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Peripheral biomarkers do not correlate with cognitive impairment in highly active antiretroviral therapy–treated subjects with human immunodeficiency virus type 1 infection

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Neuropsychological (NP) impairments in human immunodeficiency virus (HIV)-infected individuals remain high despite the introduction of highly active antiretroviral therapy (HAART). We sought to determine whether or not a monocyte gene expression profile along with other peripheral factors would correlate with neuropsychological impairment among HIV-infected individuals. Forty-four HIV-1–seropositive subjects (HIV+) on HAART and 11 HIV-1–seronegative controls (HIV−) had NP testing and blood drawn for monocyte gene expression analysis. All HIV+ subjects were assessed for CD4 counts, apolipoprotein E (ApoE) genotype, viral load, and plasma lipopolysaccharide (LPS) and soluble CD14 (sCD14). NP scores were normalized to age, gender, and education. Twenty-five percent of HIV+ individuals showed abnormal NP testing results (>1.5 SD below normal in two domains). HIV+ individuals had deficits in attention/working memory, verbal learning, and information processing speed compared to HIV− controls. There was no correlation between overall NP impairment and plasma viral load, level of education, age, ethnic diversity, sCD14, plasma LPS, CD4 cell count, ApoE genotype, or years of infection. However, greater years of infection had worse visual learning performance. sCD14 and CD4 nadir positively correlated with information processing speed and fine motor skills, respectively. LPS correlated with viral load but not cognitive impairment. Monocyte gene expression confirmed a chronic inflammatory profile that correlated with viral load but not cognition. No blood index or profile was associated with overall NP impairment. *Journal of NeuroVirology* (2010) **16,** 115–124.

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Introduction

Despite the ability to suppress viral replication with antiretroviral therapy, human immunodeficiency virus (HIV)-associated neurocognitive disorders (HAND) can still be detected in up to 30% of HIV-seropositive (HIV+) subjects (Cysique *et al*, 2006). Cognitive dysfunction before initiating or in the absence of antiretroviral treatment is primarily the result of neuronal dysfunction or neurotoxins associated with HIV-activated monocyte/macrophages (M/MΦ), whereas that in patients receiving antiretroviral therapy appears to be associated at least in part with inflammation driven by chronic low-level infection. Contributing factors may include genetic differences in HIV, human host genetics, and aging of patients (Clifford, 2008).

Monocyte/macrophages are one of the reservoirs of HIV in the periphery. HIV appears to enter the

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central nervous system (CNS) via infiltrating monocytes (Fischer-Smith *et al*, 2001; Liu *et al*, 2000) and when activated, M/MΦ release neurotoxins that initiate a neurodegenerative process that may end in neurocognitive impairment (Grant *et al*, 1987; Masliah *et al*, 1992). This may result in a variety of CNS dysfunctions. An updated diagnostic criterion has been published (Antinori *et al*, 2007) for HAND, which includes asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD). HAD and MND occur in 10% to 15% of chronically infected HIV individuals in the United States (McArthur, 2004; McArthur *et al*, 2005). Despite the introduction of highly active antiretroviral therapy (HAART), the prevalence of HIV cognitive impairment keeps increasing, as HIV-infected individuals are living longer (Grant *et al*, 2005; McArthur, 2004).

The sensitivity of neuropsychological (NP) testing to brain dysfunction is well established (Lezak, 2004). With HIV infection, the results of NP testing show robust associations with structural and functional brain imaging (Jernigan *et al*, 1993; Stout *et al*, 1998) as well as with postmortem neuropathology findings (Cherner *et al*, 2002; Everall *et al*, 1999; Masliah *et al*, 1997; Moore *et al*, 2006). In nontreated HIV+ nondemented individuals, HIV-related NP impairment was mild and often affected attention, speed of information processing, and learning efficiency (Heaton *et al*, 1995). A recent study showed that nontreated HAD individuals had NP impairments in a variety of domains (Valcour *et al*, 2007).

HAART has significantly decreased viral load in most HIV+ subjects; however, a significant number of individuals continue to have high viral loads (HVL; plasma HIV virus ≥10,000 RNA copies/ml) due to viral resistance or drug holidays. The need for a peripheral blood, laboratory-based biomarker for CNS functional impairment is still a high priority.

