomes (total learning, delayed free recall, recognition),
207 SDMT, Trail Making Test (TMT-Part B), Stroop-3, LNS-working memory and Animal fluency
208 (*P*'s<0.05; **Figure 2, Table 1**). In contrast, the proportions of MNC CD4+ and CD8+ T cells or
209 CD4:CD8 ratio, were not associated with any of the NP or mental health outcomes (*P*'s>0.09;
210 **Supplemental Table 2**).
211
212 To better understand the associations between total monocytes and NP function, we conducted a
213 series of correlations in the Baltimore cohort focusing on: 1) how the proportions of MNC
214 monocyte subsets (classical, intermediate and non-classical) correlate with each other and MNC
215 TLR2+ monocytes (total monocytes) and 2) how the proportions of MNC monocyte subtypes
216 correlate with NP outcomes. When correlating the proportion of each MNC monocyte subset to

the proportion of MNC TLR2+ monocytes, a higher number of classical monocytes correlated with a higher proportion of TLR2+ cells (Spearman's Rho[rs]=0.93, $P<0.001$, **Figure 3A, Supplemental Table 3**). There were no significant associations between the proportions of MNC monocyte subsets (P's>0.16). This suggests that as the proportion of TLR2+ monocytes increase in blood, there is either an increase of classical monocytes egressing from the bone marrow or a lack of classical monocytes trafficking into the tissues in response to an inflammatory signal. When correlating the proportion of MNC monocyte subsets with NP outcomes, there were positive associations observed with the classical monocyte subset, that closely mirror the associations observed with MNC TLR2+ cells. A higher proportion of MNC classical monocytes was associated with higher global NP function and higher performance on all HVLT-R outcomes (total learning, delayed free recall, recognition), TMT-A and B, SDMT, Letter and Animal fluency, Stroop Trial 3, and LNS working memory (P's<0.05; **Figure 3B, Table 1**). These data provide additional evidence that the positive outcomes observed with higher proportions of TLR2+ MNC in whole blood are primarily driven by the classical monocyte fraction.

A higher percentage of the intermediate monocyte subset is associated with worse cognitive function

Assessing neuropsychiatric outcomes in relation to the proportions of all MNC elucidates whether the proportion of each cell type compared to all cells is related to these outcomes. However, this type of analysis can minimize the contribution of small cellular populations such as intermediate and non-classical monocytes, which often make up less than 1% of all MNC. To compensate for this possibility, we completed an alternate analysis that assessed neuropsychiatric outcomes in relation to the percentage of each monocyte subset within the monocyte cellular fraction alone

(**Figure 1G**). This is an important comparison as assessing the percentage of monocyte subsets, using CD14/CD16 expression to distinguish cell types, is the most common way of analyzing these cells and would allow for comparisons between multiple cohorts. Additionally, it is important to know if absolute monocyte numbers are associated with cognitive function or if the percentages of monocyte subsets, within the monocyte system, are reflective of NP performance.

When assessing the percentages of TLR2+ monocyte subsets (classical, intermediate, non-classical) within the Baltimore cohort, a higher percentage of intermediate monocytes ($CD14^{+}CD16^{+}$) was associated with lower global NP function (rs=-0.54, *P*=0.006; **Figure 4A; Table 1**). When examining each of the NP outcomes separately, a higher percentage of intermediate monocytes was also associated with lower performance on all outcomes on the HVLT-R (total learning, delayed free recall, recognition), TMT-Part B, SDMT, Animal fluency, LNS working memory condition, and the Grooved Pegboard dominant hand (*P*'s<0.05). However, the percentage of non-classical monocytes was not associated with any NP outcomes (*P*'s>0.12). Thus, when all CD16+ monocytes (intermediate + non-classical) are combined, as has been reported in previous analyses(2, 3, 22-24, 27, 28, 38), it results in no significant associations with NP outcomes, except for TMT-Part B (rs=-0.44, *P*=0.02) and Animal fluency (rs=-0.49, *P*=0.01; **Supplemental Table 4**). Although the percentage of classical monocytes was not associated with global NP function (*P*=0.11), a higher percentage was associated with higher performance on TMT-Part B (rs=0.44, *P*=0.03), and Animal fluency (rs=0.54, *P*=0.006). Mental health measures were not significantly associated with any of the monocyte subsets (*P*'s>0.27).

To confirm our findings, we assessed monocyte subsets in relation to NP and mental health outcomes in a secondary validation and predictive cohort. In the Bronx cohort, monocyte subset percentages were assessed in PBMCs and neuropsychiatric assessments performed approximately one year later (median). In this cohort, a higher percentage of intermediate monocytes was also associated with lower global NP function (rs=-0.54, *P*=0.02; **Figure 4B; Table 2**). When examining each of the NP outcomes separately, a higher percentage of intermediate monocytes was also associated with lower performance on TMT-Part B (rs=-0.68, *P*=0.003) and SDMT (rs=-0.65, *P*=0.003). Similar to the Baltimore cohort, classical and non-classical monocytes were not associated with global NP function in the Bronx cohort (*P*'s>0.65). There were no associations between the percentage of classical or non-classical monocytes and any NP outcome (*P*'s>0.14). Mental health measures were not significantly associated with any of the monocyte subsets (*P*'s>0.11). These data suggest that the percentages of the intermediate monocytes within the monocyte cellular fraction, is reflective of worse cognition both concurrently and over time while classical monocytes do not appear to play a role long term.

