Csf Lymphocyte And Monocyte Activation And Trafficking In Primary Hiv Infection

Aveline Xiang Li

Follow this and additional works at: https://elischolar.library.yale.edu/ymtdl

Part of the Medicine and Health Sciences Commons

Recommended Citation
https://elischolar.library.yale.edu/ymtdl/3512

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.
CSF Lymphocyte and Monocyte Activation and Trafficking in Primary HIV Infection

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By

Aveline Xiang Li

2019
ABSTRACT

Aveline Xiang Li¹, Fangyong Li², Anji Yi², Brinda Emu³, Richard Price⁴, Elizabeth Sinclair⁴, and Serena Spudich¹

¹ Department of Neurology, Yale University School of Medicine, New Haven, CT, USA
² Yale Center for Analytical Sciences, New Haven, CT, USA
³ Section of Infectious Disease, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA
⁴ University of California San Francisco, San Francisco, CA, USA

Trafficking of immune cells to the central nervous system (CNS) is hypothesized to facilitate HIV entry and immune-induced neuronal injury, and is mediated by cell surface proteins such as chemokine receptors and integrins. We longitudinally assessed immune cell activation and surface marker expression in cerebrospinal fluid (CSF) and blood and their relationship with CSF HIV RNA during primary HIV infection (PHI) before and after combination antiretroviral therapy (ART). Longitudinal paired blood and CSF were obtained in initially ART-naïve PHI (<12 months since infection) participants; some subjects independently initiated ART during follow up. Multiparameter flow cytometry was used to determine activation (% CD38⁺HLADR⁺) and chemokine receptor expression (% CCR5⁺ and CXCR3⁺) on CD4⁺ and CD8⁺ T cells, and subtype and α4 integrin expression (% and mean fluorescence intensity (MFI) of CD49d⁺) on monocytes. Analyses employed Spearman correlation and linear mixed models. A total of 51 participants enrolled at a median of 3.3 months post-infection, with 168 total visits (113 untreated, 55 on ART) during a median of 6.5 months follow up (range 0-40). Pre-ART, the rate of increase in T-cell activation was 3 times higher in CSF than blood. In univariate longitudinal analysis, both CSF CD4⁺ and CD8⁺ T-cell activation correlated with CSF HIV RNA (all p≤0.01); in multivariate analysis CSF CD4⁺ but not CD8⁺ T-cell
activation was an independent predictor of CSF HIV RNA. CSF monocyte subtypes and α4 expression did not correlate with CSF HIV RNA. Monocyte α4 MFI correlated with CD4+ and CD8+ T-cell activation in blood pre-ART, and in both CSF and blood while on treatment (all p<0.05). During follow up on ART, blood but not CSF T-cell activation declined with days on treatment (slope= -0.06, p=0.001). In conclusion, during untreated PHI, T-cell activation increases faster in CSF than blood, and CSF CD4+ T-cell activation but not monocyte activation correlates with CSF HIV RNA. Intrathecal T-cell activation does not decline during early follow up on ART. This study suggests a level of irreversibility to the neuroinflammation that develops in PHI, implicates CD4+ T cells as a likely source for trafficking HIV into the CNS, and highlights possible monocyte-T cell interactions that warrant further exploration.
ACKNOWLEDGEMENTS

This work was supported by research grants from the National Institute of Health and National Institute of Mental Health (grants R01 MH081772, K23 MH074466, R01 MH095613, R01 NS084911, and R01 MH062701) awarded to Dr. Serena Spudich and collaborators, the Yale School of Medicine Summer Research grant, and the G.D. Hsiung Ph.D, Student Research Fellowship at Yale School of Medicine.

I would like to thank all the study participants, without whom this research could not be accomplished, as well as the co-authors, our collaborators at USCF, and the Spudich lab. I am immensely grateful to Dr. Serena Spudich for being a marvelous mentor and an inspirational physician-scientist during my time on this project.
INTRODUCTION

HIV-Associated Neurocognitive Disorders in the Modern Era

Modern advancements in HIV treatment have greatly improved life expectancy and changed HIV into a chronically managed disease, yet long-term sequelae such as HIV-associated neurocognitive disorders (HAND) continue to impact the daily life of individuals living with the virus. The introduction of combined antiretroviral therapy (ART) in the late 1990s led to a decline in severe forms of HAND, such as HIV-associated dementia, from roughly 10-15% down to 2%[1]. Milder neurocognitive manifestations have in turn increased, maintaining a steady prevalence of HAND overall. Studies estimate that almost 40% of HIV-infected individuals on ART continue to have some form of neurocognitive impairment[2, 3]. Hence, the neurologic facet of chronic HIV persists as a subject of both clinical and epidemiologic relevance in the era of ART.

According to formal classifications established for research purposes, HAND diagnoses of varying severity can be established using neuropsychologic testing and reported changes in daily functioning (Table 1)[4]. Additionally, HAND can only be diagnosed in the absence of other attributable neurologic comorbidities. The combined ART era has seen notable changes in the types of neurocognitive deficits detected in HAND patients, namely a shift from previously described impairments in processing speed and motor domains toward deficits in the memory/learning and executive function domains[5]. These research measures can translate into real world difficulties with medication adherence[6, 7], unemployment[8, 9], finance management[10], and driving[11] for patients living with HAND. Given the persistence of neurologic symptomatology in the setting of effective viral suppression, further understanding of the pathophysiology of
HIV-related neuronal injury is required for effective prevention and treatment of HAND.

**HIV Reservoirs in the Central Nervous System**

The presence of HIV in the central nervous system (CNS) may play a crucial role in the pathogenesis of HAND. HIV enters the CNS early on during infection, and HIV RNA, the standard measure of viral load\[13\], is detectable in the cerebrospinal fluid (CSF) as soon as 8 days post-transmission\[14\]. While the virus may reach the CNS initially as part of a spreading systemic HIV infection, over time, a genetically distinct CNS viral reservoir can develop. One example is the evolution of HIV variants that have adapted to enter different target cells. The predominant HIV variant found during early infection is T cell-tropic, requiring the CCR5 co-receptor and a high density of surface CD4 molecules, such as that seen on activated CD4\(^+\) T cells, for entry into its target\[15\]. In comparison, macrophage-tropic HIV variants found in some individuals during later stages of CNS infection are able to efficiently infect cells with a lower surface CD4 density, such as that seen on monocyte-derived macrophages or microglia\[15, 16\].

### Table 1. Diagnostic criteria for classification of HAND

<table>
<thead>
<tr>
<th>HAND Classification(^a)</th>
<th>Neuropsychologic testing(^b)</th>
<th>Effect on daily functioning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic neurocognitive impairment</td>
<td>Impairment in ≥ 2 domains with ≥ 1 SD below mean</td>
<td>No noticeable interference</td>
</tr>
<tr>
<td>Mild neurocognitive disorder</td>
<td></td>
<td>Mild interference</td>
</tr>
<tr>
<td>HIV-associated dementia</td>
<td>Impairment in ≥ 2 domains with ≥ 2 SD below mean</td>
<td>Marked interference</td>
</tr>
</tbody>
</table>

\(^{a}\) HAND diagnosed in the absence of evidence of any other preexisting cause of neurocognitive impairment (i.e. mood disorder, substance use disorder, CNS infection, CNS neoplasm, cerebrovascular disease, or delirium)

\(^{b}\) Neuropsychologic testing assesses at least the domains of verbal/language, attention/working memory, abstraction/executive function, memory/learning, processing speed, and sensory/motor skills. Testing means established using age-education appropriate norms

\(^{c}\) Adapted from Antinori et al. (2007) and Farhadian et al. (2017)
adaptation allows sustained HIV infection and replication in longer-lived cell lines in the CNS and may contribute to compartmentalization of the virus.