We hypothesized that neurocognitive impairment would correlate with high viral load, low CD4 count, high plasma sCD14 and lipopolysaccharide (LPS) levels, longer years of HIV infection, and certain gene expression patterns. We thus recruited HIV-infected individuals with high and low (LVL; plasma HIV virus <10,000 RNA copies/ml) viral loads and collected these data. It was our intent to correlate these data with NP performance with the goal of identifying peripheral biomarkers related to neurocognitive dysfunction.

Results

Participant's demographic, clinical, and laboratory data

A total of 55 subjects were included in this study. All participants were male, predominantly Caucasian (62%) and African American (22%), middle aged (50.9 ± 7.4 years old) and well educated (14.5 ± 2.1 years of education) (Table 1). The mean CD4 count was 491.3 ± 410.0/μl, with a log10-transformed mean plasma viral load of 3.63 ± 1.54 copies/ml. All participants were not current drug users or alcohol abusers and were on HAART or on a treatment interruption. HIV-seronegative (HIV−) subjects (n = 11) were slightly older (53 ± 4.2 years old) than HIV+ subjects (n = 44, age 50.3 ± 7.9 years old) but not significantly (P = .289). HIV− subjects had more education (15 ± 2.4 years of education) than HIV+ subjects (14.4 ± 2.1 years of education), but not significantly (P = .257).

Table 1 Demographic characteristics of age, education, and ethnic diversity

Items	*N*	Percent	Mean (± SD)
Age	55		50.9 (± 7.4)
Education	55		14.5 (± 2.1)
Ethnicity			
Caucasian	34	62	
African American	12	22	
Hispanic	5	9	
Asian	2	3.5	
Other	2	3.5	

Note. Age and education means are in years.

Subjects were also divided into three groups according to HIV serostatus and NP testing results: HIV− with normal NP (C; n = 11), HIV+ with normal NP (NPN; n = 27), HIV+ with impaired NP (NPI; n = 17) (Table 2). There was no significant difference between any two of the groups on age, ethnic diversity, years of education, and CD4 cell count. There was no significant difference within the NP groups between plasma viral load (log transformed) and years of infection. Viral load and CD4 count were calculated using Spearman's coefficient; rho = −.5932 (P = .00017).

NP data and correlations to demographic variables and other clinical parameters

Seventeen of 44 (39%) HIV+ individuals had abnormal NP testing results, with decreased scores in attention/working memory, information processing speed, and/or verbal learning/memory (Table 3). HIV+ older individuals demonstrated lower performance on measures of visual learning and attention/working memory. Higher education levels were associated with higher general IQ, attention/working memory, and information processing speed in HIV+ individuals (Table 4). Greater years with HIV infection correlated with lower performance in worse visual learning ability. Soluble CD14 and a low CD4 nadir positively correlated with symbol digit written performance and fine motor skills in the nondominant hand in HIV+ individuals, respectively.

To inspect possible medication interferences, we also collected HAART medication history at

Table 2 Descriptive statistics by neurological impairment

		HIV		
	Controls	NPN	NPI	*P*
N	11	27	17	
Age	53.0 (± 4.2)	49.3 (± 8.4)	52.0 (± 7.0)	.276
Education	15.0 (± 2.4)	14.7 (± 2.0)	13.9 (± 2.1)	.187
CD4 count	1037.8 (± 313.8)	342.9 (± 291.1)	373.3 (± 332.9)	.759
Viral load (log10)		3.43 (± 1.49)	3.94 (± 1.61)	.305
Years infection		16.0 (± 5.4)	18.4 (± 4.7)	.125
LPS (EU/ml)[§]	1.85 (± 0.65)	3.69 (± 1.97)	3.52 (± 1.56)	.783
sCD14 (μg/ml)[§]	1.66 (± 0.27)	2.38 (± 0.73)	2.61 (± 1.02)	.421
ApoE (%E4)	45.50%	26.90%	25.0%	1.000

Note. Values are presented as mean (± SD) except for ApoE. NPN = HIV+ NP normal; NPI = HIV+ NP impaired. NA = not applicable. *P* value stands for comparison of NPN with NPI using Student's *t* test, Wilcoxson's nonparametric test, or Fisher's exact test where appropriate. [§]$n = 9$ in controls, $n = 25$ in NPN, and $n = 14$ in NPI subject samples measured. ApoE E4 includes ApoE 3,4 and ApoE 4,4.

