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Transcriptome analyses identify key cellular factors associated with HIV-1 associated neuropathogenesis in infected men

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Abstract

Objective—HIV-1 viral proteins and host inflammatory factors have a direct role in neuronal toxicity in *in vitro*, however, the contribution of these factors *in vivo* in HIV-1 associated neurocognitive disorder (HAND) is not fully understood. We applied novel Systems Biology approaches to identify specific cellular and viral factors and their related pathways that are associated with different stages of HAND.

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Conceived and designed the experiments: VA, NJV, LW, SJ, SBM, ZBJ. Performed the experiments: NJV, LW, SBM. Analyzed the data: VA, NJV, SJ, ZBJ, LW, AC. Wrote the paper: VA, NJV, SJ, ZBJ, CR, AR, ES, AL, JB, EM, NS. All authors read and approved the final manuscript.

Design—A cross-sectional study of individuals enrolled in the Multicenter AIDS Cohort Study (MACS) including HIV-1 seronegative (N=36) and HIV-1 seropositive individuals without neurocognitive symptoms (N=16), or with mild neurocognitive disorder (MND) (N=8) or HIV-associated dementia (HAD) (N=16).

Methods—A systematic evaluation of global transcriptome of peripheral blood mononuclear cells (PBMCs) obtained from HIV-1 seronegative individuals and from HIV-1 positive men without neurocognitive symptoms, or MND or HAD was performed.

Results—MND and HAD were associated with specific changes in mRNA transcripts and miRNAs in PBMCs. Comparison of upstream regulators and TimePath analyses identified specific cellular factors associated with MND and HAD, while HIV-1 viral proteins played a greater role in HAD. Additionally, expression of specific microRNAs – miR-let-7a, miR-124, miR-15a and others were found to correlate with mRNA gene expression and may have a potential protective role in asymptomatic HIV-1 seropositive individuals by regulating cellular signal transduction pathways downstream of chemokines and cytokines.

Conclusions—These results identify signature transcriptome changes in PBMCs associated with stages of HAND and shed light on the potential contribution of host cellular factors and viral proteins in HAND development.

Keywords

HIV-1 associated neurocognitive disorder; host factors; mild neurocognitive disorder; HIV-1 associated Dementia; Systems biology

Introduction

HIV-1 associated neurocognitive disorder (HAND) is one of the major co-morbidities of HIV-1 infection. HAND includes a spectrum of clinical manifestations associated with cognitive and behavioral impairments, based on increasing severity is classified as asymptomatic neurocognitive disorder (ANI), mild neurocognitive disorder (MND) and HIV-associated dementia (HAD) [1]. These clinical manifestations are the consequence of progressive loss of neurocognitive function [2]. Nearly half of the HIV-1 infected population is known to have some degree of HAND [3, 4] and understanding how HIV-1 contributes to neuronal dysfunction remains a priority. HIV-1 infiltrates the central nervous system (CNS) early in the infection crossing the blood brain barrier via a “Trojan horse,” mechanism involving infected monocytes/macrophages [5, 6]. This enables infection of other CNS resident monocytic cells including microglia and perivascular macrophages [7–9]. Infiltration of monocytes into brain and associated inflammatory and metabolic toxicity is the hallmark of HAND that results in neuronal degeneration, apoptosis and associated changes [10]. HAART has drastically reduced dementia and brain pathology, however the prevalence of ANI and MND remains high in HIV-1 positive individuals [3, 4]. The factors contributing to neuropathogenesis and/or HAND are not fully characterized. While neurons are not primary target cells of HIV-1 infection, they express HIV-1 coreceptors (CCR5, CXCR4). HIV-1 viral proteins (such as Tat, Vpr and Env-gp120) and inflammatory factors (TNF- α , IL-8, IL-1 β , IL-6 and others) contribute to neuronal toxicity from Ca⁺⁺ reflux, nitric oxide production, and excitotoxicity [11–15]. *In vitro* studies have identified the

neurotoxic effects of viral proteins, inflammatory and metabolic agents; however, critical factors contributing to neuropathogenesis *in vivo* are not completely understood. Polymorphism in TNF- α gene that results in increased secretion in response to bacterial lipopolysaccharide has been observed with increased frequency in HIV-1 seropositive individuals with dementia [16], whereas, mutation in CCL2 (at position 2578G), CCR5 32 and other host factors have been identified as neuroprotective [1, 17–19]. While recent work has studied the expression of microRNAs (miRNA) in HAND response [20–24] relatively little is known about their regulatory role and their targets.