Independent HIV replication in the CNS is further evidenced by the phenomenon of CSF escape, when an individual on ART has detectable levels of CSF HIV RNA despite undetectable plasma HIV RNA, or a ≥1 log higher viral load in the CSF than in plasma\cite{17}. Multiple case series have illustrated such discrepancies between CSF and blood viral loads in neurologically symptomatic HIV+ individuals on ART\cite{17, 18}. A majority of these participants were also found to harbor CNS HIV strains with resistance mutations against their systemic antiviral regimen, suggesting the development of distinct CNS HIV lineages\cite{17, 18}. Other studies have suggested that CSF escape may occur earlier and more frequently among the HIV+ population, such that up to 10% of neurologically asymptomatic, virally suppressed HIV+ individuals on ART have detectable HIV RNA in the CSF \cite{19}. To better understand the natural progression of viral compartmentalization during early untreated HIV, one study analyzed HIV replication using single genome amplification and phylodynamics, and found that episodes of independent HIV replication in the CNS were detectable as early as 4 months post-HIV transmission and in up to 30% of ART-naïve HIV+ participants\cite{20, 21}. The results from said study suggested that an elevated CSF viral load derives either from independent HIV replication in the CNS, resulting in compartmentalized virus, or from HIV trafficked from the periphery into the CNS by infected immune cells, leading to equilibrated viral strains between compartments\cite{21}. The persistence of a CNS HIV reservoir not only poses a problem for viral eradication by ART, but also reflects the complex interactions between HIV and host immune cells within the CNS.
**Neuroinflammation in HIV**

Persistence of HIV in the CNS compartment drives immune activation, which in turn contributes to ongoing neuroinjury. Though the inflammatory response to HIV would be expected to subside with initiation of treatment, studies in both primates and humans on ART have suggested that episodes of CSF escape or rebounding may be associated with the development of lymphocytic infiltrates in the brain parenchyma\textsuperscript{[18, 22]}. Additionally, a subset of HIV+ individuals who are virally suppressed on ART continue to show higher than normal levels of CSF neopterin, a biomarker of CNS macrophage and microglia activation\textsuperscript{[23-25]}. Among HIV+ individuals who are on treatment, participants with neurocognitive impairment exhibit higher CSF neopterin levels compared to those without impairment\textsuperscript{[24]}. In turn, elevated CSF neopterin correlates with increased CSF levels of neurofilament light chain (NFL)\textsuperscript{[24]}, a sensitive marker of neuronal injury, and with detectable CSF HIV RNA\textsuperscript{[25, 26]}, suggesting that low amounts of HIV replication or release from the CNS may invoke continued inflammation and eventual neuroinjury.

While studies of chronic HIV infection (CHI) have provided a basis for investigating the role of inflammation in HIV neuropathology, the past decade has seen an increased focus on the earlier stages of HIV infection. HIV reaches the CNS during acute HIV infection (AHI, defined as the first 2-4 weeks after HIV transmission), accompanied by CSF leukocytosis and increased inflammation, as measured by neuroimaging and biomarkers such as neopterin and the soluble chemokines CCL2 and CXCL10\textsuperscript{[14]}. Extending into the period of primary HIV infection (PHI, defined as the first year of HIV infection), studies of ART-naïve participants have demonstrated correlations
between elevated CNS viral loads and increased CSF white blood cell (WBC) counts\textsuperscript{[27]}. The systemic and CNS immune activation that begins during the early phases of HIV infection increases until initiation of ART, at which point the inflammation plateaus but does not significantly decrease\textsuperscript{[28, 29]}. This escalating and persistent inflammation supports a push-pull model where systemic HIV infection leads to increased immune cell extravasation to the CNS while CNS HIV infection establishes an inflammatory milieu that enhances further recruitment of activated immune cells. Elucidating the cellular mechanisms involved in this process requires additional examination of the specific immune cell types, such as T lymphocytes and monocytes, involved in HIV neuroinflammation.

**CD4\textsuperscript{+} T cells as HIV Hosts and Immune Regulators**

CD4\textsuperscript{+} T cells are well-established targets of HIV infection, and the immunodeficiency that is characteristic of severe HIV/AIDS results from a severe depletion of CD4\textsuperscript{+} T-cell population. Studies of T-cell activation in the HIV field predominantly use the markers CD38 and HLA-DR, two surface molecules known to be upregulated following \textit{in vitro} induction of T-cell activation\textsuperscript{[30-32]}. These markers also hold clinical relevance, given that CD38\textsuperscript{+} HLA-DR\textsuperscript{+} CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell levels are significantly elevated in HIV-infected individuals and correlate with viral load and disease severity\textsuperscript{[33-36]}. One explanation for this relationship posits that activated CD4\textsuperscript{+} T cells are more susceptible to HIV infection. Analysis of lymph node tissue from untreated HIV\textsuperscript{+} participants revealed that CD38\textsuperscript{+} HLA-DR\textsuperscript{+} CD4\textsuperscript{+} T cells contain higher concentrations of HIV RNA compared to other CD4\textsuperscript{+} T-cell populations, and contribute a
median of 86% of total HIV RNA copies produced in a given sample of CD4+ T cells. Another study found that activated CD4+ T cells are twice as likely to be productively infected with HIV compared to non-activated cells, and that HIV infection of lymphoid tissue induces activation of both infected and uninfected CD4+ T cells. This relationship suggests that HIV may boost its own replication by expanding the pool of nearby target cells, namely activated CD4+ T cells, which are more easily infected and more productive of virus. Notably, said study measured activation in terms of HLA-DR and CD25, another surface molecule known to be upregulated during lymphocyte activation and proliferation. Given the significance of CD4+ T cells as host and replication resource for HIV in the peripheral blood and lymphoid tissues, CD4+ lymphocytes may also be expected to play a similar role in the CNS.

Though the CNS has historically been thought of as an immune-privileged site, CD4+ T cells are present in this compartment and may affect both HIV replication as well as regulation of the immune response against HIV infection. Lymphocytes are the most common immune cell population in the CSF, with CD4+ T cells predominating in the CSF of healthy individuals and CD8+ T cells in the CSF of HIV+ individuals. Though the fraction of CD4+ T cells decreases after HIV infection, the absolute counts of CSF CD4+ T cells do not differ significantly between infected and uninfected groups, likely due to the leukocytosis that accompanies HIV-infection. The minimal change in CD4+ T-cell counts, however, does not necessarily signify a static cell population. In both CSF and blood, HIV+ participants show a higher frequency of T-cell activation compared to uninfected participants. The percentage of activated CD4+ T cells in the CSF correlates with that seen in blood, suggesting that the systemic inflammation may
promote trafficking of activated CD4\(^+\) T cells into the CNS\[^{40}\]. Compared to in blood, the CSF of HIV\(^+\) individuals contains a significantly higher percentage of CD4\(^+\) T cells that express the chemokine receptors CXCR3 and CCR5, as well as a higher concentration of the chemokine CXCL10, ligand to CXCR3\[^{41}\]. The chemokine receptors may facilitate extravasation of activated T cells into local tissues in response to inflammatory signals.

Once in the CNS, the dynamics between immune cells and HIV further supports the role of CD4\(^+\) T cells as a crucial host to the virus. A study of viremic HIV\(^+\) participants who were either untreated or failing treatment found that <1\% of total CSF T cells produce HIV, but notably, the percentage of HIV-producing CD4\(^+\) T cells is significantly higher in CSF than in blood\[^{42}\]. Furthermore, the concentration of HIV-producing CD4\(^+\) T cells in the CSF correlates with CSF viral load, and these two factors also correlate with CSF CD4\(^+\) T-cell activation levels\[^{42}\]. These relationships suggest that infected CD4\(^+\) T cells may sustain low amounts of HIV replication in the CSF. However, further studies are needed to clarify whether the correlation with CD4\(^+\) T-cell activation stems from the virus inducing activation of CSF CD4\(^+\) T cells or if it results from sustained trafficking of HIV within activated CD4\(^+\) T cells. CD4\(^+\) T lymphocytes make up only one part of a complex and interconnected immune response in HIV infection, and their CD8\(^+\) counterparts hold both similar and contrasting relationships with the virus.

**CD8\(^+\) T cells as Viral Controllers and Inflammatory Effectors**

Comprising the cytotoxic arm of cellular immunity, CD8\(^+\) T cells have been studied with regard to their function in viral control and in generating inflammatory damage during HIV infection. Blood CD8\(^+\) T-cell activation increases over the course of untreated
HIV, correlating with disease progression\textsuperscript{[35]}. Additionally, CD8\textsuperscript{+} T-cell activation associates strongly with levels of CD4\textsuperscript{+} T-cell depletion and is predictive of progression to AIDS and death\textsuperscript{[43-45]}. Unlike CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells are not targets for HIV infection, leading to the question of what elicits activation of this cell population during HIV infection. On one hand, a higher frequency of HIV-specific CD8\textsuperscript{+} T cells have been observed among the activated CD8\textsuperscript{+} T-cell population\textsuperscript{[34]}, indicating that the inflammation may represent an effort by these cytotoxic cells to control viral replication. Other studies have suggested that a more non-specific stimulus from the infected host may explain the increased activation. In a study of participants with HIV rebound due to treatment interruption, increased viral load correlated with higher CD8\textsuperscript{+} T-cell activation regardless of if the T-cell receptor was HIV-specific or non-HIV-specific\textsuperscript{[46]}. Further investigation revealed that in response to microbial antigen or HIV, antigen presenting cells produce inflammatory cytokines that strongly stimulate CD8\textsuperscript{+} T-cell activation in an antigen-independent manner\textsuperscript{[46]}. Other \textit{in vitro} studies have also supported the role of cytokines in CD8\textsuperscript{+} T-cell activation\textsuperscript{[47]}. Early on in HIV infection, these factors in the peripheral blood likely drive activation of CD8\textsuperscript{+} T cells and contribute to increased immune cell trafficking to the CNS.