The proportion of MNC monocytes (particularly classical) and the percentage of intermediate monocyte subset were associated with cognitive function even among virally suppressed

To ensure that our pattern of significant associations were not being driven by the few participants with low levels of viremia (HIV RNA <250 copies/ml), we re-ran our correlations after removing these individuals. After removing the five participants with HIV RNA between 20-238 copies/ml in our Baltimore cohort, we observed the same pattern of associations between %MNC TLR2+, classical monocytes (%MNC and %subsets), and cognitive function as we did in the full cohort

285 (**Supplemental Table 5)**. The same pattern of associations remained between the % intermediate
286 monocyte subsets and cognitive outcomes except for the Grooved Pegboard dominant hand. We
287 also re-ran our analyses in the Bronx cohort and again no change was observed in the pattern of
288 associations after removing the three women with HIV RNA less than 100 copies/ml (data not
289 shown). These data suggest that low level viral blips are not the driving force of the monocyte
290 subset associations with cognitive function observed in the vsWWH.
291
292 **Discussion**
293 In this study, we identified a cognitive biomarker that can be readily measured in blood and may
294 be used to assess neuropsychiatric function in WWH. Here, we examined associations between
295 total monocytes, monocyte subsets, T cells, and neuropsychiatric function in WWH. First, in two
296 independent cohorts, higher proportions of intermediate monocytes ($CD14^{+}CD16^{+}$) were
297 associated with lower global cognitive function as well as lower executive function (mental
298 flexibility) and processing speed. These associations were present when assessing monocytes
299 concurrently with (Baltimore cohort), and approximately one year before (Bronx cohort), the NP
300 assessment. Our analysis indicated that the percentage of intermediate monocytes within the
301 monocyte fraction of cells, and not the proportion of MNC intermediate monocytes, was associated
302 with lower cognitive function. Second, when assessing monocytes and NP function concurrently
303 there were a number of other cognitive correlates with intermediate monocytes, including those
304 that assess verbal learning and memory, semantic fluency, working memory and motor. Third, the
305 opposite patterns of associations were observed when examining associations between total MNC
306 monocytes, classical monocytes (subset percentage and MNC proportion), and cognitive function,
307 a greater number of total and classical monocytes were associated with better cognitive function.

Fourth, there were no significant associations between monocytes and mental health outcomes and as expected, no associations between CD4+, CD8+ T cells and CD4:CD8 ratio and cognitive function. Fifth, small detectable viral blips (20-250 copies/ml) do not drive the observed associations with cognitive function as we found that removal of participants with detectable viral blips primarily strengthened the pattern of associations. Overall, our findings suggest that the proportion of the intermediate monocyte subset in blood provides insight into immune-brain connections, specifically cognitive function in virally suppressed WWH.

It is widely accepted that lentiviral infection of the CNS targets cells of monocyte-macrophage-microglial lineage, causing immune activation and blood brain barrier disruption(2, 16, 29). The association of these events with increases in monocyte migration into brain and the emergence of intermediate monocytes ($CD14^{+}CD16^{+}$) in the blood has been previously been reported(3, 22, 23). However, the proportion of intermediate monocytes in blood of virally suppressed WWH has never been directly associated with cognitive function. Currently, markers of chronic immune activation, such as circulating monocytes, show the most promise as blood biomarkers to measure NP function during viral suppression. Circulation of intermediate monocytes infected with HIV contributes to chronic immune activation and these monocytes are thought to transport HIV into the brain(25), where HIV proteins, cytokines and chemokines damage cells and tissue. While numerous studies demonstrate that higher levels of monocyte inflammatory markers (sCD163, sCD14) in blood are associated with lower NP function in PWH(39-43), including women(41, 42), no study to date has demonstrated links between specific monocyte subsets and global or domain-specific NP function in virally suppressed WWH. Previous studies assessing monocyte subsets in relation to cognitive function have either combined subsets (e.g., all CD16+ cells, (2, 3, 22-24, 27,

28, 38)), focused on markers on monocyte cells (e.g., CCR2, CCR5, CD38, CD163(24, 44, 45)), or measured peripheral pro-inflammatory proteins (e.g., sCD163, sCD14(41, 43)) as a surrogate. One study assessed HIV DNA levels within monocyte subsets and correlated DNA levels with cognition; however, correlations with the actual monocyte subsets and cognition were not included(46). An additional study assessed HIV DNA levels in PBMCs and found a correlation with CCR2 expression on intermediate monocytes and HAND(24).

Our study focused directly on the proportion of monocyte subsets as a biomarker rather than downstream indicators. In addition to CD14 and CD16, we have used TLR2 as a marker to more specifically separate monocytes from NK cells and granulocytes(6, 7). Including TLR2 as a marker provides a more accurate assessment of total monocytes and proportions of monocyte subsets. Additionally, we analyzed each monocyte subset independently, as each subset provides information about inflammation and homeostasis in the host while grouping monocyte subsets together obscures the function of each subset. Recent studies have shown that the intermediate and non-classical monocytes are transcriptionally and functionally distinct(47), and that the intermediate monocytes are the cells that preferentially migrate across the blood brain barrier(3, 27). In fact, if we combine both of the CD16+ monocyte subsets (intermediate and non-classical monocytes) in our dataset, the previously observed associations between monocytes and cognition are lost except for Animal Fluency and TMT-B. Finally, the use of fresh samples, as was done with both cohorts, allows us to account for all monocytes present in the blood at the time of draw. Freezing and subsequent thawing of PBMCs can lead to preferential loss of inflammatory cell types(48). Together, these alternative methods of acquisition and monocyte subset analysis may