Table 3 Summary of neuropsychological tests

		HIV		*P* value	
Tests	Controls	NPN	NPI	HIV vs C	NPN vs NPI
General IQ					
WAIS-R III Information	13.2 (± 2.0)	12.1 (± 1.9)	11.2 (± 2.5)	.061	.178
Executive function					
WCST total errors	43.2 (± 9.1)	44.2 (± 10.6)	35.4 (± 11.2)	.528	.012*
WCST perservative	50.5 (± 16.3)	44.8 (± 10.3)	39.1 (± 10.4)	.055	.084
Stroop Word	51.0 (± 7.0)	53.0 (± 6.9)	47.7 (± 7.2)	.993	.018*
Attention/working memory					
WAIS-R III Digit Span	58.5 (± 8.3)	51.5 (± 9.5)	50.8 (± 6.2)	.012*	.802
Brown Peterson 18	58.2 (± 4.6)	45.1 (± 15.7)	36.1 (± 13.9)	.001*	.062
Brown Peterson 36	56.5 (± 9.7)	49.3 (± 12.4)	38.9 (± 15.9)	.020*	.019*
Information-processing speed					
Symbol Digit Oral	56.4 (± 12.4)	53.1 (± 9.6)	41.9 (± 8.6)	.047*	.000*
Symbol Digit Written	59.1 (± 10.4)	51.8 (± 9.2)	43.0 (± 9.9)	.003*	.005*
Stroop Color	48.4 (± 12.6)	47.6 (± 6.8)	39.8 (± 5.8)	.200	.000*
Fine motor skills					
GP Dominant Hand	62.5 (± 14.5)	56.7 (± 10.9)	48.4 (± 14.8)	.051	.040*
GP Non-Dominant Hand	60.7 (± 11.8)	57.4 (± 10.5)	51.4 (± 12.0)	.151	.084
Verbal Associate Fluency					
COWA Test	50.3 (± 8.5)	53.4 (± 12.5)	47.2 (± 9.2)	.846	.088
Verbal learning/memory					
CVLT total 1–5	61.4 (± 10.2)	53.9 (± 10.9)	48.2 (± 8.3)	.007*	.072
CVLT LDFR	59.1 (± 9.4)	52.6 (± 8.5)	47.9 (± 5.3)	.004*	.050*
Visual learning/memory					
BVMT trials 1–3	53.4 (± 10.4)	52.7 (± 8.7)	32.3 (± 10.3)	.057	.000*
BVMT delay	52.0 (± 9.5)	55.6 (± 5.5)	35.7 (± 15.4)	.368	.000*

Note. Average of scores is presented as mean (± SD). NPN = HIV+ NP normal; NPI = HIV+ NP impaired. All scores have been converted to *T*-score (mean = 50, SD = 10) except WAIS-R III Information (mean = 10, SD = 3). *$P < .05$.

blood-draw time and a 2-year period before that. All HIV+ subjects were on HAART or on treatment interruption. There was no difference found between NCI and NCN for the better, intermediate, or worse categories of the medicines (according to their CNS penetration; Koopmans *et al*, 2009) used either at blood-draw time or during the 2-year period before blood-draw. There was also no correlation between NCI and NCN for subjects on medication interruption.

Monocyte gene expression profiles

CD14+ monocytes isolated from 55 subjects were analyzed by cDNA microarrays. Seventy-eight genes showed 2-fold changes, with significant *t* test between the NP normal and NP impaired groups; however, no genes passed the Benjamini-Hochberg multiple-comparison correction. Gene classification analyses were also not able to find genes capable of differentiating the groups.