Neuropathogenesis is determined by both neuroprotective and neurotoxic factors from infiltrating immune cells from the periphery, as well as resident microglial cells, astrocytes, neural support cells, and cells of the blood brain barrier. Diverse viral quasispecies in infected individuals also play a role [25, 26]. Thus, outcome reflects numerous host and viral factors that interact with their cellular partners in multiple cell types. Most molecular interactions, especially those regulating basic cellular functions, are well conserved across multiple cell types; however, the functional effect is determined by epigenetic changes associated with different cell types. Therefore, it is plausible that neuroregulatory factors involved in HAND pathogenesis may be observed in immune cells of the peripheral blood compartment, as previously reported [27, 28]. Exposure of cells to neurotoxic or neuroprotective factors may result in transcriptome changes that include both short term and long-term changes. Here, we performed global transcriptome analyses of peripheral blood mononuclear cells (PBMCs) obtained from HIV-1 seropositive individuals without clinical neurocognitive symptoms on standard neuropsychological testing, from those identified as MND, and as HAD and from HIV-1 seronegative controls. Based on the results, we have defined the transcriptome changes in PBMCs associated with different stages of HAND and identified the potential contribution of host cellular factors and viral proteins in regulating HAND development.

Results

Comparative analysis of upstream regulators in HIV-1 seropositive individuals without HAND and those with MND and HAD identified both neurotoxic and neuroprotective factors associated with different stages of HAND

To characterize factors associated with HIV-1 seropositive individuals with different stages of HAND, we compared transcriptome profiles of HIV-1 seronegative individuals with HIV-1 seropositive individuals with no clinically identifiable HAND symptoms or with MND or HAD. Only the genes consistently detected ($p < 0.01$) in the groups compared were included in the analyses. Comparison of the differentially regulated genes among the three HIV-1 seropositive groups (no HAND, MND and HAD) relative to HIV-1 seronegative genes identified specific changes in mRNA transcripts in PBMCs (Fig. 1A–D) (Fig S1, Table S1, <http://links.lww.com/QAD/B34>). Ingenuity Pathway Analysis (IPA) based comparison of upstream regulators including genes unique for the HAND groups, suggest that multiple factors are regulated in opposite direction in HIV-1 seropositive who do not have HAND and those who have MND and HAD.

Results presented in Figure 1E indicate that for HIV-1 positive individuals with MND or HAD - IFN γ , TNF- α , IL2, CSF2, IL27, IL1 β , CD40LG, IL15, IFNA2, TGF β 1 and others cytokines are expressed at higher levels when compared to HIV-1 seronegative individuals. In addition, the activity of cytokines including TNF- α , IL2, CSF2, IL1 β , IL15, IFNA2, TNFSF10/11 and IL18 progressively increases with more severe forms of HAND (Z score increases >40%). In contrast, the activity of IFN γ , IL27, CD40LG, KITLG and IL6 remain unchanged with the two different stages of HAND (change in Z score <40%). Interestingly, increased activity of LIF is observed only during MND and is not identified in HAD. TIMP1, which encodes for tissue inhibitor of metalloproteinases is predicted to be inhibited in HAD positive individuals. Activity of Prolactin was observed to be reduced (29% reduction in Z-score) in HAD relative to MND. Specific factors such as IL5, IL17A, CXCL2, AIMP1, IL1 α , CCL2, IL12B, PF4, and IL18 are predicted to be active only during HAD. Conversely reduced activity of these cytokines and chemokines is associated with the HIV-1 seropositive individuals who never developed clinical HAND.