353 explain why we observed a strong phenotype with intermediate monocyte subset percentages and
354 cognition that has been previously overlooked in other studies.
355
356 Our findings provide evidence that the percentage of the intermediate monocyte subset is
357 associated with cognitive function in WWH. We demonstrate that a greater proportion of total
358 TLR2+ monocytes present in blood is associated with better concurrent cognition whereby the
359 magnitude of associations across all NP outcomes were similar suggesting a general rather than
360 domain specific association with cognitive function. Additionally, higher total TLR2+ monocytes
361 directly correlates with an increase in the classical monocyte cell type, as expected given that
362 classical monocytes typically comprise 80-95% of total monocytes in the blood(8) . A larger
363 proportion of classical monocytes is indicative of homeostasis and low levels of inflammation, as
364 classical monocytes are relatively immature in the blood and do not mature until they receive
365 inflammatory signals that recruit them to injured tissues(49, 50). Therefore, a higher proportion in
366 the blood suggests a lack of recruitment to tissues and lower inflammation. In HIV specifically,
367 classical monocyte levels are lower in ART suppressed PWH compared to HIV-uninfected
368 individuals(51, 52). Therefore, it is possible that the WWH in our cohort with a higher proportion
369 of classical monocytes are more similar to healthy individuals and thus associated with better
370 cognitive function.
371
372 In contrast, the percentage of the intermediate monocyte subset significantly correlates with
373 impaired concurrent and predictive cognitive function. Additionally, intermediate monocytes do
374 not strongly correlate with total TLR2+ monocytes or classical monocytes. This suggests that an
375 increase in the percentage of the intermediate monocyte subset in the blood is a result of a) classical

monocytes trafficking out of blood into tissue altering the proportions of cells in the blood, or b) a greater number of classical monocytes maturing into intermediate monocytes, not an increase in total monocytes in blood. Further, the proportion of intermediate monocytes to all MNC in the blood is not associated with cognitive function. This suggests that it is not the absolute number of intermediate monocytes that is important but the proportion of intermediate monocytes to the other monocyte subsets. However, it is also possible that the MNC analysis may mask the effects of very small cell populations. Intermediate monocytes have a higher capacity to secrete cytokines in the blood and are reflective of an inflammatory environment(49, 50). Therefore, a change in their proportion within the monocyte subsets would likely be associated with a change in inflammation in the host. Additionally, when comparing monocyte subset proportions between ART suppressed PWH and HIV-uninfected individuals, it has been shown that intermediate monocyte levels are the same while classical and non-classical monocyte proportions differ between the two groups(51, 52). This reinforces the conclusion that the subset percentage, and not the absolute number, of intermediate monocytes is important for cognitive function.

When assessing monocytes and cognition concurrently, the strongest NP outcomes that correlate with intermediate monocytes included the HVLT-R, Animal fluency, TMT-Part B, SDMT, and the LNS working memory condition. The deficits observed in these tasks most likely relate to changings in brain function as chronic low-level inflammation in PWH may lead to the migration of intermediate monocytes into corticolimbic brain regions necessary to perform these NP tests(53-57). When we assessed monocytes approximately one year before NP testing, the two NP tests that remained significant included TMT-Part B and SDMT. Functional neuroimaging studies of the NP tasks that are significant in both cohorts implicate the prefrontal cortex as being required to

399 complete each of these tasks(53, 54). Therefore, elevated intermediate monocytes may be
400 predictive of prefrontal function throughout HIV infection despite ART suppression.
401
402 Despite the small sample size, both cohorts were acquired, processed, and analyzed independently,
403 and therefore the reproducibility of our concurrent and predictive findings gives weight to the
404 associations observed. In fact, the strength of the correlations observed, despite the small sample
405 size in each cohort, is suggestive of an important and relevant finding. The generalizability of our
406 findings may be limited given the cohorts are composed of predominately low-income women of
407 color with HIV. However, this is an important and relevant HIV population, given that people of
408 color comprise the majority of HIV infections in the US and around the world. Studies
409 incorporating men and healthy individuals would determine the generalizability of our findings.
410 We are not suggesting that this finding is specific to WWH as previous work has shown that the
411 percentages of CD16+ monocytes in blood of men and women with HIV do not differ(28),
412 suggesting that these cells are present in men at a similar frequency and may relate to cognition in
413 this population as well. Additionally, it should be noted that in this study women were defined by
414 self-report biological sex and the terms woman and female are used interchangeably in this study.
415 This is the first study to directly assess intermediate monocyte percentages in blood and cognition
416 in any virally suppressed group living with HIV and will need expansion. The effect of ART
417 regimens on monocyte subset and/or cognition was not addressed in this study due to small sample
418 sizes and the relatively heterogeneous treatment regimens within each cohort. Finally, our findings
419 focus on cognition and HIV, however, it is likely these findings will extend beyond HIV and
420 contribute to other neurological diseases and disorders as $CD14^{+}CD16^{+}$ monocytes have emerged
421 as a principal driving cell type for a number of pro-inflammatory conditions.

This study has provided evidence of a cognitive biomarker that can be readily measured in blood in WWH. We report that the percentage of the intermediate monocyte subset significantly correlates with impaired cognitive function, and that the levels of intermediate monocytes may have a long lasting effect on cognition in WWH. These findings warrant future larger-scale studies to identify the optimal cut-point for the percentage of intermediate monocytes that predict cognitive impairment in PWH.

Methods

Study Cohorts and Procedures

The initial study cohort (Baltimore cohort) included 25 WWH enrolled in a Phase 0 clinical trial between December 5, 2018 to March 3, 2020 focused on the effects of glucocorticoids on cognition (https://clinicaltrials.gov/ct2/show/NCT03237689; R01 MH113512). These participants passed a phone screen and were scheduled for their study enrollment visit, which consisted of informed consent, completing a neuropsychological (NP) test battery, questionnaires (including demographic and mental health screeners), a urine toxicology screen, and a blood draw. The enrollment visit occurred prior to study randomization. Initial inclusion criteria were: ages 18 to 65, female, living with HIV, English as a first language, able to give informed consent and travel to the study site for study participation. Exclusion criteria were: current use of hormone-based contraceptives, currently pregnant, post-partum, or lactating, currently regular use of steroids, closed head injury, history of schizophrenia or schizoaffective disorder, current untreated hypertension or diabetes, history of dementia or any other neurologic CNS or AIDS-defining disorder, substance use disorder in the past six months, positive urine toxicology screen (except