Table 4 Significant correlations of NP tests with other factors in HIV-infected subjects

Factors	Correlating NP tests	rho	*P* value
Age			
	Visual learning		
	BVMT trials 1–3	−.335	.026
	Attention/working memory		
	Brown Peterson 36	−.334	.027
Education			
	General IQ		
	WAIS-R III Information	.458	.000
	Attention/working memory		
	Brown Peterson 18	.275	.040
	Information-processing speed		
	Stroop Word	.299	.025
Years of infection			
	Visual learning		
	BVMT trials 1–3	−.312	.039
Soluble CD14			
	Information-processing speed		
	Symbol Digit Written	.321	.047
CD4 nadir			
	Fine motor skills		
	GP Non-Dominant Hand	.326	.031

Note. Spearman correlation test was used to test associations between factors and NP tests. Rho is the correlation coefficient of Spearman's test.

On the other hand, when comparing the groups based on viral loads, 99 differentially expressed (DE) genes were found between subjects with high versus low viral loads. Of the 99 genes, 55 have gene ontology (GO) annotations in the GO database (http://www.geneontology.org/). Gene ontology analyses show that nine categories were significantly enriched (Table 5A). Most of the genes are related to defense response, immune response, stress, and signal transduction. This confirms our previous report that chronic high HIV viral load affects the peripheral immune response (Pulliam *et al*, 2004). These genes have been implicated in chemotaxis (CCL2, CCR5, and DEFB1), inflammation (CCL2, CCR5, IP-10, and SN), neurotoxicity (IP-10), and interferon induction (IFI27, IFI44, and IFI44L) (Table 5B). These genes have been confirmed by polymerase chain reaction (PCR). Microarray data have been uploaded to NCBI Gene Expression Omnibus database and can be accessed by using accession number GSE18464.

Table 5A Selected GO for DE genes from comparison of HVL with LVL

GO category	Number of genes	*P* value
Defense response	49	.000
Immune response	46	.000
Signal transduction	39	.034
Response to Stress	37	.000
Apoptosis	14	.007
Cell proliferation	12	.038
Inflammatory response	11	.000
Chemotaxis	10	.000
Cell migration	7	.007

NP impairment and ApoE allele frequency

Apolipoprotein E (ApoE) genotyping was performed on 53 of the 55 samples. In the 53 genotyped subjects, 15 (28.3%) were ApoE 3,4; 1 (1.9%) was ApoE 4,4; and 1 (1.9%) was ApoE 2,4. In all of the 106 alleles, 13.2% were ApoE ϵ2; 69.8% were ApoE ϵ3; and 17% were ApoE ϵ4. The percentages of ApoE genotypes in our cohort are within normal ranges. Our cohort had slightly higher ϵ2 and ϵ4 allele percentages than found in a normal population, which are 5% to 10% and 10% to 15%, respectively (Hill *et al*, 2007). In HIV+ subjects, ApoE 3,4 or 4,4 were found in 7 out of 26 ApoE-genotyped NP normal subjects and 4 out of 16 ApoE-genotyped NP impaired subjects. ApoE genotyping analysis showed no difference in the NP impaired group compared to the NP normal subjects (Fisher's exact test, $P = 1.000$). No differences in ApoE genotypes were found between the HVL and LVL groups.

NP impairment, viral load, sCD14 and LPS levels

Of the subjects measured for LPS, HIV+ (N = 38) subjects (3.63 ± 1.82 EU/ml) had significantly higher plasma LPS than controls (n = 11, 1.85 ± 0.65 EU/ml; $P = .006$); sCD14 in HIV+ subjects (2.47 ± 0.84 μg/ml) was also significantly higher than controls (1.66 ± 0.27 μg/ml; $P = .007$). There was no difference