Similar analyses of kinases, phosphatases and associated signaling factors suggest that in individuals without HAND, EIF2AK3 and EIF2AK2 kinases exhibit reduced activity, while MAP4K4 has increased activity, similarly phosphatase SOCS3 and DUSP1 also had increased activity (Fig. 1F). In individuals with MND, EIF2AK3 is activated but MAP4K4 and SOCS3 are reduced. In individuals with HAD, EIF2AK2 is active to a greater extent than in MND. Similarly, the activity of the TFs – MYC, RELA, STAT1, XBP1, NFATC2 and IRF5 increases with the severity of HAND, while the activity of CEBPA was reduced (more than 40% change in Z-score between MND and HAD). The activity of ZFP36 and SMAD7 are inhibited in HAD, whereas, the activity of TFs- STAT3, IRF7, IRF3, IRF1, SREBF1, TRIM24, CNOT7 and NKX2–3 did not change between MND and HAD individuals (Fig. 1G). Together, these results identify multiple specific factors including cytokines, transmembrane proteins, receptors and associated transcription factors as the critical upstream regulators of genes that are associated with different stages of HAND.

mRNA and miRNA co-expression validates the upstream analysis and identifies neuroprotective miRNAs

MicroRNAs along with other host cellular molecules including TFs and associated signaling molecules, regulate host gene expression. Next, we evaluated the changes in miRNAs expression level, and also analyzed the correlation between the expression of miRNAs and their predicted mRNA targets across the groups being compared. MiRNAs that were detected with CT value less than 36 cycles in at least 75% of the individuals within the group were included in the analysis. Comparison identified 74 miRNAs that were down regulated in HAD relative to HIV-1 seropositive group with no HAND, of which, 37 miRNAs decreased in HAD but increased in no HAND relative to HIV-1 seronegative. Five miRNAs decreased to a greater extent in HAND than in HAND negative group. Whereas, 32 miRNAs increased in both the HAND negative and HAD groups with the increase in HAND negative group greater than HAD group leading to an apparent down regulation of miRNAs in HAND (Fig. S2, Table S4, S5, <http://links.lww.com/QAD/B34>).

Correlating miRNA and mRNA expression data between HIV-1 seropositive individuals with no neurocognitive disorder and with HAD, identified 15 miRNAs that are both significantly dysregulated and regulate gene targets that are also significantly altered (Fig. 2A, Table S6, <http://links.lww.com/QAD/B34>). MiR-15a had 17 mRNA targets and miR-124-3p targeted 14 mRNAs. MiRNAs including miR-210, miR-124-3p, and let-7a-5p that were predicted as upstream regulators in HAD by IPA (Fig. 2B), were also validated as differentially dysregulated in our transcriptome analyses. Evaluating the association of the miRNAs and their gene targets with biological function identified their potential role in inhibiting chemokine signaling specifically IL8, CXCR4 and Integrin (Fig. 2C). Similarly, correlative analysis of mRNA:miRNA expression in HIV-1 seropositive individuals in comparison to HIV-1 seronegative individuals identified 21 miRNAs targeting 98 mRNAs in the no HAND group; while 4 miRNAs targeting 10 mRNA and 5 miRNAs targeting 30 mRNAs were found in MND and HAD groups. Further analysis of miRNA gene target with biological function determined that miRNAs upregulated in HAND negative group are associated with multiple gene targets downstream of CSF2, IL5, RELA, p38 MAPK, IL3, IL1B, TLR4, CXCR4, and IL8 signaling (Fig. 2D, Table S7, <http://links.lww.com/QAD/B34>), suggesting that the miRNAs upregulated in HIV-1 seropositive patients who do not develop HAND can potentially neutralize the downstream effect of many of these neuroinflammatory factors associated with HAND pathogenesis. In summary, these results suggest that certain miRNAs have a protective role in HAND where the upregulation of specific set of miRNAs is associated with reduced HAND pathogenesis.