445 marijuana) or breathalyzer and/or any evidence of acute intoxication or withdrawal. All Baltimore
446 cohort samples were fresh, never frozen, whole blood samples. The validation study cohort (Bronx
447 cohort) included 18 WWH over the age of 18 that enrolled in a previous published study at the
448 Bronx WIHS site between June 25, 2012 and October 4, 2013 that focused on monocytes and HIV-
449 associated neurocognitive disorder (HAND(23)). These participants completed the same NP
450 battery, questionnaires, urine toxicology screen, and blood draw. Exclusion criteria included any
451 crack, cocaine, and/or heroin use in the past six months or hepatitis C antibody positive. While the
452 blood draw and NP test battery were done concurrently in the Baltimore cohort, the NY cohorts'
453 NP testing and mental health screeners were completed 0 to 1.55 years later (median=1.14,
454 IQR=0.58). All Bronx cohort samples were fresh, never frozen, PBMCs.
455
456 **Neuropsychiatric Outcomes**
457 **NP Test Battery**
458 Both cohorts of women completed the same NP test battery. The NP test battery included the
459 Hopkins Verbal Learning Test-Revised (HVLT-R; outcomes= total learning, delay free recall,
460 recognition), Letter-Number Sequencing (LNS; outcomes=experimental [working memory] and
461 control [attention] conditions total correct), Trail Making Test- (TMT; outcomes=time to complete
462 Part A and B), Stroop (outcome=time to complete Trials 1 [color reading], 2 [color naming], and
463 3 [color-word]), Symbol Digit Modalities Test (SDMT; outcome=total correct), Letter-guided
464 verbal fluency (Controlled Oral Word Associations Test (COWAT; outcome=total correct words
465 generated across three trials [F, A, S]), Animal fluency (outcome=total correct animals generated),
466 and Grooved Pegboard (GPEG; outcomes=time to completion, dominant and non-dominant hand).
467 Timed outcomes were log transformed to normalize distributions and reverse scored so higher

equated to better performance. Demographically-adjusted Z-scores were calculated for each outcome using data from HIV-uninfected WIHS women(35, 36). Global NP function was calculated as the average of the demographically-adjusted Z-scores for each individual outcome measure.

Mental health screeners

Both cohorts of women completed the: 1) Center for Epidemiological Studies Depression scale (CES-D) which assesses depressive symptoms(58), 2) Perceived Stress Scale (PSS)-10 which measures the degree of uncontrollability, unpredictability, and overload in the respondents life(59, 60), and 3) Post-Traumatic Stress Disorder (PTSD) Checklist-Civilian Scale (PCL-C) which assesses post-traumatic symptom burden(61). Higher scores on each of these measures indicate more symptomatology.

FACS

For the Baltimore cohort, whole-blood samples were stained with pretitered amounts of monoclonal antibodies using 100µl of whole blood at room temperature for 20 min. The antibody panels consisted of anti-CD3 V500 (clone SP34-2; BD Biosciences), anti-CD4 PerCP-Cy5.5 (clone L200, BD Biosciences), anti-CD8a BV570 (clone RPA-T8; BioLegend), anti-CD159a APC (clone NKG2A; Beckman Coulter), anti-TLR2 AF488 (clone 11G7; BD Biosciences), anti-CD14 BV650 (clone M5E2; BD Biosciences), and anti-CD16 AF700 (clone 3G8; BioLegend). Whole-blood samples were then lysed and fixed in 2 ml of FACS Lysing Solution (BD Biosciences, San Jose, CA) for 10 min at room temperature. Samples were collected in a centrifuge at 400 × g for 5 min, washed in 2 ml of 1× phosphate-buffered saline (PBS), and then resuspended in 0.5 ml of

PBS for analysis. Flow cytometry was performed on a BD LSRFortessa (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo 10.0.8 software (FlowJo, LLC, Ashland, OR). See **Figure 1(A-F)** for gating scheme. Mononuclear cells (MNC) were counted as agranulocytes in the whole blood sample, summing both TLR2+ monocyte gating, which separates monocytes from granulocytes, and TLR2- lymphocytes as smaller, agranular lymphocytes. Both were gated post debris and doublet removal. TLR2+ monocytes, CD4+ and CD8+ T cell populations were expressed as a percentage of MNC. Monocyte subsets were express as either percent of TLR2+ or percent of MNC (**Figure 1G**).

For the Bronx cohort, details on methods regarding cell isolation and monocyte identification by flow cytometry have been previously published(23). In brief, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation, and the cells stained with a cocktail of fluorochrome-coupled monoclonal antibodies specific for human CD14 (clone M5E2), CD16 (clone 3G8), CD3 (clone HIT3a), CD19 (clone HIB19), CD56 (clone B159), CD66b (clone G10F5), HLA-DR (clone G46-6), or corresponding isotype-matched, negative control antibodies (all anitbodies were purchased from BD Biosciences). PBMC ($2–5 \times 10^5$) were washed with calcium- and magnesium-free PBS (Gibco, Grand Island, NY, USA), supplemented with 1% BSA (Thermo Scientific, Waltham, MA, USA), and were incubated in the dark on ice for 30 min with the appropriate antibodies. Following staining, PBMCs were washed once with PBS/1% BSA and fixed with 0.2 mL 2% paraformaldehyde. Flow cytometry was performed on a BD FACS Canto II flow cytometer and data were analyzed using FlowJo. Monocytes were defined according to forward- and side-scatter characteristics, and were identified as CD14 and HLA-DR positive and CD3, CD19, CD56, and CD66b negative as described previously(23).