Table 5B Selected differentially expressed genes by microarray analysis

Symbol	Name	GenBank	Fold change					
			NPI/NPN	NPI/C	NPN/C	HVL/C	HVL/LVL	LVL/C
CCL2	MCP-1	NM_002982	0.8	2.8*	3.5	5.4*	5.2*	1.0
CCR5	Chemokine receptor	BC038398	1.0	1.8	1.8	2.4*	2.0*	1.2
SN	Sialoadhesin	NM_023068	0.8	7.5*	9.4*	15.2*	7.1*	2.2
DEFB1	Beta 1 defensin	NM_005218	2.1	5.8	2.7*	6.5*	4.8*	1.3
IP-10	CXCL10	NM_001565	0.9	2.7*	2.9*	4.0*	2.4*	1.7*
IFI27	Interferon, alpha-inducible protein 27	NM_005532	1.5	63.0*	41.8*	88.5*	7.7*	11.4
IFI44	Interferon-induced protein 44	NM_006417	1.0	2.9*	2.9*	4.3*	2.8*	1.5*
IFI44L	Interferon-induced protein 44-like	NM_006820	1.1	5.6*	5.3*	8.9*	4.4*	2.0

Note. C = HIV − control; NPN = HIV+ NP normal; NPI = HIV+ NP impaired. HVL = HIV+ high viral load (≥10,000 copies/ml), LVL = HIV+ low viral load (<10,000 copies/ml). *P <.05.

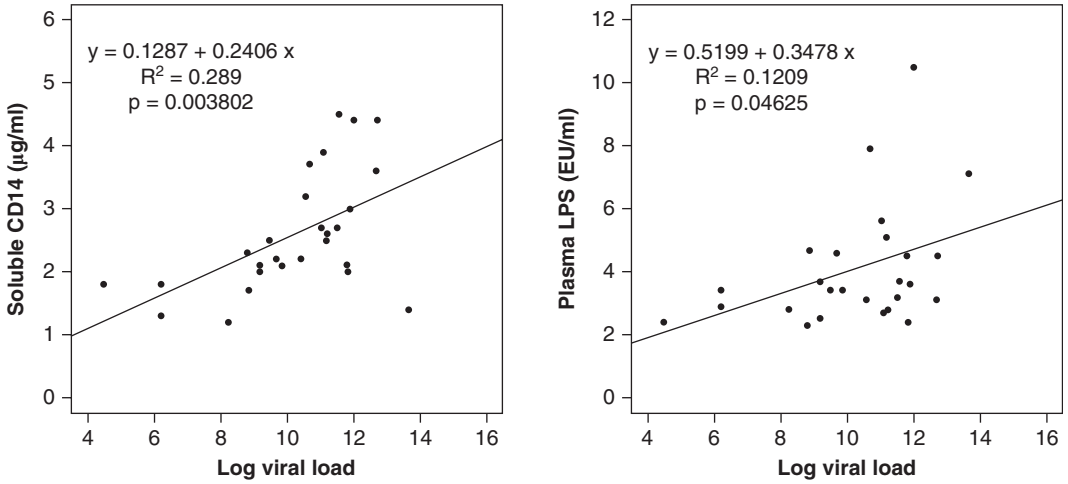

Figure 1 Linear regression of viral load (log) and plasma sCD14 or LPS. The correlations are mild but significant. Spearman's rank correlation was used.

between the NPI and NPN groups in sCD14 or LPS (Table 2). Plasma sCD14 and LPS levels in HIV+ subjects correlated with plasma viral load significantly (Figure 1).

Discussion

Although frank HIV dementia is now rare in HAART-treated HIV-infected subjects, cognitive impairment is still prevalent. In one recent study, 34% of HIV+ individuals demonstrated cognitive decline during HAART treatment with undetectable viral load (Cysique *et al*, 2006). In another study, 21% of HIV+ subjects developed NP impairment while on HAART treatment with viral load suppression (Robertson *et al*, 2007). In a large cohort study, about 1.44% (222 of 15380) patients developed HAD with undetectable viral load (Bhaskaran *et al*, 2008). Thus, there is consistent evidence that NP impairment in HIV patients can occur despite effective viral suppression.

Our initial hypothesis was that HIV+ individuals with high viral load would have a monocyte profile that would correlate with neurocognitive dysfunction. Before HAART, several reports pointed to neurotoxic soluble factors produced from activated M/MΦ of HIV+ individuals, including tumor necrosis factor alpha (α) (Giulian *et al*, 1990; Pulliam *et al*, 1997). With the introduction of HAART, the M/MΦ does not have a proinflammatory phenotype; however, there are still elevated genes present that are associated with chronic inflammation and distinct differences in the phenotype based on viral load (Pulliam *et al*, 2004). In this cohort, M/MΦ from HIV+ individuals with HVL up-regulated CCL2, CCR5, SN, β-defensin, interferon-inducicble protein (IP)-10, and several interferon-induced genes. Monocytes from individuals with LVL had a pattern similar to uninfected subjects except for an increase in IP-10 and interferon gene IFI44. This profile has remained consistent with our previous findings 5 years ago (Pulliam *et al*, 2004).