CD8\textsuperscript{+} T lymphocytes constitute a major part of the CNS inflammation associated with HIV and HAND. In the CSF of HIV\textsuperscript{+} individuals, CD8\textsuperscript{+} T cells are the most frequent immune cell type and are significantly heightened in number compared to what is seen in the CSF of uninfected persons\textsuperscript{[27, 40]}. Based on both this disparity in cell counts as well as the strong correlation between CSF CD8\textsuperscript{+} T-cell activation and CSF WBC concentration, activated CD8\textsuperscript{+} T cells make up the majority of the CSF leukocytosis associated with
HIV\cite{27, 40}. Similar to their CD4\(^+\) counterparts, activated CD8\(^+\) T cells have been noted to upregulate expression of chemokine receptors CXCR3 and CCR5, in addition to adhesion molecules such as \(\alpha 4\beta 1\) integrin, to facilitate trafficking to the CNS in response to elevated levels of inflammatory chemokines\cite{41}. Because this CD8\(^+\) T-cell predominant inflammation persists in the CNS during untreated HIV, the interactions between CD8\(^+\) T cell and virus likely affects neurologic outcomes.

Within the CNS, CD8\(^+\) T cells have been implicated in the seemingly opposing roles of viral suppression and neuroinjury. In HIV\(^+\) individuals off of or failing treatment, high IFN-\(\gamma\) expression by CSF CD8\(^+\) T cells strongly associates with the severity of neurocognitive impairment, while CSF CD8\(^+\) T-cell expression of CD107, a marker of cytolytic activity, negatively correlates with impairment\cite{48}. Such duality may suggest the existence of two phenotypes of CD8\(^+\) T cells, one that promotes CNS damage by production of inflammatory cytokines and one that controls HIV replication and spread by lysing infected cells. The commonality in these cases is that both CSF CD8\(^+\) T-cell production of IFN-\(\gamma\) in neurologically impaired participants and cytolytic activity in unimpaired participants correlate with CSF CXCL10 concentration\cite{48}. Thus, this chemokine attracts and activates CD8\(^+\) T cells regardless of phenotype, which may be determined by other currently unidentified factors. From a temporal perspective, CSF CD8\(^+\) T-cell activation correlates with CSF viral load and inflammation markers such as neopterin and CXCL10 during AHI, in contrast to associating with plasma viral load, markers of neuroinjury, and markers of microglial activation in CHI\cite{49}. Activated CSF CD8\(^+\) T cells in AHI are further characterized by upregulation of T-cell effector functions and TCR signaling, and the distinctive feature that HIV-specific CD8\(^+\) T cells in the CSF
recognize of a wider range of HIV antigens compared HIV-specific CD8⁺ T cells in blood[49, 50]. These findings depict an early CD8⁺ T-cell population that responds to HIV-induced inflammatory signals in the CNS, employs cytolytic functions to control HIV replication, and clonally expands to better recognize compartmentalized HIV epitopes in the CNS. The activated CD8⁺ T cells in chronic HIV infection corresponds well with the phenotype described in neurocognitively impaired individuals. Driven by residual or resistant HIV in the blood, CD8⁺ T-cell activation in these cases contribute to neurologic injury and persistent activation of local immune cells through IFN-γ. Based on these studies, early treatment initiation may preserve the function of protective, cytolytic CD8⁺ T cells in the CNS and prevent switching to a more injurious phenotype.

**Monocytes as Viral Reservoirs**

Monocytes are another immune cell population implicated in systemic and CNS HIV infection. Of myeloid origin, monocytes circulate in the peripheral blood for up to three days before migrating into local tissues to differentiate into tissue resident macrophages or dendritic cells. While all monocytes express CD14, they are further divided into three subtypes: CD14⁺CD16⁻ classical monocytes, CD14⁺⁺CD16⁺ intermediate monocytes, and CD14⁻CD16⁺⁺ non-classical monocytes[^51]. Treatment of monocytes with macrophage colony stimulating factor has shown a possible developmental relationship between these subtypes, such that classical monocytes mature into a transitional intermediate monocyte state before reaching the non-classical monocyte stage[^51, 52]. In studies of sepsis and autoimmune disease, classical monocytes have been characterized as phagocytic and non-inflammatory, while non-classical
monocytes act as producers of inflammatory cytokines and antigen\textsuperscript{[53, 54]}. Because the formal monocyte nomenclature was established in 2010, much of the earlier literature defines all CD14\textsuperscript{+}D16\textsuperscript{+} monocytes, both intermediate and non-classical subtypes, as activated monocytes.

As CD4 expressing cells, monocytes present another viable target for HIV. Activated monocytes comprise only 5-10\% of all circulating monocytes in healthy individuals, but this number increases up to 40\% in the setting of HIV infection\textsuperscript{[55]}. Furthermore, these activated monocytes have been characterized as more permissive to HIV entry and replication compared to nonactivated monocytes\textsuperscript{[56]}. Infected monocytes and macrophages have been hypothesized to act as a latent HIV reservoir, as replication-competent virus can be isolated from CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes even from individuals virally suppressed on ART\textsuperscript{[57-59]}. Not only do monocytes demonstrate lower viral decay rates compared to CD4\textsuperscript{+} T cells, but treatment may not inhibit HIV replication in monocytes with the same effectiveness that it does in CD4\textsuperscript{+} T cells\textsuperscript{[58, 60]}. By migrating into local tissues, infected monocytes can differentiate into macrophages and serve as a long-lived source of viral infection. Of particular interest is the ability of monocytes to migrate into the CNS.

Infected monocytes that enter the CNS may contribute to both viral trafficking as well as immune activation in this compartment. Elevated levels of chemokines such as CCL2 and CXCL12 in the CSF of HIV\textsuperscript{+} individuals have been implicated in attracting monocytes and facilitating the transmigration of these cells across the blood brain barrier\textsuperscript{[61-64]}. Once the virus reaches the CNS, infection and activation of resident microglia, macrophages, and astrocytes can induce pro-inflammatory cytokines as well as
additional CCL2 and CXCL12 to attract more activated monocytes. The role of monocytes in facilitating neuroinjury, whether directly or indirectly, is further supported by studies of monocyte as potential biomarkers for HAND. The HIV DNA content of activated monocytes in blood correlates with worse cognitive performance in both treated and untreated HIV+ individuals, including those with undetectable plasma viral loads.

Further emphasizing the role of monocyte trafficking, a lower plasma level of non-classical monocytes expressing CCR2, the receptor for CCL2, correlates with a worsened score on neuropsychologic testing. Counterintuitively, the depletion of CCR2+ non-classical monocytes in the plasma may reflect increased migration of this subset into the CNS, an interpretation substantiated by the inverse association between CCR2+ CD14+CD16++ monocytes and CSF neopterin. The literature implicates activated monocytes as a viral reservoir, a conveyor of HIV into the CNS, and a contributor to CNS inflammation and neuroinjury.
STATEMENT OF PURPOSE

Despite ART, persistent HIV and immune activation in the CNS contributes to chronic neuroinjury and development of HAND. HIV entry into the CNS may be mediated by infected immune cells that traffic in response to inflammatory signals, and neuroinjury may also result from migration of activated effector immune cells. The literature shows conflicting findings with regard to the roles of CD4+ and CD8+ T-lymphocytes and monocytes in CNS HIV disease. Additionally, cross-sectional studies fail to fully capture the immunologic sequence of events during early untreated HIV infection, and to date, no longitudinal study has addressed such a question. This study aims to determine the natural progression of the immune response during untreated PHI; to investigate the association between activated T-lymphocytes, monocytes, and virus in the CNS; and to examine the effect of ART initiation on these processes. We hypothesized that CD4+ T cells may act as the main mediator of HIV trafficking to the CNS. This study analyzed paired blood and CSF from a longitudinally followed cohort of PHI participants, looking at flow cytometry data of T-cell activation markers (CD38 and HLA-DR) and chemokine receptors (CCR5 and CXCR3), monocyte activation (CD14 and CD16) and trafficking markers (alpha 4 integrin/CD49d), as well as correlating clinical and biomarker data.
Hypothesis

- Individuals with untreated chronic HIV infection (CHI) will show higher levels of activated T cells and monocytes in the CSF than individuals with PHI at baseline, and both groups will show higher activation than HIV uninfected control individuals.

