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CD14+ Enriched Peripheral Cells Secrete Cytokines Unique to HIV-associated Neurocognitive Disorders

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Abstract

Monocytes play a vital role in HIV-associated neurocognitive disorder (HAND), postulated to transport HIV into the brain and secrete pro-inflammatory cytokines. We analyzed cytokines released by cultured peripheral blood mononuclear cells enriched with the CD14+ marker isolated from HIV-infected individuals with HAND and normal cognition (NC) in combination antiretroviral therapy (cART)-naïve and after one year on treatment. Interleukin-8 and monocyte chemoattractant protein-1 (MCP-1) levels were higher in HAND compared to NC at baseline ($p=0.002$ and $p<0.0001$). These cytokines remained higher in HAND patients one year after cART and was significant when NC patients who were initially HAND were excluded ($p=0.012$ and $p=0.002$). Both correlated with baseline CD14+ PBMC HIV DNA levels supporting the role of HIV DNA reservoir size and monocyte cytokines in HAND persistence.

Keywords

HIV; Neurocognitive; Cognition; Cytokines; DNA

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INTRODUCTION

Despite access to effective combination antiretroviral therapy (cART), HIV-associated neurocognitive disorder (HAND) continues to affect 35-50% of HIV-infected individuals. HIV-infected monocytes, particularly those with an activated phenotype are theorized to traverse the blood brain barrier, a means of HIV transport to the brain [1, 2]. It is thought that, once in the brain, they contribute to an inflammatory milieu that ultimately results in neuronal damage and contributes to HAND [3, 4]. These events are likely influenced by autocrine and paracrine responses of cytokines that are secreted by monocytes, resident macrophages and microglia. In turn, the cytokines attract more inflammatory cells including monocytes and activate macrophages and microglia to secrete detrimental cytokines. This cytokine overproduction causes neuronal damage and a loss of function.

The persistence of HIV is due to the establishment of viral reservoirs that may include lymphocytes, monocytes and macrophages. While T-cells may provide a significant contribution to viral reservoirs in general, monocyte and macrophage reservoirs are thought to be the primary contributors to HAND because they are the key cell types that are infected with HIV-1 in the central nervous system[1]. In previous studies, we quantified HIV DNA in CD14+ enriched PBMCs and have demonstrated a tight link to HAND [5-7]. We hypothesize, that the persistence of HAND is in part due to these reservoirs, which provide a constant source of inflammation through cytokine production.

Several cytokines such as IL-8 and IFN-γ have been implicated in the neuropathogenesis of HIV [8]. These studies typically analyze cytokines measured from plasma samples. In contrast, the current study investigated cytokines produced in cultures of CD14+ enriched PBMCs to more accurately investigate the role of monocyte secretory products and to provide a tighter link to concurrently measured HIV DNA levels. Since discordant results have been described with plasma cytokines [9], we further hypothesized that CD14+ PBMC supernatants may be a more reliable indicator of monocyte-related pathways. In this study, inflammatory cytokines that were shown to have involvement in HAND [10-16] were measured in supernatants from cultured peripheral monocytes by isolating CD14+ enriched PBMC. We previously reported that CD14+ PBMC subsets were higher in patients with HAND [17] and now demonstrated that supernatants from isolated CD14+ in culture have markers of immune activation in patients with HAND.

METHODS

Patient Cohort

Participants were enrolled in SEARCH011 (NCT00782808) and provided consent approved by the Institutional Review Boards (IRB) of UCSF, Chulalongkorn University, Phramongkutklao Hospital and the University of Hawaii. Selection criteria were previously reported [17]. Briefly, community physicians referred participants if they met the Thai Ministry of Public Health criteria for treatment that included CD4<350 cells/mm^3 or symptomatic disease. A total of 63 participants were enrolled but two were excluded at entry due to opportunistic CNS infections. Subjects started first-line cART typically with lamivudine (3TC) + nevirapine (NVP) + either stavudine (d4T) or zidovudine (ZDV) or

tenofovir (TDF). Participants intolerant to this regimen were changed based on clinical acumen, most commonly to efavirenz (EFV) for nevirapine complications.

CD14+ PBMC Isolation

Blood was collected at entry and one year after cART in ACD tubes and processed within four hours using a Ficoll Histopaque (Sigma, St Louis, MO) gradient to isolate PBMCs. CD14+ cells (monocytes) were separated from the PBMC using MACS magnetic bead positive selection kit (Miltenyi Biotec, City, ST) and frozen in 10% DMSO/FBS. Purity of CD14+ cells was measured by multi-parameter flow cytometry on every fifth sample for the first 42 cases and the median purity was determined to be 91.9% (min 76.9%; max: 98.7%) [17]. Purity data did not show any significant differences between HAND and NC groups, p = 0.48 (two sample Wilcoxon test). HAND: median (IQR) = 94.3 (89.8, 95.4), n = 12 vs. NC: median (IQR) = 94.0 (89.0, 97.2), n = 11.