HIV-1 viral proteins play a major role in modulating the transcriptome changes associated with HAD

We next explored the contribution of HIV-1 viral proteins using TimePath analysis [29]. Results (Figure S4, <http://links.lww.com/QAD/B34>) identified CCND3, CDK4, CCND1, ESR1 and RB1 as the top 5 regulators of the transcriptome changes observed in MND (Table S8, <http://links.lww.com/QAD/B34>). It can also be noted that HIV-1 Env is ranked higher than the other viral proteins at rank 26, with Gag-pol at 33 and Rev at 37. Similarly analyses of the HAD stage, with the restriction to include the cellular networks associated with HIV-1 seropositive group and MND, shows that the viral proteins are ranked relatively high (between ranks 20–39), suggesting that the viral proteins and/or virus infection may play a major role in progression of disease from MND and HAD. Other proteins that ranked high include the host protein CD4, which is the main receptor of HIV-1 virus along with transcription factors including TP53, EP300, RELA, RB1, and ESR1, which are known to regulate virus replication, further strengthening the association of virus replication/infection with HAD (Figure 3). Additionally specific HIV-1 viral proteins were identified to regulate pathways: TRAF→CD40→RNF31, CREBBP→SREBF1→MYH9, CEBPB/SUMO1→HSF1→HSPH1 (Table S9, <http://links.lww.com/QAD/B34>), which have been previously identified to regulate monocyte/macrophage chemotaxis, inflammation and regulation of intracellular signaling, these were identified during HAD. Interestingly, other significant pathways (Table S9, <http://links.lww.com/QAD/B34>) regulated by HIV-1 viral proteins, especially those regulating NRG1 and CIRBP were identified in patients who did not have HAND symptoms while the rest of the other significant pathways were enriched in HAD (Table S9, <http://links.lww.com/QAD/B34>), suggesting that some of the early

molecular events associated with neurological pathogenesis caused due to HIV-1 viral proteins are observed in PBMC in the absence of any HAND symptoms. The HIV-1 proteins regulating these pathways in HAD were due to Nef, Vpu and Env, while the changes in NRG1 and CIRBP in HIV seropositive subjects with no HAND can be attributed to Tat, Vpr, Vif, Vif, Nef and Gag-Pol.

Discussion

Identifying host and viral factors that influence neurological progression is critical to minimize neurocognitive morbidity. The balance of neurotoxic and neuroprotective factors secreted by brain microvascular endothelial cells, pericytes, astrocytes, neurons and associated neural support cells determine the severity of pathology. HIV-1 viral proteins also directly affect metabolism, function and survival of these cells, but host determinants such as polymorphism and genetic allele variations have also been identified as relevant to individual differences in risk of neurocognitive impairment [1, 16]. As shown in Figure 4, mononuclear cells in brain microcapillaries and veins are also exposed to viral proteins and to host derived neurotoxic and neuroprotective factors. The mononuclear cells in the microvasculature are continuous with the peripheral blood compartment, thus evaluating the transcriptome changes in the peripheral blood mononuclear cells will provide an indication of neuronal insult. While systematic analysis can reflect the factors influencing these changes, the analysis is limited by the fact that canonical pathways can be shared between multiple factors and a single factor can induce multiple transcriptome changes. We evaluated global transcriptome changes in cell populations of PBMC since such an approach will enrich for transcriptome changes that occur across diverse cell types, particularly changes, which are overlapping with diverse cells of the central nervous system. Zhou et al [30] reported that the transcriptional changes in PBMCs obtained from HIV-1 patients free of neurological disease were enriched in neurodegenerative pathways suggesting that PBMCs associated gene changes can be reflective of early HIV-1 induced changes. Our results also found transcriptional changes in PBMCs from patients who are HIV-1 seropositive, and HAND negative that overlap with genes associated with neurological pathology; however, the differentially regulated genes in PBMCs from individuals clinically identified as MND and HAD positive are enriched for genes related to neuropathogenesis and these genes are dysregulated to a greater extent (Figure S2, Table S3, <http://links.lww.com/QAD/B34>). Our results suggest that MND and HAD are associated with distinct transcriptional changes in peripheral compartment that overlaps changes in transcriptome observed in other related neurological diseases [31–35]. The changes observed in the peripheral compartment may have either a direct or indirect role in neuropathogenesis and these changes in the peripheral compartment may help us to identify the factors influencing HAND onset and progression. Though the percentage of CD4⁺ cells during HIV-1 infection as determined by CD4 surface expression is significantly different (Table 1), there is no major difference in the number of genes detected that can be attributed to reduced surface expression of CD4 molecules in T cells associated with different groups of HIV-1 seropositive individuals. Imaging studies evaluating the changes in white and grey matter of the brain during progression of HAND also reported loss of CD4⁺ T cells with severe forms of HAND [36].