- In the absence of ART over time, an increasing HIV load in the CNS will associate with pleocytosis and increased activation of immune cells. These cells, especially CD8+ T cells, may mediate an increasing inflammatory response in the CNS that leads to increasing levels of neuroinjury over time and eventual symptoms of neurocognitive disorders.

- In study participants that start treatment during the follow up period, CSF immune cell activation will decrease after ART, but will still remain at levels higher than in uninfected individuals

Aims

- Quantify levels of CD4+ T cell, CD8+ T cell, and monocyte activation in the CNS in PHI, CHI, and uninfected study participant groups.

- Examine the longitudinal changes in markers of cellular immune activation in PHI participants prior to and after treatment with ART.

- Investigate correlations between longitudinal changes in intrathecal inflammation, neuroinjury, virus load, and neuropsychological evaluations of these participants over time.
METHODS

Study Participants
This study includes 51 PHI, 32 CHI, and 11 HIV-negative participants. Participants were enrolled between 2005 to 2010 as part of the Primary Infection Stage CNS Events Study (PISCES), a prospective, observational study in San Francisco. PHI participants were enrolled within the first 12 months of infection, and date of HIV transmission was estimated per previously described methods[28, 29]. All PHI participants were ART-naïve at baseline; some participants subsequently started treatment at variable times during follow up, independent of this study. CHI and HIV-negative participants were recruited for baseline comparison as described in previous studies[29]. The control group was age and gender matched, recruited from the same community as study participants, and had no known neurologic diseases. PHI participants were followed longitudinally, with visits at baseline, 6 weeks, and every 6 months thereafter. Some individuals initiated ART during follow up because of personal choice or recommendations by their providers due to immunological, clinical, or virological disease markers.

Sample Collection, Laboratory Studies
Paired blood and CSF samples were obtained at baseline for PHI, CHI, and HIV-negative groups, and also at follow-up visits for PHI participants. Fresh blood samples were analyzed for CD4+ and CD8+ lymphocyte counts by flow cytometry, and CSF samples for WBC count, protein, and albumin. To quantify viral load, HIV-1 RNA concentration was measured in previously frozen (-70°C) cell free samples, with paired CSF and plasma samples in the same polymerase chain reaction (PCR) run using the ultrasensitive
Amplicor HIV Monitor (version 1.5; Roche Molecular Diagnostic Systems, Branchburg, NJ) or Abbott RealTime HIV-1 (Abbott Laboratories, Abbot Park, IL) assay. CSF concentration of chemokine CXCL10 was measured in previously frozen samples using commercial ELISA (R&D Systems).

Flow cytometry

Paired blood and CSF samples were obtained from study participants, and samples were prepared as previously described. Multiparameter flow cytometry was used to measure blood and CSF samples for the percentage of activated CD4⁺ and CD8⁺ T cells, as determined by CD38 and HLA-DR co-expression, as well as for expression of chemokine receptors CCR5 and CXCR3. Blood and CSF monocytes were classified by CD14 and CD16 expression and analyzed for expression of alpha-4 integrin, measuring both the percentage of CD49d⁺ monocytes as well as the mean/median fluorescence intensity (MFI) of CD49d staining. Only a subset of samples were analyzed for T-cell data and a different subset for monocyte data (see supplementary information). Monoclonal antibodies included CD3, CD4, CD8, CD38, HLA-DR, CCR5, CXCR3, CD45, CD14, CD16, and CD49d conjugated to allophycocyanin (APC), phycoerythrin (PE) peridinin chlorophyll protein (PerCP), fluorescein isothiocyanate (FITC), and tandem conjugations with cyanine (ACP-Cy7, PE-Cy7) and Texas Red (PE-Texas Red). Blood samples were stained with fluorescence-minus-one controls in which one antibody was omitted; an unstained control and single-stained samples were also prepared as compensation controls. Samples were run on a FACS DIVA (BD Biosciences, San Jose, CA) and flow cytometry data analyzed with FlowJo (TreeStar, Ashland, OR).
Flow cytometry antibody-dye panels were switched halfway through the study in 2008, such that participants already enrolled continued to be analyzed under Panel 1 while newly enrolling participants were analyzed under Panel 2 (Table S1 and S2). Given significant differences in some measurements between panels, data from the 2 panels were analyzed separately throughout this study. Only Panel 1 included analysis of CCR5⁺ and CXCR3⁺ expression in T cells, and only Panel 2 included alpha-4 integrin (CD49d⁺) in monocytes. Baseline and longitudinal analyses for T cells used Panel 1 data, for monocytes used Panel 2 data, and for comparison of monocyte alpha-4 integrin expression in relation to T-cell activation used Panel 2 data.

**Statistical Analyses**

Nonparametric methods were used to analyze baseline visit data, including the Mann-Whitney U test, Kruskal-Wallis test with post hoc testing using Dunn’s multiple comparisons, and Spearman rank correlation coefficient. Longitudinal data was analyzed using linear mixed models, which accounts for differences in total number of visits. Univariate and multivariate mixed-effect regression modeling and Sobel’s test were used for mediation analysis, to clarify direct and indirect effects of variables on the outcome. Relationships between variables were also checked using within-subject correlation, to account for possible errors that stem from repeated measurements of the same participant. Statistics were performed and graphics generated using IBM SPSS Statistics 25 (IBM, Armonk, NY) or Prism 7 (GraphPad Software Inc, La Jolla, CA).
Participant recruitment, sample collection, and flow cytometry were performed by Dr. Spudich and collaborators at University of California San Francisco. Statistical analysis was primarily performed by the author, with the assistance of the Yale Center for Analytical Sciences. Interpretation of the analysis was performed by the author with the guidance of coauthors on this thesis.
RESULTS

Table 2. Demographic and clinical characteristics of PHI study participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Primary HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Participants (n)</td>
<td>51</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36 (31, 45)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male (100%)</td>
</tr>
<tr>
<td>Estimated time post HIV transmission (months)</td>
<td>3.2 (2.4, 5.6)</td>
</tr>
<tr>
<td>CD4⁺ T cell count (cells/µl)</td>
<td>581 (429, 738)</td>
</tr>
<tr>
<td>CD8⁺ T cell count (cells/µl)</td>
<td>985 (691, 1336)</td>
</tr>
<tr>
<td>Plasma HIV RNA (log₁₀ copies/ml)</td>
<td>4.37 (3.80, 4.86)</td>
</tr>
<tr>
<td>CSF HIV RNA (log₁₀ copies/ml)</td>
<td>2.31 (1.69, 3.10)</td>
</tr>
<tr>
<td>CSF WBC count (cells/mm³)</td>
<td>6 (2, 11)</td>
</tr>
<tr>
<td>CSF protein (mg/dL)</td>
<td>41.0 (35.5, 51.3)</td>
</tr>
<tr>
<td>Follow-up duration (months)</td>
<td>6.5 (0, 22.7)</td>
</tr>
<tr>
<td>Total Visits</td>
<td>168</td>
</tr>
<tr>
<td>Pre-ART</td>
<td>113</td>
</tr>
<tr>
<td>On ART</td>
<td>55</td>
</tr>
</tbody>
</table>

*Median (interquartile range) shown unless otherwise indicated

Study participant characteristics

Baseline characteristics of PHI study participants are shown in Table 2. Longitudinal data on the PHI group was collected from a median follow-up duration of 6.5 months and 168 total visits, 113 while participants were treatment-naïve and 55 visits after ART initiation.
Baseline measures of T-cell activation and chemokine receptor expression in blood and CSF

As little is known about how the level of T cell-related inflammation in PHI compares to that seen in CHI and HIV-negative individuals, we first assessed the percentage of activated CD4^+ and CD8^+ T cells in blood and CSF among the three groups at baseline.

Compared with HIV^+ individuals, PHI participants showed increased T-cell activation in peripheral blood and CSF (Figure 1), although activation was only significantly elevated in blood. The CHI group showed significantly higher T-cell activation in blood and CSF when compared with HIV-negative controls (Figure 1). No significant differences were found between PHI and CHI groups.
As cellular trafficking to peripheral tissues reflect another aspect of activated immune cells, we also assessed the frequency of T-cell expression of inflammation-related chemokine receptors CCR5 and CXCR3. Notably, all three groups demonstrated high levels (>95%) of CXCR3 expression among T cells in the CSF (Figure S1). Given these findings, we were interested in the role that chemokines such as CXCL10, a ligand to CXCR3, may play trafficking of activated T cells into the CNS during HIV infection. In PHI participants at baseline, no significant correlation existed between CSF CXCL10 levels and CXCR3 expression on CSF CD4+ or CD8+ T cells (Figure S2). CSF CXCL10 concentrations positively correlated with CSF CD4+ and CD8+ T-cell activation, as well as with CSF HIV RNA levels (Figure S2). While these cross-sectional analyses give an idea of the immune response to HIV during PHI, the findings give only a snapshot of the natural progression of the developing inflammatory response in the CNS.