Cytokine Measurement

CD14+ cells were initially isolated from PBMC and were stored at subzero for HIV DNA analyses at a later time point. Any available cells which were left over after the aliquot was frozen for HIV DNA testing were available for the cytokine tests. Some patients did not have enough cells to establish cell cultures for the cytokine analyses. Thus the number of tests performed varied depending on the available cells from patients. Isolated CD14+ PBMC were cultured in a 96-well plate overnight in RPMI1640 with 2% FBS and 1% pen/strep at 37°C. The plates were then centrifuged and the cell culture supernatants collected and stored at −80°C. The cells were resuspended and frozen in 10% DMSO/FBS. The chemokine and cytokines in the cell culture supernatants were analyzed using a custom 10-plex Milliplex MAP kit (EMD Millipore, Billerica, MA) that detected Fractalkine, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, IP-10, MCP-1, and TNF-α. Cell Culture supernatants and quality controls were prepared and run as described in the manufacturer's manual with one hour incubation with beads at room temperature and 30 minute incubation with detection antibodies at room temperature. A Luminex 100 system (Luminex, Austin, TX) was used to analyze the samples and cytokine concentrations in the samples were determined using a spline curve-fitting method.

HIV DNA Quantification

Frozen CD14+ PBMCs were thawed in 20%FBS/DMEM and DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) as per guidelines by the manufacturer and eluted in 20 uL TE buffer. HIV DNA copies per million were determined through the amplification of regions in the *gag* and *b-globin* genes as previously described with a limit of detection of 10 copies per million cells [5].

Statistics

Comparisons between cytokines produced from HAND and NC groups were examined by Wilcoxon rank-sum tests and the comparisons among multiple groups were evaluated by Kruskal-Wallis tests. CD14+ HIV DNA and cytokine association analyses were examined through nonparametric Spearman correlation. All statistical analyses were conducted in SAS

version 9.3 (SAS Institute, Cary, NC). A two-sided $p<0.05$ was regarded as statistically significant. Benjamini–Hochberg correction ($q<0.05$) was conducted to account for multiple testing.

RESULTS

Participants

The study group consisted of 61 HIV-infected Thais; 28 with an average age of 34.0 years were diagnosed with HAND and 33 with an average age of 35.3 years with NC at baseline (**Table S1**). After beginning cART, the cognitive status of 18 individuals improved from HAND to NC whereas none of the individuals with baseline NC developed HAND after cART. Three cases were not seen at follow-up resulting in a total of 58; 10 of which met HAND criteria and 48 who had NC [17].

Cytokine Analyses

Three of the measured chemokines and cytokines (IFN-γ, IL-2, IL-4) were excluded from analyses because greater than 50% of the samples were below the limit of detection. Of the seven chemokines and cytokines measured in the supernatants, only IL-8 and MCP-1 levels were significantly higher in HAND individuals compared to those with NC at baseline ($p=0.002$ and $p<0.0001$, respectively), **Figure 1a-1b, Supplement Table S2**. The levels of both chemokines remained higher in the supernatants in HAND individuals after one year of treatment; but, this only met our level of statistical significance for IL-8 **Figure 1c-1d**. Statistical significance was met for both IL-8 and MCP-1 when analyses exclude participants with NC at 12 months who initially were diagnosed with HAND, **Figure 1e-1f, Supplement Table S3**. Although treatment was associated with improved cognitive status for 18 HAND individuals who became NC, the levels of IL-8 and MCP-1 secreted by their monocytes were still higher than the individuals with NC ($p=0.012$ and $p=0.002$, respectively), **Figures 1e-1f**. IL-8 and MCP-1 supernatant cytokine levels did not correlate with plasma or CSF IL-8 ($r=0.039$, $p=0.775$ and $r=-0.226$, $p=0.178$ respectively) and MCP-1 cytokine levels ($r=0.115$, $p=0.402$ and $r=-0.005$, $p=0.976$ respectively), **Supplement Table S4.**

CD14+ enriched PBMC HIV DNA

At entry, HIV DNA levels positively correlated with MCP-1 in supernatants ($r=0.39$, $p=0.003$) and with IL-8 ($r=0.22$, $p=0.012$; correlation was still significant when corrected for multiple testing (Benjamini–Hochberg)), **Figures 2a-2b, Supplement Table S5**.

DISCUSSION

This study measured the differences in cytokine expression from isolated CD14+ enriched PBMC in HIV-infected HAND and NC individuals naïve to cART and after one year on treatment. Among cytokines in our panel, both IL-8 and MCP-1 were significantly associated with HAND at both pre- and post-cART time points. Although previous studies demonstrated the importance of MCP-1 in HAND [18, 19], emerging studies in recent years reveal IL-8 to be equally important [10, 15, 20]. They both have been reported to be higher in

HAND and remain high despite antiretroviral therapy [15, 20-22]. The uniqueness of our findings is that the cytokines were measured from the supernatants of CD14+ enriched isolated monocytes from patients, providing a link to monocyte-associated neuropathogenesis. Other studies reported cytokines in plasma or CSF, which likely represent total cytokines secreted from a broader array of cells as demonstrated by the fact that no correlations were found between the levels of IL-8 and MCP-1 in plasma or CSF. However, a limitation of this study is the monocyte purity which varied between 76.9-98.7%; therefore, contaminating cells such as lymphocytes could have contributed to some of the secreted cytokines that were measured.