HAART can arrest and to some extent reverse CNS dysfunction (Navia and Price, 2005), improve NP (Sacktor *et al*, 2006) and neurological performance, and decrease neurological complications

(Sacktor *et al*, 2003; Schmitt *et al*, 1988). The HIV+ cohort that we studied was either on HAART or on a treatment interruption. The frequency of NP impairment in HIV+ HAART-treated subjects was higher (39%) compared to previous reports (20%) (Grant *et al*, 2005). When examining individual tests, we found that HIV infection was associated with lower attention/working memory, information processing speed, and verbal learning. These results are consistent with previously published data that in the pre-HAART era, attention and learning were the most impaired functions in HIV-infected subjects (Grant, 2008; Heaton *et al*, 1995). These domains remain the most affected ones in the HAART era. The combination of antiviral drugs used in our HIV+ cohort was quite diverse. Thus, we were not able to determine individual antiviral effects on NP performance. However, we grouped drugs used according to their CNS penetration ability (Koopmans *et al*, 2009), and found no correlation between drugs that penetrate the CNS and neuropsychological evaluations.

When exploring potential risk factors such as CD4 and viral load with individual NP tests, we found, relatively weak but statistically significant, correlations between nadir CD4 and NP results (manual dexterity and speed). A study from Nigeria showed that a low CD4 count was associated with memory impairment and worse nondominant hand fine motor skills (Odiase *et al*, 2007). The Grooved Pegboard challenge, which was used to detect this effect in our study is sensitive to basal ganglia dysfunction. Recent studies have shown that CNS injury markers, such as gliosis, are more evident in basal ganglia (Paul *et al*, 2007). Actually, low CD4 nadir and high viral load have been shown associated with parenchymal brain damage (Everall *et al*, 2009). We also found longer HIV infection seemed to negatively impact visual learning performance. Although our cohort is from the United States, we did not test for clade diversity of the HIV strains. Results from international studies suggest that severity of NP impairment is dependent on HIV clade differences (Clifford *et al*, 2007; Gopukumar *et al*, 2008).

In this study, sCD14 and LPS weakly correlated with viral load. Likewise, the elevation of the M/MΦ genes CCL2, CCR5, SN, DEFB1 and alpha interferon–associated genes correlated with an increase in sCD14 or LPS which are all related to increased viral load. A previous report showed an increase in LPS in subjects with HAD and an increase in sCD14 in neurocognitively impaired AIDS patients, and in comorbidities such as substance abuse (Ancuta *et al*, 2008; Ferrier *et al*, 2006) and hepatitis C virus coinfection (Ancuta *et al*, 2008; Caradonna *et al*, 2002; Dolganiuc *et al*, 2007). Our cohort had no subjects with HAD and had lower viral loads than previous cohorts. This may partially explain why elevated LPS was not found to correlate with NP tests in the present study. Elevated sCD14 was found to correlate positively with information processing speed in HIV+ individuals (Table 4); however, absolute levels of sCD14 from NPN and NPI subjects did not differ (Table 2). This finding needs to be confirmed in a larger cohort, since the sample size is limited and the cross-sectional characteristics of this study. Because this is an exploratory study, the *P* values are reported without any correction for multiple comparisons and therefore warrant independent verification. However, we note that 8 out of 45 correlations were significant at the 5% level, which is 6 more than that expected by chance. In previous studies, LPS, together with M/MΦ activation markers sCD14, CCL2, and interleukin (IL)-6, was associated with neurocognitive impairment and HAD (Ancuta *et al*, 2008; Ryan *et al*, 2001). These studies involved subjects in late-stage disease or with coinfections. Individuals with HIV-1 have activated M/MΦ with elevated levels of LPS; however, whatever M/MΦ activation product(s) were present before HAART are being suppressed because the number of subjects with HIV dementia is considerably decreased. We did not see an increase in monocyte IL-6, CD16, CD69, or TNFα gene expression in subjects with HVL HIV, which in the past may have significantly contributed to neurocognitive impairment (Pulliam *et al*, 1997).