514

515 **Statistical Analyses**

516 A series of Spearman's Rho correlations (rs) were conducted to examine associations between
517 total monocytes, monocyte subsets, T-cell populations, T-cell ratios and NP performance.
518 Adjusted analyses were not necessary as the measured sociodemographic (e.g. age, education),
519 behavioral (e.g., substance use, smoking), clinical factors (e.g., CD4+ count, **Supplemental Table**
520 **1**), and female-specific factors (menopausal stage) were not related to both biomarkers and NP
521 performance. All analyses were conducted in IBM SPSS Statistics for Windows (Version 25.0.
522 Armonk, NY: IBM Corp). Significance was set at $P<0.05$.

523

524 **Study approval**

525 Data collected as part of the primary cohort (Baltimore) was approved by the Johns Hopkins
526 University Institutional Review Board. Data collected as part of the validation cohort (Bronx) was
527 approved by the Institutional Review Board at the Montefiore Medical Center, Albert Einstein
528 College of Medicine, and the Mount Sinai Program. Informed written consent was obtained from
529 all participants prior to enrollment into each study site. The study was conducted according to
530 Declaration of Helsinki principles.

531

Author contributions

RTV and LHR conception and design of the study. RTV, DWW, ENS and JEC designed experiments. DWW, ES, EF, and CMA performed experiments. RTV, DWW, ENS, and LHR analyzed the data. JMC, and TTB collected specimens. LHR, JEC, JWB, and KA provided materials. RTV and LHR wrote the initial drafts of the manuscript. All authors (ENS, CMA, EF, JMC, TTB, PMM, KA, JWB, JEC) provided critical review of the manuscript for important intellectual content and contributed to and approved the final manuscript.

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Figures

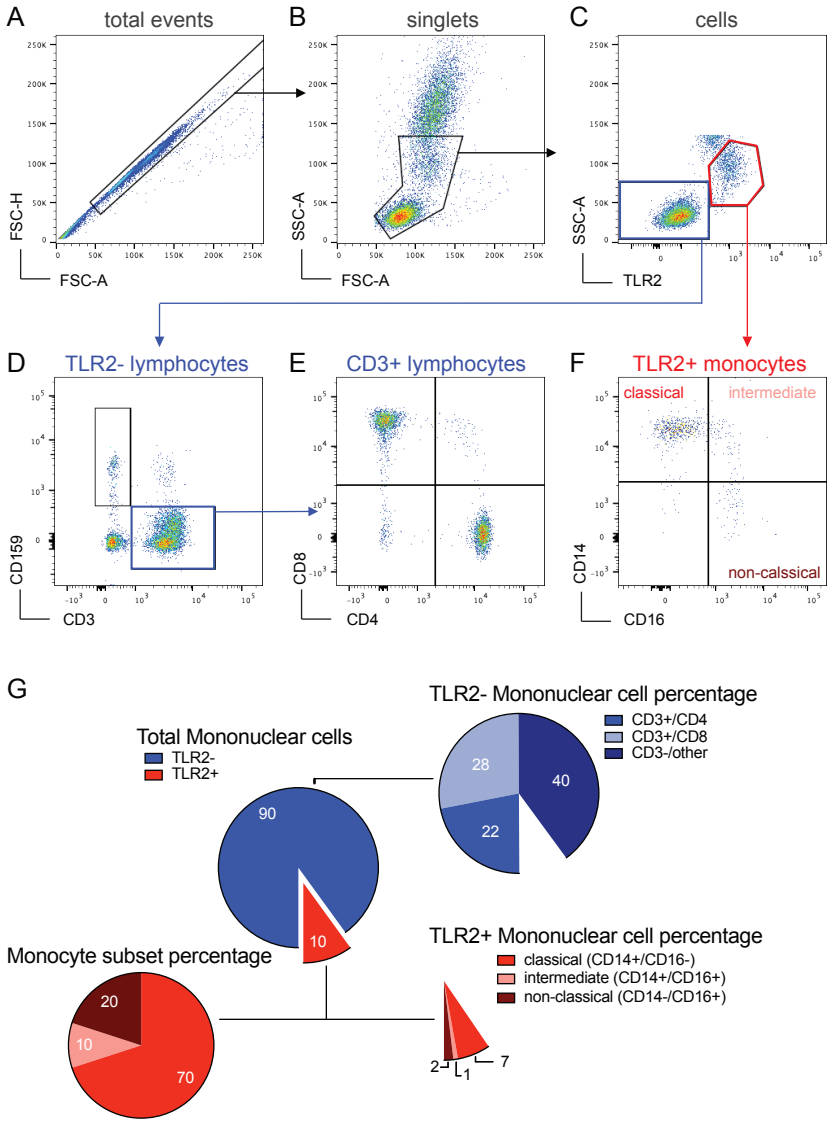

Figure 1. Monocyte subset and mononuclear cell calculation for Baltimore Cohort. (A-F) A representative FACS gating scheme. (A) Doublets were excluded using FSC-A and FSC-H measurements, and (B) debris was gated out by drawing a gate on cell-sized events using FSC-A and SSC-A. (C) TLR2+ cells were gated as monocytes, and non-granulocyte TLR2- cells were gated as lymphocytes. (D) TLR2- lymphocytes were gated as CD3+ T cells or CD159a+ NK cells. (E) CD3+ cells were then gated as CD4+ or CD8+ T cells. (F) TLR2+ monocytes were gated based

on the expression of CD14 and CD16 and classified as classical (CD14+/CD16), intermediate (CD14+/CD16+), or non-classical monocytes (CD14-/CD16+). (G) Mononuclear cells (MNC) were counted in the whole blood sample, by summing both TLR2+ monocyte gating, which separates monocytes from granulocytes, and TLR2- lymphocytes as smaller, agranular lymphocytes. Both were gated post debris and doublet removal. TLR2+ monocytes, CD4 and CD8 T cell populations were expressed as a percentage of MNC. Monocyte subsets were express as either percent of TLR2+ or percent of MNC.