**Longitudinal T-cell activation and chemokine receptor expression in early untreated infection**

In PHI participants who did not start treatment immediately after diagnosis, we assessed the longitudinal trajectory of T-cell activation during early HIV infection in the absence of ART. While T-cell activation significantly increased over time in both blood (CD4+ T cells only) and CSF (both CD4+ and CD8+ T cells), the CSF showed a three-fold greater rate of increase in percent T-cell activation compared to that seen in blood (Figure 2). Mixed model analysis of the longitudinal data showed that both CSF CD4+ and CD8+ T-cell activation correlated significantly with CSF HIV RNA levels (Table 3). Biologically, CD4+ T cells are known targets for HIV infection and hypothesized to bring virus into
Figure 2. Longitudinal changes in blood and CSF CD4+ and CD8+ T-cell activation (% CD38+ HLA-DR+) in untreated PHI participants (n=21). Data points from the same participant over time are connected. Solid line represents significant regression slope and dashed line non-significant regression slope.

Table 3. CSF CD4+ and CD8+ T-cell activation (% CD38+ HLA-DR+) as univariate and multivariate predictors of CSF viral load (log₁₀ CSF HIV RNA).

<table>
<thead>
<tr>
<th>Univariate Analysis</th>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Regression Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF CD4+ T-cell activation</td>
<td>CSF HIV RNA (log₁₀ copies/mL)</td>
<td>0.0436</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>CSF CD8+ T-cell activation</td>
<td>CSF HIV RNA (log₁₀ copies/mL)</td>
<td>0.0274</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>CSF CD8+ T-cell activation</td>
<td>CSF CD4+ T-cell activation</td>
<td>0.496</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multivariate Analysis</th>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Regression Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF CD4+ T-cell activation</td>
<td>CSF HIV RNA (log₁₀ copies/mL)</td>
<td>0.0383</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>CSF CD8+ T-cell activation</td>
<td>CSF CD4+ T-cell activation</td>
<td>0.0088</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

* T-cell activation is defined as % CD38+ HLA-DR+
the CNS, while activated CD8+ T cells are thought to be attracted to the CNS in response to antigens and inflammatory signals. Multivariate analysis was used to clarify whether activation of both T-cell types independently associate with CSF HIV RNA levels. While CSF CD4+ T-cell activation remained an independent predictor of CSF HIV RNA in multivariate analysis, CSF CD8+ T-cell activation was not significantly associated with CSF HIV RNA (Table 3). In addition, this analysis confirmed that the CSF CD4+ T-cell activation level has significant mediation effects, accounting for 69% of the association between CSF CD8+ T-cell activation and CSF HIV RNA (Figure S3).

Given these longitudinal changes in T-cell activation, we examined whether chemokine receptors expression displayed a corresponding trend during PHI. Similar to markers of T-cell activation, chemokine receptor expression in CSF leukocytes may reflect the role of trafficking T cells in increasing intrathecal inflammation. While a relative decrease in CXCR3+ cells was seen in blood for CD8+ T cells, the percent of CXCR3+ T cells remained elevated in CSF and even increased significantly for CSF CD4+ T cells (Figure S4). In the CSF, longitudinal analysis showed a decrease in the percentage of CCR5+ T cells, though only significant for CD8+ T cells, as well as a decrease in CCR5+CXCR3+ populations and an increase in CCR5−CXCR3+ populations for both CSF CD4+ and CD8+ T cells (Figure S4). The presence of longitudinal immune activation in untreated PHI leads to the question of how antiretroviral treatment can alter the course of immune dysregulation.
Figure 3. Longitudinal changes in blood and CSF CD4⁺ and CD8⁺ T-cell activation (％CD38⁺ HLA-DR⁺) in PHI participants (n=11) after initiation of ART.

Longitudinal changes in T-cell activation and chemokine receptor expression after initiation of ART

A central question regarding the increasing immune activation in PHI is whether early ART initiation can halt or reverse the observed CNS inflammation. A subset of PHI participants (n = 11) initiated treatment independent of the study at a median of 479 days after estimated infection. Blood and CSF samples collected during the first 500 days post-ART initiation showed significant decline in blood CD8⁺ T-cell activation, but no
significant change in blood CD4⁺, CSF CD4⁺, or CSF CD8⁺ T-cell activation (Figure 3).

After ART initiation, no significant changes were observed in expression of any combinations of chemokine receptor CCR5 and CXCR3 in either blood or CSF T cells over time (Figure S5).

**Baseline monocyte subtypes and α4 integrin expression**

Monocytes are another component of the immune response to HIV that has not been well characterized in the CSF during PHI. At baseline, the populations of classical (CD14⁺ CD16⁻), intermediate (CD14⁺⁺ CD16⁺), and non-classical (CD14⁺ CD16⁺⁺) monocyte subtypes did not differ significantly between PHI, CHI, and HIV-negative groups in blood or CSF (Figure 4).

![Figure 4](image)

*Figure 4.* Baseline comparison of classical (CD14⁺ CD16⁻), intermediate (CD14⁺⁺ CD16⁺), and non-classical (CD14⁺ CD16⁺⁺) monocyte subtypes in blood and CSF of HIV-negative, PHI, and CHI participants.
Monocyte ability to traffic into local tissues was assessed by expression of α4 integrin, both in terms of percentage of monocytes expressing α4 integrin as well as the expression intensity through MFI measures. Compared with the HIV-negative group, PHI monocytes showed significantly higher α4 integrin expression both in blood, by MFI, and in CSF, by percentage (Figure 5). Significant differences also existed between CHI and HIV-negative groups in these same measures, whereas no significant differences were found between the three groups in terms of α4 integrin percent expression in blood or in terms of MFI in CSF (Figure 5).
Longitudinal monocyte activation and α4 integrin expression in early untreated infection

Given the detectable difference in monocyte α4 integrin expression at baseline, changes in monocyte activation and trafficking over the natural course of untreated PHI may further elucidate the roles of this immune cell type. Mixed model analysis showed no significant changes to the proportion of intermediate, classical, and non-classical monocyte subpopulations in blood or CSF over time, nor did monocyte subgroups correlate with CSF HIV RNA or with T-cell activation in blood or CSF (Table S3). Monocyte α4 integrin expression, by either percentage or MFI, did not show significant changes over time or correlate with CSF HIV RNA levels (Table S3). In blood but not CSF, monocyte α4 integrin expression intensity by MFI correlated with CD4+ and CD8+ T-cell activation (Figure 6). Analysis of monocyte α4 integrin by percent expression did not show significant correlation with T-cell activation in blood or CSF (Table S3).
Figure 6. Correlation between monocyte α4 integrin expression intensity (CD49d MFI) and CD4⁺ and CD8⁺ T-cell activation in blood and CSF, using longitudinal data from untreated PHI participants (n=27).
**Longitudinal changes in monocyte activation and α4 integrin expression after ART**

The relationship between monocytes and T cells in treatment-naïve PHI evokes the question of whether this correlation can be disrupted by initiation of ART. A significant decrease in blood monocyte α4 integrin expression intensity was found after initiation of treatment (Table S3). The proportion of each monocyte subtype and monocyte α4 integrin percent expression did not change significantly in blood post-ART, nor did any CSF monocyte measures show changes over time post-treatment (Table S3). Monocyte α4 integrin expression intensity continued to show a positive correlation with CD4+ and CD8+ T-cell activation post-treatment, and a significant correlation was detected for CSF monocyte α4 integrin MFI and CSF CD4+ T-cell activation (Figure 7). Due to insufficient variance in the data, the relationship between CSF monocyte α4 integrin MFI and CSF CD8+ T-cell activation could not be determined. In contrast to treatment-naïve results, blood intermediate and classical monocytes showed a positive correlation with T-cell activation, and α4 integrin percent expression showed a negative correlation with T-cell activation post-treatment (Table S3).
Figure 7. Correlation between monocyte α4 integrin expression intensity (CD49d MFI) and CD4⁺ and CD8⁺ T-cell activation in blood and CSF, using longitudinal data after ART initiation (n=17).
DISCUSSION

The nature of this study is unique because of its longitudinal nature, focus on a PHI cohort, access to a sizable amount of follow-up data during untreated HIV infection, and the availability of paired CSF and blood samples allowing for study of CNS-specific immune response. Our findings demonstrate a significant increase in CSF T-cell activation over time during untreated PHI that is not completely reversed by initiation of ART. This study also indicates that activated CSF CD4+ T cells, but not monocytes, correlate with CSF HIV RNA, suggesting that T cells are the main cell types trafficking HIV into the CNS during PHI. We reveal a notable role for CXCR3 and its ligand CXCL10 in T-cell trafficking to the CNS and for α4 integrin expression as a likely marker of inflammation in monocytes.