HIV-1 invasion and replication in the central nervous system compartment is associated with release of neurotoxic cytokines and chemokines including IL-1 β , TNF- α , IL-8, IL-6, CCL2, and others. These factors damage the blood brain barrier, which triggers the chemokine feedback loop and further enhances the recruitment of additional inflammatory cells - mainly monocytes/macrophages and associated neuronal toxicity. Furthermore, HIV-1 proteins Tat, Env (gp120), Nef and Vpr induce neuronal apoptosis via direct and indirect mechanism [2–4, 9, 11–13, 37, 38]. Thus, there is an overlap in the mechanism of neurotoxicity induced by both viral proteins and inflammatory factors. While our study is an association study, results identify many factors such as TNF α , IL1 β , IL6, TGF β and CCL2 that are well established to have a role in HAND pathogenesis. Further, CSF1 and CSF3, which are involved in differentiation and growth of monocyte/macrophage lineage are also identified. This supports results from simian neuroHIV studies, which report that SIV infected monocytes originating from bone marrow migrate to the brain and the onset of simian encephalitis correlated directly with the viral load in bone marrow [39].

EIF2AK3 activation by HIV-1 envelope is reported to induce proinflammatory cytokines in microglial cells and has been identified as a contributor for apoptosis of neurons in *in vitro* experiments and is also identified in our analyses. Interestingly, prolactin has an inverse relation with HAND progression. All the factors included in Fig.4, are pharmacologically amenable targets and are good leads in development of novel therapeutics. MicroRNAs – miR-124-3p, let-7a-5p and miR-210 were identified in upstream analysis and were differentially regulated. The relative increase in the expression of these miRNAs in HIV-1 seropositive individuals who do not have HAND symptoms, suggests a neuroprotective role for these miRNA. Previous results from *in vitro* experiments as well as SIV model suggests that miR-124-3p has an anti-inflammatory role and was found in higher levels in brain of monkeys with HIVE. Additionally in rheumatoid arthritis and primary pulmonary fibrosis models in mouse, miR-124a, was shown to prevent monocyte migration and reduced inflammation, suggesting that miR-124-3p along with other closely related miRNAs in PBMCs inhibit the migration of monocytes across blood brain barrier in response to CCL2 [40, 41].

TimePath evaluation of the role of HIV-1 viral proteins identified a greater role for viral proteins in the transcriptome changes associated with HAD development and is further supported by the observation indicating decreased incidence of HAD in patients under combination anti-retroviral treatment. Additionally, TimePath analysis identified specific HIV-1 viral proteins associated with pathways involved in regulation of proteins identified to be associated with other neurological disease. In summary, based on the correlation of our results with previously identified factors that have a role in *in vitro* and animal experiments, we believe a role for these factors in HAND onset and progression. Our analysis cannot rule out other source of these factors that are identified to induce transcriptome changes in PBMCs, though the role of these factors originating from other compartments like gastrointestinal tract, respiratory tract, bone marrow or other areas can potentially increase the risk for onset, progression, and severity of HAND. Though our analyses identified the changes in PBMC, it should be noted that monocyte/macrophage associated factors and other factors that can cross the BBB or regulate its integrity, may have a greater role in HAND pathogenesis. One of the limitations of our study is the missing group of ANI

individuals resulting from technical challenges involved in clinically identifying individuals with ANI. Systematic follow up studies prior to HAND onset may help deduce algorithms that can predict neurocognitive disease outcomes based on transcriptome changes in PBMCs. Follow up studies in animal models of HIVE and in *in vitro* experiments of novel factors associated with different stages of HAND will help to confirm the role of these factors and will aid in development of novel therapeutics that can prevent and/or delay the onset, progression and severity of HAND.