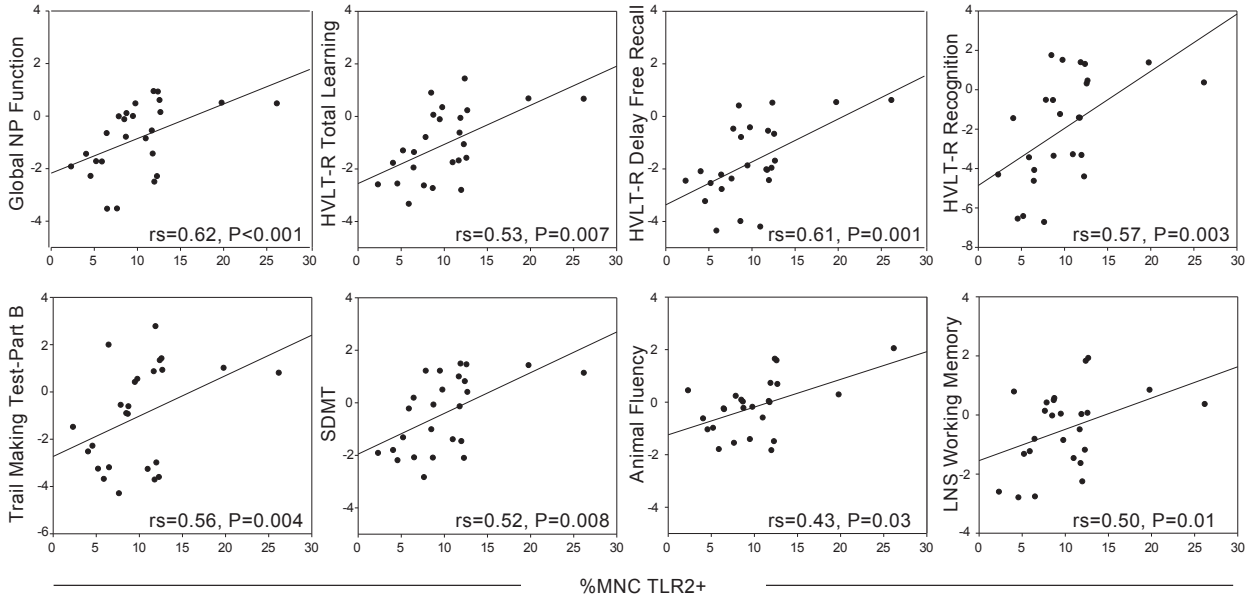

Figure 2. A higher percentage of TLR2+ mononuclear cells (MNC) is associated with higher cognitive function. Percent TLR2+ MNC was measured concurrently with cognitive function in women with HIV (WWH; n=25) living in Baltimore, MD; Stroop Test Trial 3 not shown (rs=0.53, p<0.001). Spearman's Rho (rs) was used to examine the associations. HVLT-R=Hopkins Verbal Learning Test-Revised; LNS=Letter-Number Sequencing; SDMT=Symbol Digit Modalities Test