T-cell activation

In our study, untreated PHI individuals at a median of 3.2 months after estimated infection showed increased levels of CD4+ and CD8+ T-cell activation compared to the HIV-negative group, though these differences only reached statistical significance in blood and not CSF. In addition, immune activation in blood and CSF was not significantly different between the PHI and CHI groups, suggesting that elevated systemic and CNS inflammation manifests even within the first year of HIV infection. Longitudinally, untreated PHI participants showed increases over time in almost all blood and CSF T-cell activation levels, except for blood CD8+ T cell, and a threefold higher rate of inflammation increase in CSF compared to blood. These findings suggest that though systemic inflammation may help promote CNS inflammation, there also exists
CNS-specific drivers of immune activation in HIV. Viral antigens may be picked up by local CNS immune cells and translated into chemokine signals and upregulation of trafficking markers on local endothelium. According to Kessing et al. (2017), levels of CSF CD8+ T-cell activation that correlates with CSF viral load rather than plasma viral load during AHI, and our findings are consistent with the idea that activation and trafficking of CD8+ T cell during PHI is primarily driven by the pull of HIV antigens and inflammatory markers, more so than the push of a systemic inflammatory state. Alternatively, the lack of significant increase in blood CD8+ T-cell activation may reflect a depletion of this subset in the periphery due to massive migration into local tissues.

The relationship between T-cell activation and CSF HIV RNA level during untreated PHI remains of interest due to the potential for activated CD4+ T cells to bring HIV into the CNS and inversely, the potential for virus to stimulate CD8+ T-cell influx into the compartment. While both CSF CD4+ and CD8+ T-cell activation correlated with CSF HIV RNA levels in univariate analysis, only CSF CD4+ T-cell activation remained an independent predictor of CSF HIV RNA in multivariate analysis. This result suggested an interaction between the two T-cell types that affects their association with CNS virus, and formal mediation analysis confirmed that CSF CD4+ T-cell activation acts as a significant mediator, accounting for 69% of the correlation effect of CSF CD8+ T-cell activation on CSF HIV RNA. One interpretation of this mediation relationship may be that CD8+ T-cell inflammation in the CNS recruits more activated, HIV-infected CD4+ T cells to the compartment, thus increasing CSF HIV levels. As this type of analysis cannot determine causality, CD4+ T-cell activation could instead mediate the effect of virus on CD8+ T cells in the CNS. CNS virus may infect, induce damage to, or activate CD4+ T
cells, which in turn produce signals that activate or attract CD8+ T cells. The presence of HIV during untreated PHI may instigate signaling between these T-cell groups that eventually becomes driven by the pro-inflammatory environment, despite viral suppression.

Given longitudinal increases in T-cell activation during untreated PHI, the putative ability of ART to halt or reverse this progressive inflammation is essential to improving clinical outcomes. Longitudinal analysis showed significant decreases in blood CD8+ T-cell activation post-treatment, but no significant changes were seen for the other T-cell groups after ART initiation. Notably, these unchanged T-cell groups, namely blood CD4+, CSF CD4+, and CSF CD8+ T cells, were the lineages that showed significant increases in activation during untreated PHI, while blood CD8+ T cells did not, so the effect of treatment may not address the correct subset of immune cells for reducing CNS immune dysregulation. Though ART may halt the escalation of T-cell activation, treatment did not seem to reverse the elevated inflammation levels in the follow-up period during this study.

**T-cell chemokine receptors**

Chemokine receptors constitute another set of surface markers whose upregulation can provide vital information about inflammation. In CD4+ T cells, CXCR3+ and CCR5+ expression are characteristic of an inflammatory Th1 response and have been associated with pathology in autoimmune conditions such as multiple sclerosis and immune reconstitution inflammatory syndrome[69-71]. In PHI, chemokine receptor expression on T cells can act as another measure of activation in addition to indicating
homing of T cells to local tissues such as the CNS.

At baseline, the PHI and CHI groups generally did not have significantly different percentages of CXCR3$^+$ and CCR5$^+$ T cells in the blood or CSF. Baseline levels also showed consistently high CXCR3 expression above 95% in CSF CD4$^+$ and CD8$^+$ T cells across PHI, CHI, and HIV$^-$ groups, which indicate that CXCR3 may be an essential component for T-cell homing to the CNS regardless of inflammatory status. This theory would suggest that changes in concentration of CXCR3 ligands, such as CXCL10, could play an important role in controlling influx of activated immune cells to the CNS. While CSF CXCL10 levels did not correlate with the percentage of CSF CXCR3$^+$ T cells, CXCL10 did significantly correlate with CSF CD4$^+$ and CD8$^+$ T-cell activation as well as CSF HIV RNA levels at baseline in PHI participants. The lack of correlation with CXCR3 is not surprising given the generally high level of CXCR3 expression in all CSF T cells, but the association with both virus and percentages of activated T cells support a possible role for CXCL10 in immune recruitment in response to HIV.

Given the findings at baseline, longitudinal changes in CCR5 and CXCR3 expression may shed light on mechanisms of T-cell recruitment and ongoing inflammation in the CNS during untreated PHI. In blood, no significant changes were seen for CD4$^+$ T-cell expression of chemokine receptors CCR5 and CXCR3, while blood CD8$^+$ T cells showed significant decreases in the fraction of CXCR3$^+$ cells. Such a change may reflect a relative depletion of inflammatory CXCR3$^+$ cells that are migrating from blood to the CNS. CNS T cells overall show a decrease in CCR5$^+$ T cells, though significant only for CD8$^+$ T cells, and increases in the already elevated CXCR3$^+$ T-cell population, significant only for CD4$^+$ T cells. Both CD4$^+$ and CD8$^+$ T cells showed
significantly decreased CCR5^+CXCR3^+ double-positive populations and increased CCR5^-CXCR3^+ populations, detailing a more specific shift reflected by the increase in CCR5^-T cells. These results imply that the highly active and inflammatory CCR5^+CXCR3^+ T cells may be short lived in the CNS and die off after performing their function. The fact that CCR5 is the receptor used for HIV entry into CD4^+ T cells may also contribute, if the depletion is of infected cells. In conjunction with the above, a continuing migration of CXCR3^+ T cells into the CNS may continue supplying the compartment with inflammatory, but not fully activated, CCR5^+CXCR3^+ cells. The role of CCR5-expression on CD8^+ T cells is not well understood, as CCR5 is often studied in conjunction with CXCR3 and subsets such as CCR5^-CXCR3^+ T cells have not been well characterized before in either CD4^+ or CD8^+ T cells.

No significant changes were detected over time on treatment for chemokine receptor expression in blood and CSF CD4^+ and CD8^+ T cells, but interpretation of these data are limited by the short length of follow up and small number of samples with these measures in the current study.

**Monocyte subtypes**

Another immune cell type of interest in HIV is the monocyte population, which has canonically been thought to bring HIV into the CNS and to constitute some of the long-term viral reservoirs in the brain[72, 73]. In addition, interactions between monocytes and T cells may also facilitate HIV replication and dissemination and contribute to the inflammatory milieu during viral infection. Conjugation between CD16^+ monocytes and CD4^+ T cells is hypothesized to promote viral replication beyond the level in T cells
alone\textsuperscript{[74]} and expression of HIV Env by infected T cells may induce lymphocyte-monocyte fusion into a heterokaryon with markers and functions of activated monocytes\textsuperscript{[75]}. SIV studies have shown that monocytes and monocyte-derived macrophages produce high levels of proinflammatory chemokines such as CXCL10, leading to increased recruitment of CCR5\textsuperscript{+}CXCR3\textsuperscript{+} CD4\textsuperscript{+} T cells in secondary lymphoid organs\textsuperscript{[76]}. This study aimed to investigate both the relationship between monocytes and HIV as well as monocytes and T cells during PHI.