In summary, we did not find a distinct monocyte phenotype associated with cognitive impairment. Although subjects with HVL had monocytes that upregulated several genes, including the chemokine CCL2, these subjects did not have cognitive impairment, re-enforcing the notion that other factors cause neurotoxicity. Previous studies have linked M/MΦ activation with an increase in CCL2 as well as a positive correlation with LPS (Ancuta *et al*, 2008); our results suggest that a lack of proinflammatory monocyte activation even with increased CCL2, and LPS did not correlate with dementia or neurocognitive dysfunction. Although this is a small study, it is exhaustive in looking at peripheral blood markers. We conclude that HIV+ subjects on HAART with cognitive impairment do not have an associated peripheral blood profile using the variables we studied.

Methods

Participants

Forty-four male HIV+ and 11 HIV− controls, matched for age, gender, education, and social position, were recruited from clinics at the San Francisco Veterans Affairs Medical Center (San Francisco, CA) through nurse recruiters, advertisement, and word-of-mouth. All participants in the study gave written consent in accordance with the Committee on Human Research of the University of California, San Francisco. Major exclusion criteria included any acute illness at the time of the blood draw or history of head injury, seizures, multiple sclerosis, active opportunistic infection including HCV,

lymphoma, cerebrovascular disease, major psychiatric illness, drug or alcohol abuse, or existing cause of brain disorder. Thirty milliliters of whole blood were collected as previously described (Pulliam *et al*, 2004). Twenty-two low–viral load (LVL; plasma HIV virus <10,000 copies/ml) and 22 high–viral load (HVL; plasma HIV virus ≥10,000 copies/ml) subjects were recruited. Subjects were aged 33 to 59 years old.

Neuropsychological testing

All subjects were tested for neuropsychological performance at the time of the blood draw or within 2 to 3 days. Neuropsychological testing was performed by the same trained psychologist (L.A.). Subjects were interviewed using the Structured Clinical Interview for DSM-IV Diagnosis (SCID) (First *et al*, 1997) to establish lifetime history of depressive illness and to diagnose exclusionary psychiatric disorders, including current major depressive disorder. Subjects completed a comprehensive neuropsychological battery that assessed seven broad domains of cognitive-motor functioning. The Information subtest for the Wechsler Adult Intelligence Scale—Third Edition (Wechsler, 1997) was used as an estimate of general intellectual functioning. The test battery included verbal associative fluency (Controlled Oral Word Association Test [Benton *et al*, 1983]); executive function (Stroop Color and Word Test [Golden, 1978], Computerized Wisconsin Card Sorting Test [Heaton *et al*, 1993]); speed of information processing (Symbol Digit Modalities Test [Smith, 1982]), Stroop Color and Word Test [Golden, 1978]); attention/working memory (Wechsler Adult Intelligence Scale-Digit Span [Wechsler, 1997], Brown Peterson Auditory Consonant Trigrams [Struss *et al*, 1987]); verbal learning and memory (California Verbal Learning Test Trials 1–5 total recall and long-delay free recall [Delis *et al*, 1987]); visual learning and memory (Brief Visuospatial Memory Test—Revised Trials 1–3 total recall and delayed recall [Benedict, 1997]); and motor speed (Grooved Pegboard Test [Heaton *et al*, 2004]). Neuropsychological evaluation results were adjusted according to educational level, gender, and age. Raw scores were transformed to age and (where applicable) education-corrected standard scores. Cognitive impairment was categorized according to scores >1.5 SD below the norm in at least two cognitive domains. One-way analysis of variance (ANOVA) was used to compare among the groups and nonparametric statistical analyses (chi-square test, Wilcoxson's rank sum test, and Fisher's exact test) were used to compare rates of significant cognitive impairment across groups. Levels of impairment between the seven cognitive domains were also compared.