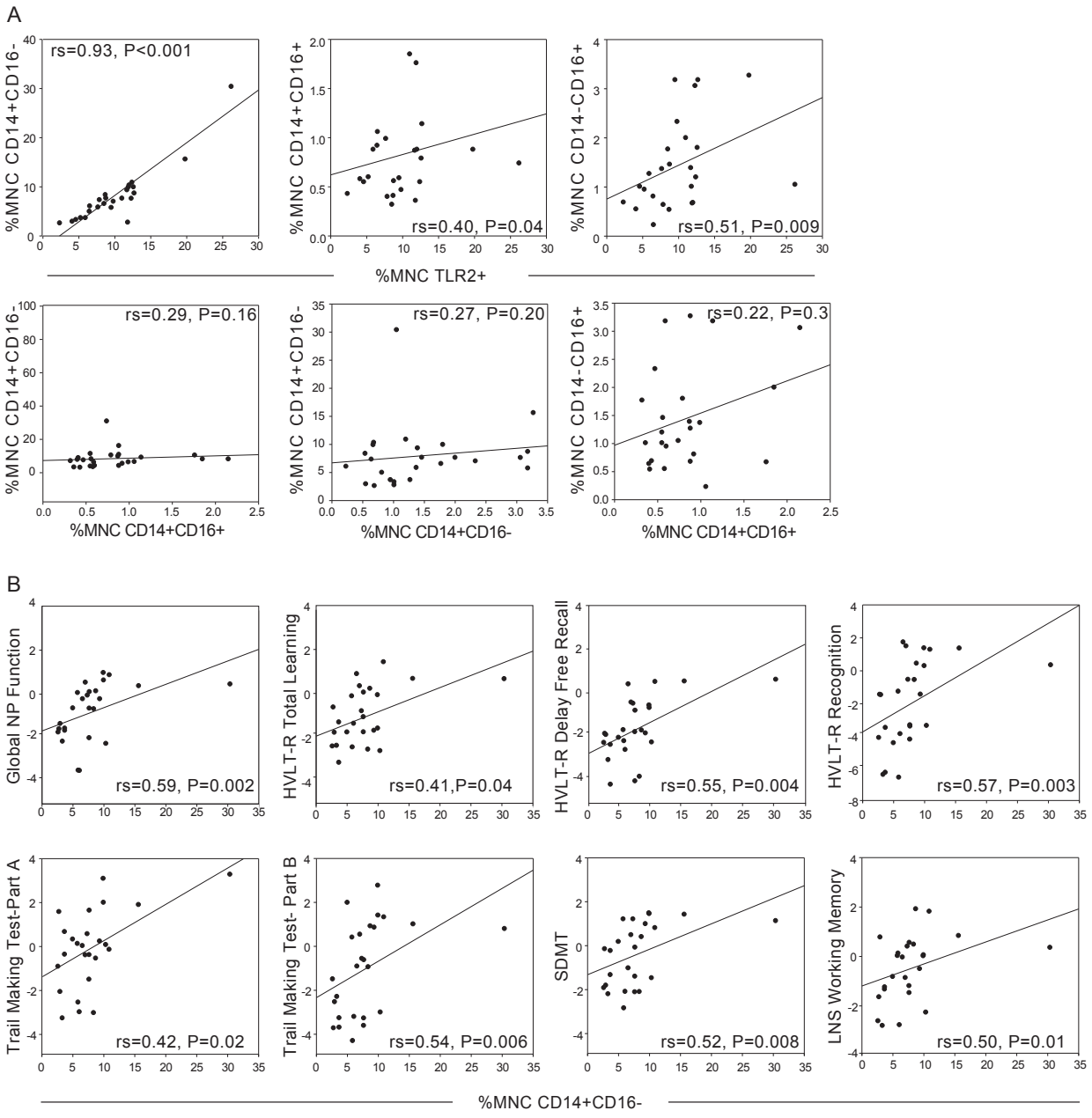

Figure 3. TLR2 numbers and associations with cognitive function are driven by an increase in classical monocytes, not maturation into other subsets. (A) Associations (using Spearman's Rho) between % mononuclear cells (MNC) TLR2+ and % MNC monocyte subsets in women with HIV (WWH) living in Baltimore, MD (n=25). (B) Statistically significant associations (using Spearman's Rho) between cognitive function in women with HIV (WWH) living in Baltimore,

MD (n=25) and concurrent measurements of the % MNC classical monocyte subset; Letter fluency (r_s=0.43, $p<0.05$), Animal fluency (r_s=0.49, $p<0.05$) and Stroop Test Trial 3 (r_s=0.47, $p<0.05$) not shown. HVLT-R=Hopkins Verbal Learning Test-Revised; LNS=Letter-Number Sequencing; SDMT=Symbol Digit Modalities Test; classical (CD14+CD16-); intermediate (CD14+/CD16+); non-classical (CD14-/CD16+)