At baseline, no significant difference was found between monocyte populations in PHI, CHI, and HIV-negative participants. Prior studies have investigated monocyte subtypes in blood, but no studies to our knowledge have compared relative proportions of monocyte subtypes in the CSF in healthy controls, HIV infection, or other neuroinflammatory diseases. While the majority of blood monocytes are of the classical subtype, the CSF contains a majority of intermediate monocytes, followed by classical monocytes as second most populous. These results suggest preferential migration of intermediate monocytes to the CNS and future research on monocyte roles in HIV would benefit from looking into the roles of this cell population. Longitudinally, no significant changes were observed over time in the percentage of any monocyte subtypes in blood or CSF during untreated PHI. Monocyte subtype did not correlate with CD4\textsuperscript{+} or CD8\textsuperscript{+} T-cell activation levels in blood or CSF, or with CNS HIV RNA over time. Though monocytes have been canonically thought to bring HIV into the CNS, the absence of a correlation in our study suggests that monocytes may not play a strong role in HIV trafficking during PHI. At the same time, a possibility exists of a relationship between monocytes and HIV outside of monocyte subtype classification.
Initiation of ART was not associated with any significant changes in monocyte subtype percentages over time. In participants on treatment, intermediate monocytes showed a significant positive association with CD4$^+$ and CD8$^+$ T-cell activation while classical monocytes showed a significant negative association with T-cell activation in blood, which suggests presence of signaling between these immune cells that had not been as strong prior to treatment. Because HIV may be a more potent driver of T-cell activation, perhaps this monocyte-T cell signaling had been disrupted or superseded by other signals during untreated PHI. Classical and intermediate monocytes seem to have the opposite relationship with T cells, which are in accordance with the characterization of classical monocytes as non-inflammatory and intermediate and non-classical monocytes as more activated and inflammatory\cite{53}, and these monocyte subtypes may have different functions when it comes to immune signaling. Though intermediate monocytes make up a much smaller fraction of blood monocytes, these cells still showed a significant positive association with T-cell activation, suggesting that small changes in this population may have larger implications for inflammation even in individuals on ART. This association was not detected in the CSF, despite the elevated proportion of intermediate monocytes in this compartment. As intermediate monocytes are thought to be a transitional subset, perhaps the abundance of these cells show an influx that will, with time, differentiate into non-classical monocytes or tissue resident macrophages.

**Monocyte α4 integrin**

Like other immune cells, monocytes express specific surface molecules to aid in trafficking from blood into tissues. Alpha-4 integrin, also known as Very Late Antigen 4
(VLA4) or CD49d, is a subunit of the transmembrane integrin protein. Integrins are required for leucocyte adhesion to endothelial cells during the process of extravasation from blood into local tissue, and alpha 4 integrin has been targeted in neuroinflammatory autoimmune diseases such as multiple sclerosis to prevent leukocytosis. Primate studies using anti-α4 antibody to block monocyte/macrophage trafficking to the brain and gut have been shown to reduce levels of SIV in the brain if administered in early infection and stabilize neuronal injury if administered in late SIV[77], but to our knowledge no similar work has been done during early infection in humans.

At baseline, PHI and CHI participants showed significantly elevated expression of α4 integrin compared to HIV-negative individuals. In blood, both control and HIV+ individuals have a large percentage of monocytes that are expressing α4, but in PHI and CHI samples there also exists a significantly higher expression level or density of α4 being expressed on each cell. A higher density of integrins could aid monocytes in extravasation to the CNS and thus would be an appropriate response to inflammation. In the CSF, the HIV+ groups show a significantly higher percentage of α4-positive cells compared to the HIV- group, suggesting that such cells may have migrated from the blood. However, the three groups do not show significantly different MFI, so the density of α4-integrin amongst monocytes that are expressing this protein is comparable regardless of group. One interpretation is that only monocytes expressing a certain level of α4-integrin can traffic into the CSF, and while these newly migrating monocytes only make up a minority in HIV-negative individuals, they are the majority in the CNS of PHI and CHI individuals due to ongoing inflammation.

Longitudinal analysis showed no significant correlations between α4-integrin
expression and days of infection or CSF viral load. Blood monocyte α4 MFI showed a significant positive correlation with blood CD4+ and CD8+ T-cell activation, further supporting the α4-integrin expression level as reflection of increased inflammation in blood. However, such correlation was not found in the CSF, which can be explained by the fact that T-cell activation and recruitment in the CSF seems closely tied with CSF HIV RNA while monocyte trafficking may not be so CNS virus-driven.

Treatment with ART led to a significant decrease in monocyte α4-integrin MFI over time in blood but not CSF. While we did not show that α4 significantly increased during untreated PHI, this signal does seem to correlate with important markers of inflammation in blood and a reduction in expression intensity should reflect a benefit of treatment. During ART, the percent of α4-expressing monocytes in blood, but not in CSF, showed significant negative correlation with CD4+ and CD8+ T-cell activation. In contrast, the α4 MFI showed significant positive correlation with CD4+ T-cell activation in blood and CSF. These results further affirm that the monocyte α4 expression intensity and T-cell activation are both inflammatory responses, and treatment may have allowed non-HIV-driven signaling to be re-established in the CSF. While the negative correlation between percentage of α4-positive monocyte and T-cell activation in blood was unexpected, this result may be due to uncertainty as to whether percent expression or MFI is a more appropriate measurement of monocyte activation and extravasation ability.

**Conclusions**

This study’s longitudinal analysis showed increasing levels of CD4+ and CD8+ T-cell activation in blood and CSF during untreated PHI, with a higher rate of inflammation
increase in the CSF compared to blood. Initiation of ART halted but did not reverse CNS inflammation during a median 6.5 months of follow-up. These results suggest some level of irreversibility of inflammation, which may contribute to long term outcomes for HIV patients even after initiation of treatment. At the same time, the change in associations between monocyte alpha 4 integrin expression and T-cell activation post-treatment may reflect that CNS T-cells are reacting to a more non-specific inflammatory stimuli after viral suppression. This study indicates that CD4+ T cells are a likely source for trafficking HIV into the CNS, as CSF CD4+ T cell but not monocyte activation correlated with CSF HIV levels. Examination of T-cell chemokine receptor expression revealed CXCR3 to have a likely role in CNS homing in both control and HIV+ participants, hence CXCR3 ligands such as CXCL10 may be relevant targets for immunotherapy in future treatment of HIV-neuroinflammation.
## SUPPLEMENTARY TABLES AND FIGURES

### Table S1. Flow cytometry markers for T cell and monocyte panels 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Panel 1 markers</th>
<th>Panel 2 markers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD3</td>
<td>CD3</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4</td>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
<td>CD8</td>
<td>CD8</td>
</tr>
<tr>
<td>CD38</td>
<td>CD38</td>
<td>CD38</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>HLA-DR</td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td></td>
<td>CD27</td>
</tr>
<tr>
<td>CXCR3</td>
<td></td>
<td>CD28</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>RA</td>
</tr>
<tr>
<td><strong>Monocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD45</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>CD14</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>CD16</td>
<td></td>
</tr>
<tr>
<td>CD16</td>
<td>CD49d</td>
<td></td>
</tr>
</tbody>
</table>

### Table S2. Participant demographics and visit information for flow cytometry panels 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Panel 1</th>
<th>Panel 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of participants (n)</strong></td>
<td>51</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td><strong>Enrollment period</strong></td>
<td>2005-2010</td>
<td>2005-2008</td>
<td>2008-2010</td>
</tr>
<tr>
<td><strong>Total visits</strong></td>
<td>168</td>
<td>89</td>
<td>79</td>
</tr>
<tr>
<td><strong>Pre-ART</strong></td>
<td>113</td>
<td>66</td>
<td>47</td>
</tr>
<tr>
<td><strong>Post-ART</strong></td>
<td>55</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td><strong>Estimated time post HIV transmission at baseline visit (months)</strong></td>
<td>3.4 (1.9, 5.6)</td>
<td>4 (2.7, 5.3)</td>
<td>2.8 (1.8, 5.8)</td>
</tr>
<tr>
<td><strong>Follow-up duration (months)</strong></td>
<td>6.9 (1, 22.9)</td>
<td>7.4 (2.1, 20)</td>
<td>6.4 (0, 24)</td>
</tr>
<tr>
<td><strong>Number of participants that initiated ART (n)</strong></td>
<td>22</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td><strong>HIV infection duration at initiation of ART (months)</strong></td>
<td>6.1 (2.3, 17.7)</td>
<td>15.7 (4.1, 18.7)</td>
<td>5.6 (2.3, 17.0)</td>
</tr>
<tr>
<td><strong>Follow-up duration on ART (months)</strong></td>
<td>12.9 (3.8, 20.4)</td>
<td>8.5 (1.2, 10.4)</td>
<td>14.6 (7.6, 21.7)</td>
</tr>
</tbody>
</table>

^Median (interquartile range) shown unless otherwise indicated
Figure S1. Comparison of chemokine receptors expression, including percentage of total CCR5+ or total CXCR3+, double positive (CCR5+ CXCR3+), singly positive (CCR5+CXCR3- or CCR5-CXCR3+), and double negative (CCR5CXCR3-) cells in blood and CSF CD4+ and CD8+ T cells of HIV-negative, PHI, and CHI participants at baseline. Compared with the HIV-negative group, PHI and CHI groups showed an increased percentage of CCR5+ and CCR5+CXCR3+ cells, and decreased percentage of CCR5+CXCR3- cells in the CD8+ T-cell population, though the difference was only significant in the blood for PHI and in both blood and CSF for CHI. The CHI group showed a significantly increased proportion of CXCR3+ and CCR5CXCR3+ cells, and decreased proportion of CCR5CXCR3- cells for blood CD4+ T cells when compared to the HIV-negative group, but no significant difference in CSF CD4+ T cells.
Figure S2. Correlation between CSF CXCL10 concentration with CSF CD4⁺ and CD8⁺ T-cell activation and CSF HIV RNA levels.