Our work further enhances our understanding of neuropathogenesis in HIV because we also linked the cytokine production to the burden of HIV DNA in these cells. This finding buttresses existing reports linking monocytes to HAND and the hypothesis that intracellular CD14+ cellular reservoirs are important in HIV neuropathogenesis [1]. These results are consistent with data demonstrating MCP-1 and IL-8 linked to increased monocyte tethering to endothelial cells through binding of E-selectin with IL-8 triggering firm adhesion [23]. Such a mechanism would permit an excessive influx of monocytes disrupting the integrity of the blood brain barrier and making it more permeable.

Although cART improves cognition in most of our subjects with HAND, the IL-8 and MCP-1 levels secreted by monocytes remained higher than those who were initially diagnosed and remained NC. This supports the hypothesis that these individuals continue to experience inflammation.

In summary, these data demonstrate a link between CD14+ HIV DNA and cytokines tightly linked to monocytes and of importance to HIV neuropathogenesis. These cytokines are elevated in HAND even after one year on cART that was initiated during chronic infection with CD4<350 cells/mm^3. The fact that inflammatory cytokine levels do not return to baseline NC levels regardless of improved cognition; suggests that cART does not completely prevent inflammation and the presence of these monocyte viral reservoirs contribute to the persistence of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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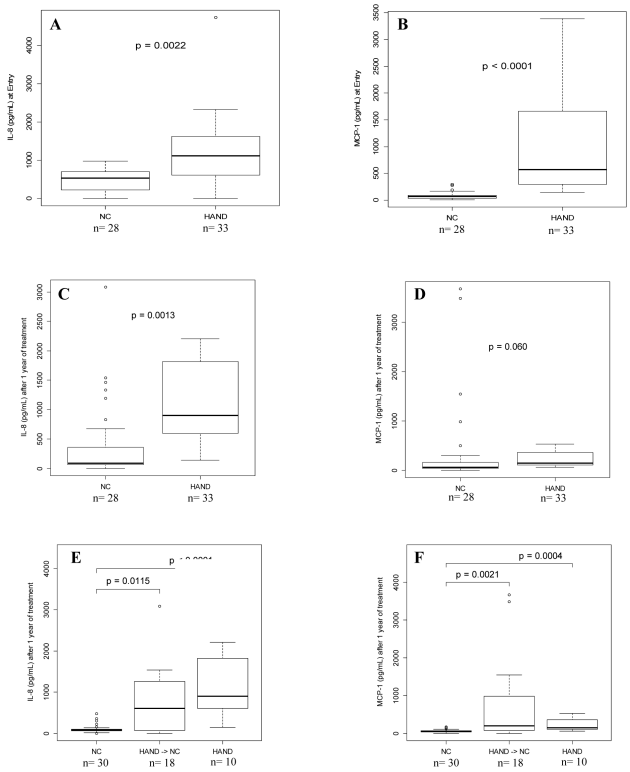

Figure 1.

A) Monocyte IL-8 secretions between HAND and NC at entry; B) Monocyte MCP-1 secretions between HAND and NC at entry; C) Monocyte IL-8 secretions between HAND and NC after one year of cART; D)Monocyte MCP-1 secretions between HAND and NC after one year of cART; E) Monocyte IL-8 secretions between HAND, NC, and HAND that become NC after one year of cART; F) Monocyte MCP-1 secretions between HAND, NC, and HAND that became NC.

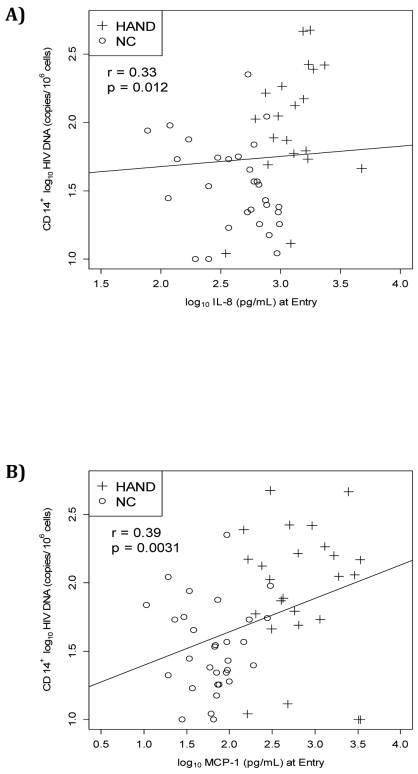

Figure 2.

A) Non-parametric Spearman correlation between HIV DNA and IL-8; B) Non-parametric Spearman correlation between HIV DNA and MCP-1.