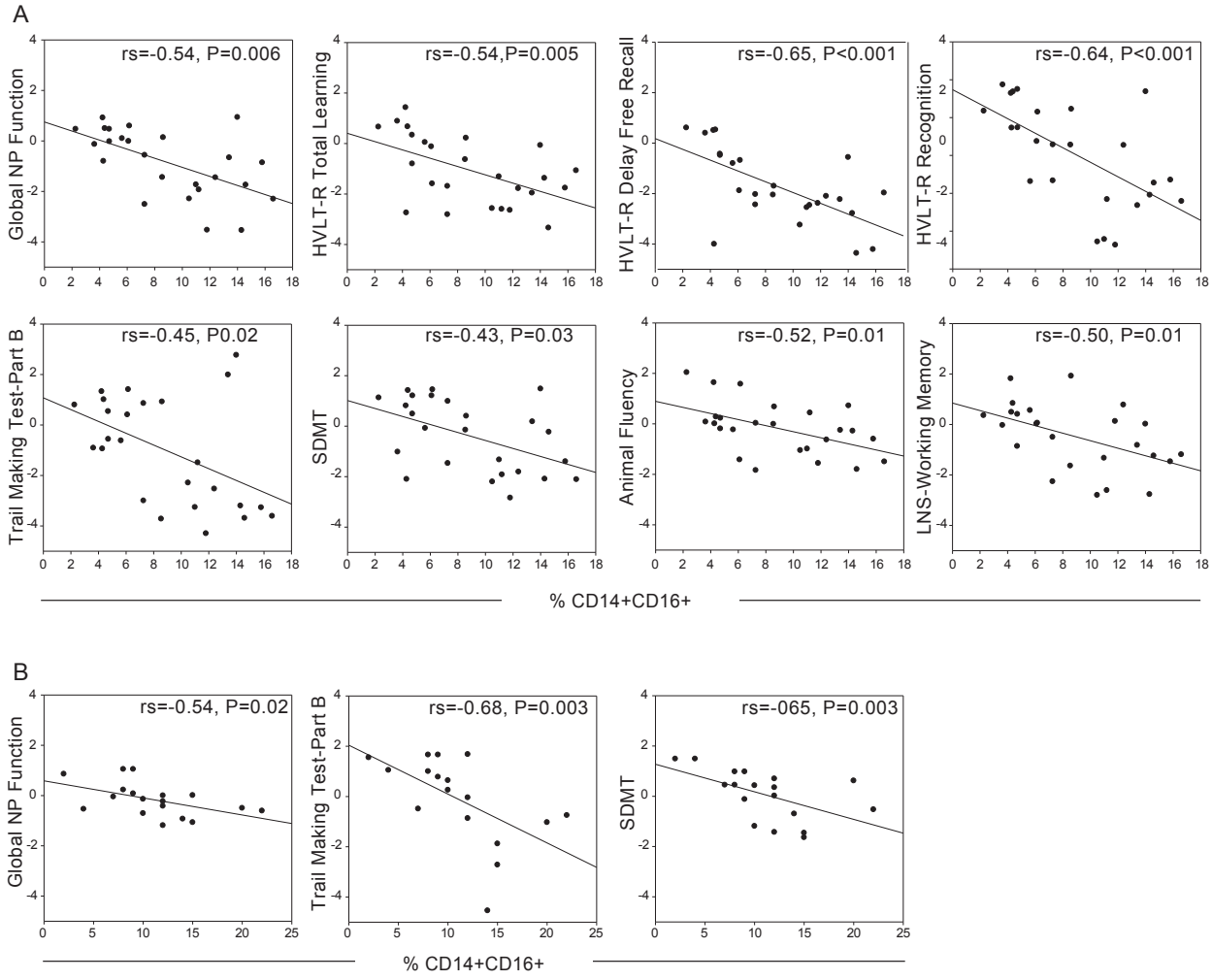

Figure 4. The percentage of intermediate monocyte subset negative correlates with concurrent and predictive cognitive function. (A) Higher numbers of intermediate monocytes are associated (using Spearman's Rho [rs]) with lower cognitive function measured concurrently in women with HIV (WWH; n=25) living in Baltimore, MD, Grooved Pegboard Dominant not shown (rs=-0.44, p<0.05). (B) Higher numbers of intermediate monocytes are associated (using Spearman's Rho) with lower executive function and processing speed whereby monocytes were cognition was assessed approximately one year following monocyte assessment (median=1.14; interquartile range 0.58) in WWH living in Bronx, NY (n=18). Note. HVLT-R=Hopkins Verbal Learning Test-Revised; LNS=Letter-Number Sequencing; SDMT=Symbol Digit Modalities Test

Table 1. Associations (Spearman's Rho) between monocyte subsets and neuropsychiatric function in women with HIV living in Baltimore, MD (n=25).

	% MNC				% subset		
	TLR2+	$CD14^+CD16^-$	$CD14^+CD16^+$	$CD14^-CD16^+$	$CD14^+CD16^-$	$CD14^+CD16^+$	$CD14^-CD16^+$
NP performance							
Global NP function	**0.62*****	**0.59****	-0.10	0.36$^{\gamma}$	0.33	**-0.54****	-0.12
HVLT-R							
Total learning	**0.53****	**0.41***	-0.17	**0.44***	0.18	**-0.54****	0.10
Delayed free recall	**0.61****	**0.55****	-0.19	0.38†	0.36‡	**-0.65*****	-0.08
Recognition	**0.57****	**0.57****	-0.25	0.27	0.39$^{\|}$	**-0.64*****	-0.09
Trail Making Test							
Part A	0.37$^{\gamma}$	**0.42***	-0.02	0.16	0.35‡	-0.33	-0.19
Part B	**0.54****	**0.56****	-0.01	0.16	**0.43***	**-0.45***	-0.31
SDMT	**0.52****	**0.51***	-0.02	0.31	0.31	**-0.43***	-0.13
Stroop Test							
Trial 2	0.20	0.18	-0.01	0.24	0.03	-0.07	0.09
Trial 3	**0.53****	**0.47***	-0.04	0.31	0.23	-0.30	-0.15
Grooved Pegboard							
Dominant	0.21	0.21	-0.30	0.28	0.12	**-0.44***	0.11
Non-dominant hand	0.24	0.20	-0.17	0.28	-0.01	-0.30	0.14
Fluency							
Letter	0.39$^{\|}$	**0.43***	-0.06	0.08	0.27	-0.33	-0.20
Animal	**0.43***	**0.49***	-0.24	0.03	**0.54****	**-0.52****	-0.30
LNS							
Attention	0.26	0.14	0.12	**0.42***	-0.27	0.05	0.26
Working Memory	**0.50***	**0.50***	-0.06	0.24	0.26	**-0.50***	-0.16
Mental health							
CES-D	-0.04	0.09	0.01	-0.16	0.23	-0.02	-0.17
PSS-10	0.00	-0.05	-0.13	0.10	-0.10	-0.11	0.17
PCL-C	-0.10	-0.16	-0.08	-0.16	-0.20	0.12	0.07

***P<0.001; **P<0.01; *P<0.05; $^{\|}$P=0.05; †P=0.06; $^{\gamma}$P=0.07; ‡P=0.08; ^{T}P=0.09; CES-D=Center for Epidemiologic Studies Depression Scale; MNC=mononuclear cells; PSS-10=Perceived Stress Scale; PCL-C=PTSD Checklist Civilian Version

Table 2. Associations (Spearman's Rho) between monocyte subsets and neuropsychiatric function in women with HIV living in Bronx, NY (n=18).

	% subset		
	$CD14^{+}CD16^{-}$	$CD14^{+}CD16^{+}$	$CD14^{-}CD16^{+}$
NP performance			
Global NP function	0.11	**-0.54***	0.06
HVLT-R			
Total learning	-0.22	0.10	0.09
Delayed free recall	-0.25	0.10	0.12
Recognition	-0.36	0.32	0.18
Trail Making Test			
Part A	0.18	-0.42‡	-0.05
Part B	0.23	**-0.68****	0.02
SDMT	0.06	**-0.65****	0.18
Stroop Test			
Trial 2	-0.19	-0.34	0.35
Trial 3	0.24	-0.37	-0.09
Grooved Pegboard			
Dominant	0.10	-0.20	-0.01
Non-dominant hand	0.04	-0.19	-0.00
Fluency			
Letter	0.05	-0.08	-0.09
Animal	0.07	-0.24	0.00
LNS			
Attention	-0.22	-0.25	0.32
Working Memory	-0.09	-0.36	0.23
Mental health			
CES-D	0.12	-0.23	0.03
PSS-10	0.14	0.30	-0.22
PCL-C	0.13	0.39	-0.29

*P<0.05; ‖P=0.05; †P=0.06; ‡P=0.08; ᵀP=0.09; CES-D=Center for Epidemiologic Studies Depression Scale; PSS-10=Perceived Stress Scale; PCL-C=PTSD Checklist Civilian Version; for the mental health measures positive associations are higher monocyte subsets associating with higher symptomatology whereas negative associations are higher monocyte subsets are associated with lower symptomatology.