Figure S3. Sobel test for mediation of the association between CSF CD8⁺ T-cell activation (independent variable) and CSF HIV RNA levels (dependent variable) by CSF CD4⁺ T-cell activation (mediator).
Figure S4. Longitudinal changes in CD4+ and CD8+ T-cell expression of chemokine receptors CCR5 and CXCR3 in blood and CSF of untreated PHI participants (n=21) over time. Expression of single markers CCR5+ or CXCR3+, or of marker combinations CCR5+CXCR3+, CCR5−CXCR3−, CCR5+CXCR3+ and CCR5−CXCR3− are shown as percentages of CD4+ or CD8+ T-cell population in blood and CSF. Blood CD8+ T cells had significantly decreased CXCR3+ and CCR5−CXCR3− subsets, as well as increased CCR5+CXCR3+ and double negative CCR5−CXCR3− subsets. In CSF, both CD4+ and CD8+ T cells showed persistently high levels of CXCR3+ expression, with declining percentages of CCR5+ and double positive CCR5+CXCR3+ cells. CSF CD4+ T cells showed significant increases in proportion of CXCR3+ and CCR5−CXCR3− and decreases in CCR5+CXCR3− subtypes. CSF CD8+ T cells showed no significant change in percentage of CXCR3+ cells, significant increase in the CCR5−CXCR3− population, and a decrease in CCR5+ and CCR5−CXCR3− populations.
Figure S5. Longitudinal changes in blood and CSF CD4+ and CD8+ T-cell expression of chemokine receptors CCR5 and CXCR3 in PHI participants (blood n=11, CSF n=8) after initiation of ART. Expression of single markers CCR5+ or CXCR3+, or of marker combinations CCR5+CXCR3+, CCR5+CXCR3-, CCR5-CXCR3+ and CCR5-CXCR3- are shown as percentage of CD4+ or CD8+ T cells in blood and CSF.
Table S3. Longitudinal changes in monocyte subtypes and α4 integrin expression, T-cell activation, and HIV RNA levels in blood and CSF of untreated and treated PHI participants.

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Blood Regression Coefficient</th>
<th>Blood p-value</th>
<th>CSF Regression Coefficient</th>
<th>CSF p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days Post-Infection</td>
<td>Classical Monocytes</td>
<td>-0.005</td>
<td>0.94</td>
<td>-0.0003</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Intermediate Monocytes</td>
<td>0.003</td>
<td>0.11</td>
<td>0.0002</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Non- Classical Monocytes</td>
<td>0.002</td>
<td>0.14</td>
<td>0.001</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Monocyte α4 integrin (%)</td>
<td>-0.002</td>
<td>0.58</td>
<td>0.004</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>Monocyte α4 integrin (MFI)</td>
<td>0.031</td>
<td>0.87</td>
<td>-0.15</td>
<td>0.67</td>
</tr>
<tr>
<td>Classical Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>-0.094</td>
<td>0.39</td>
<td>0.020</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>0.001</td>
<td>1.00</td>
<td>-0.078</td>
<td>0.44</td>
</tr>
<tr>
<td>Intermediate Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>0.097</td>
<td>0.48</td>
<td>-0.015</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>0.16</td>
<td>0.54</td>
<td>0.10</td>
<td>0.32</td>
</tr>
<tr>
<td>Non-Classical Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>0.068</td>
<td>0.70</td>
<td>-0.16</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>-0.30</td>
<td>0.40</td>
<td>-0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Monocyte α4 integrin (%)</td>
<td>CD4+ T-cell activation</td>
<td>0.006</td>
<td>0.93</td>
<td>0.015</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>0.071</td>
<td>0.65</td>
<td>0.058</td>
<td>0.44</td>
</tr>
<tr>
<td>Monocyte α4 integrin (MFI)</td>
<td>CD4+ T-cell activation</td>
<td>0.004</td>
<td>0.01</td>
<td>0.001</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>0.008</td>
<td></td>
<td>0.003</td>
<td>0.28</td>
</tr>
<tr>
<td>Classical Monocytes</td>
<td>CSF HIV RNA</td>
<td></td>
<td></td>
<td>0.00005</td>
<td>0.99</td>
</tr>
<tr>
<td>Intermediate Monocytes</td>
<td></td>
<td></td>
<td></td>
<td>-0.004</td>
<td>0.92</td>
</tr>
<tr>
<td>Non-Classical Monocytes</td>
<td></td>
<td></td>
<td></td>
<td>0.015</td>
<td>0.61</td>
</tr>
<tr>
<td>Monocyte α4 integrin (%)</td>
<td></td>
<td></td>
<td></td>
<td>-0.002</td>
<td>0.60</td>
</tr>
<tr>
<td>Monocyte α4 integrin (MFI)</td>
<td></td>
<td></td>
<td></td>
<td>0.00004</td>
<td>0.76</td>
</tr>
<tr>
<td>Monocyte α4 integrin (%)</td>
<td>Monocyte α4 integrin (MFI)</td>
<td>12.28</td>
<td>0.004</td>
<td>2.70</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Treated

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Blood Regression Coefficient</th>
<th>Blood p-value</th>
<th>CSF Regression Coefficient</th>
<th>CSF p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days on Treatment</td>
<td>Classical Monocytes</td>
<td>0.006</td>
<td>0.19</td>
<td>-0.026</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Intermediate Monocytes</td>
<td>-0.003</td>
<td>0.47</td>
<td>0.024</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Non- Classical Monocytes</td>
<td>-0.002</td>
<td>0.42</td>
<td>0.0001</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Monocyte α4 integrin (%)</td>
<td>0.003</td>
<td>0.68</td>
<td>-0.007</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>Monocyte α4 integrin (MFI)</td>
<td>-0.51</td>
<td></td>
<td>0.03</td>
<td>-0.22</td>
</tr>
<tr>
<td>Classical Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>-0.19</td>
<td></td>
<td>0.03</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>-1.14</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Intermediate Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>0.22</td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>1.36</td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>Non-Classical Monocytes</td>
<td>CD4+ T-cell activation</td>
<td>-0.026</td>
<td></td>
<td>0.93</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>-0.88</td>
<td></td>
<td>0.91</td>
<td>21.20</td>
</tr>
<tr>
<td>Monocyte α4 integrin (%)</td>
<td>CD4+ T-cell activation</td>
<td>-0.21</td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>-0.69</td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Monocyte α4 integrin (MFI)</td>
<td>CD4+ T-cell activation</td>
<td>0.005</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>CD8+ T-cell activation</td>
<td>0.018</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Monocyte α4 integrin (%)</td>
<td>Monocyte α4 integrin (MFI)</td>
<td>-13.47</td>
<td></td>
<td></td>
<td>0.031</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

α4        alpha 4 integrin
AHI       acute HIV infection
AIDS      acquired immune deficiency syndrome
ART       antiretroviral therapy
CHI       chronic HIV infection
CNS       central nervous system
CSF       cerebrospinal fluid
HAND      HIV-associated neurocognitive disorder
HIV       human immunodeficiency virus
MFI       mean fluorescence intensity
NFL       neurofilament light chain
PHI       primary HIV infection
PISCES    Primary Infection Stage CNS Events Study
SIV       simian immunodeficiency virus
TCR       T-cell receptor
WBC       white blood cell
REFERENCES


43. Sousa, A.E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z., and Victorino, R.M. 2002. CD4 T cell depletion is linked directly to immune activation in the