Methods

Study Population

Frozen PBMCs were obtained from participants of the Multicenter AIDS Cohort Study (MACS), as per the protocol [20, 42]. The study population comprised of HIV-1 seronegative controls (N=36), well-characterized HIV-1 seropositive individuals who did not have any clinical neurocognitive symptoms on standard clinical neurological testing (N=16) and those who were identified as MND (N=8) or HAD (N=16), based on well-established clinical evaluation. Details of the subjects including CD4 counts (average and range), and viral load (average and range) at the time of sample collection are included in Table 1. All the subjects were men of unknown ethnicity.

mRNA profiling and data analysis

Total RNA was isolated from PBMCs using the MirVANA kit (Applied Biosystems), as suggested by the manufacturer and was profiled with HT-12 V4 array bead chips (Illumina, San Diego, CA, USA) as described previously [20, 43]. Datasets will be deposited in GEO database upon publication. Genome Studio was used to analyze the data and identify the differentially regulated gene transcripts. Rank invariant method and no background subtraction was included to normalize the data. Additionally, the missing samples were excluded. A detection cut-off of $p < 0.01$ was used. For calculating differential expression, the Illumina custom model was included along with multiple testing corrections using Benjamini and Hochberg False Discovery Rate. $q < 0.05$ was considered as the cut-off to identify significantly regulated gene transcripts. Ingenuity Pathway Analysis (IPA) was used to determine gene interactions and correlation networks. Ingenuity knowledge base was included as the reference set and both direct and indirect interactions that were experimentally verified in humans or other animals including mouse, rat were considered for the analysis.

MicroRNA profiling and data analysis

The human microRNA microfluidic card set v3.0 (Applied Biosystems) was used for miRNA profiling of samples, as described previously [20]. Detectability threshold for miRNA assays was set to C_T value less than or equal to 36 in at least 75% of all samples in each group. Differentially expressed miRNA between different groups were identified using parametric *t*-test or LIMMA (one factor analysis) with Benjamini-Hochberg false discovery rate (FDR) method adjusted q -values < 0.05 . MicroRNA target filter analysis from IPA was used to identify experimentally verified targets for the dysregulated miRNAs, and correlated with the expression of dysregulated genes identified between the comparison groups. IPA

based canonical pathway and upstream analysis were used to identify the networks associated with miRNA targets and predict associated biological functions.