Monocyte gene microarrays and quantitative real-time reverse transcription PCR

CD14+ monocytes were analyzed for differential gene expression using microarray as previously described (Pulliam *et al*, 2004). Briefly, whole blood was collected using CPT tubes (BD, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMCs) were enriched by centrifugation. CD14+ monocytes were isolated using magnetic beads conjugated with anti-CD14 antibody (Miltenyi Biotech, Auburn, CA). Total RNA was isolated from monocytes using the RNeasy Micro Kit (Qiagen, Valencia, CA). The integrity of RNA was evaluated on the Agilent Bioanalyzer 2100 using a RNA 6000 Pico LabChip (Agilent Technologies, Palo Alto, CA). Complementary RNA (cRNA) was synthesized and labeled with biotin using iExpress iAmplify kit (Applied Microarrays, Tempe, AZ). cRNA were then hybridized to Codelink Whole Human Genome Bioarrays (Applied Microarrays). The slide was scanned and the image was analyzed using Codelink Expression Analysis software (Applied Microarrays).

Validation of microarray gene expression was performed using quantitative real-time reverse transcription PCR (ABI, Applied Biosystems, Foster City, CA) protocols as previously described (Pulliam *et al*, 2004). Relative expression of target RNA was calculated in relation to glutaldehyde 3-phosphate dehydrogenase (GAPDH).

ApoE genotyping

To identify the ApoE genotype, we applied a previously described method involving amplification of the target sequence by PCR and endonuclease HhaI digestion (Hixson and Vernier, 1990). Chromosome DNA was isolated from PBMCs using DNeasy Tissue Kit (Qiagen, Valencia, CA), yielding 200 μl of purified DNA with an average concentration of 0.2 mg/ml. For the PCR reaction, 50 ng of template DNA was diluted into a 50-μl reaction volume that included the primers F4 (5′-ACAGAATTCGCCCCGGCCTGGTACAC-3′) and F6 (5′-TAAGCTTGGCACGGCTGTCCAAGGA-3′) at a final concentration of 400 nM, 200 μM for each dNTP, 1.5 mM $MgCl_2$, and 2.5 units of Platinum Taq polymerase (Invitrogen). The amplified PCR product was digested (10:1) with endonuclease HhaI (Invitrogen) at 37°C for 1 h. Digested samples were loaded onto a DNA 500 LabChip and then fractionated using a 2100 Bioanalyser (Agilent). Genotype was determined from the resulting fragmentation pattern.

Plasma LPS and sCD14

LPS levels in subject plasma were quantified using the Pyrogene Recombinant Factor C Endotoxin Detection System (Lonza, Walkersville, MD) according to manufacturer's protocol. Briefly, plasma samples were diluted 1:50 in pyrogen-free water before incubation with rFC working reagent. Fluorescence was measured at time 0 and then again at 1 h with a SpectroMax microplate reader. Endotoxin concentrations in plasma samples were extrapolated from a standard curve. Soluble CD14 levels in plasma samples were measured using Quantikine

Human sCD14 Immunoassay (R&D Systems, Minneapolis, MD) according to the manufacturer's protocol.

Statistical analysis

NP testing analyses were discussed in the relative method section. Quantitative reverse transcriptase–PCR (RT-PCR) analysis was run in triplicate. Statistical analyses and significance were determined by Student *t* test or analysis of variance (ANOVA) using a Tukey post hoc test in SPSS software package. Microarray data were normalized with loess normalization using R (Ihaka and Gentleman, 1996) and Bioconductor package (Gentleman *et al*, 2004). Differential gene expression significance and Benjamini and Hochberg false discovery rate multiple testing correction was determined using GeneSpring GX 7.3 software package (Agilent). Classification and regression analyses of genes using random forests, prediction analysis for microarrays (pamr) and support vector machine (svm) were accomplished under R using appropriate packages. We initially hypothesized that neurocognitive impairment may correlate to high viral load, low CD4 count, low nadir CD4 count, and high sCD14 and LPS levels. Thus we chose Spearman's rank correlation to test these hypotheses in R. Medication history contingency tables were tested using χ^2 in R.

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