TimePath

TimePath reconstructs dynamic signaling and regulatory networks by integrating several diverse data sources, please see [29] for details of methodology, and Figure S4 for additional details. These include condition specific data (viral-host interactions and gene expression data) and general data (protein-protein and protein-DNA interactions) obtained from BIOGRID, HPRD and VirHostNet datasets [44–46]. To apply TimePath to this dataset, we constructed a gene expression series with the HIV seronegative, HIV seropositive individuals who did not have HAND symptoms or MND or HAD in that order. Each phase consisted of 1 time point starting with the HIV seropositive with no HAND, time point for a total of 3 phases. Following prior work [29], we extracted the top 200 DE genes for each phase relative to the previous phase. These DE genes were used as targets for the TimePath IP function. All the HIV-1 viral proteins were included as sources in the analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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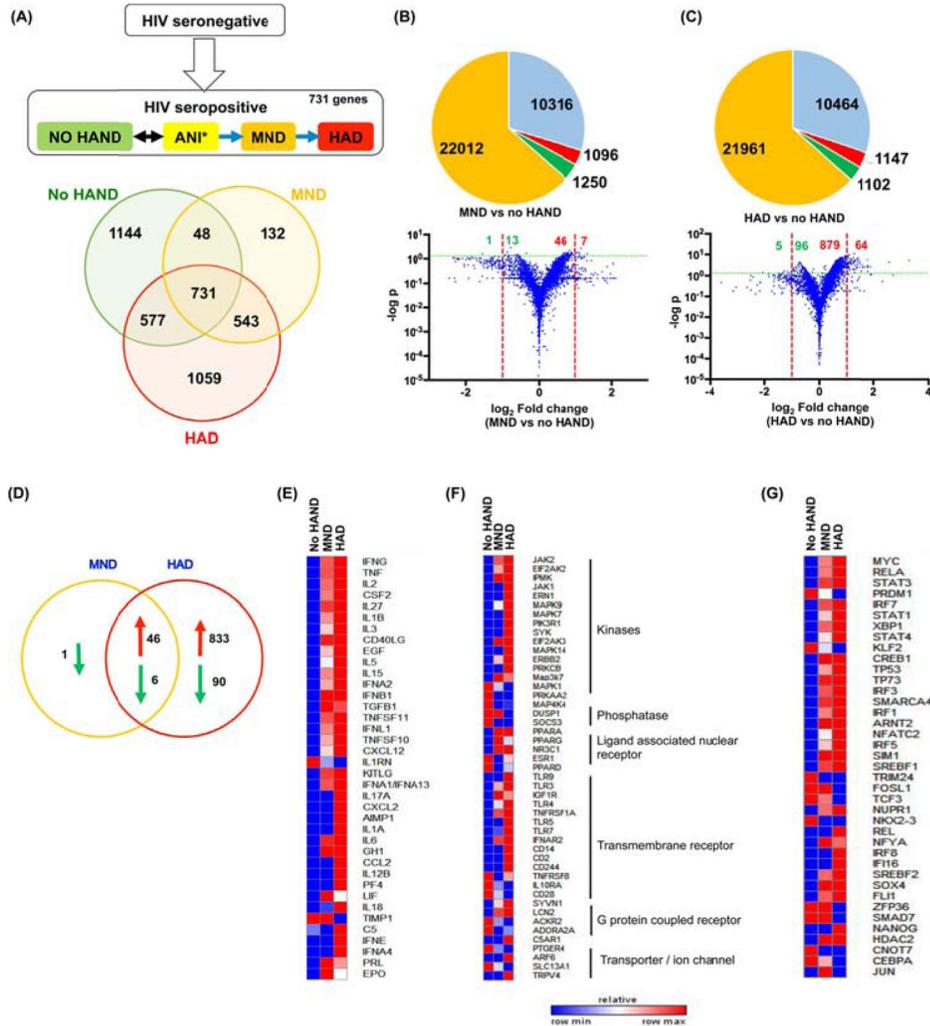


Figure 1. Comparison of differentially regulated genes ($q < 0.05$, FDR adjusted) in HIV-1 seropositive individuals without HAND, with MND or HAD relative to HIV-1 seronegative patients
(A) Schematic representation of relationship between different groups included in the study. HIV-1 seronegative individuals upon HIV-1 infection become HIV-1 seropositive and they can either be symptom free (no HAND) or can develop ANI, MND or HAD. The Venn diagram displays the number and overlap of significantly differentially expressed gene transcripts ($q < 0.05$, FDR adjusted) among HIV-1 seropositive subjects without HAND symptoms, with MND or HAD compared to HIV-1 seronegative group. Total RNA isolated from PBMCs in these donors was characterized by Illumina HT-12 V4 array beadchips and the data were analyzed using genome studio. Multiple probes for a single gene were combined together. A detection cut-off of $p < 0.01$ was used to identify transcripts that were detected consistently in each group. The expression of RNA transcripts were compared between HIV-1 seropositive individuals with MND and without HAND **(B)** or with HAD and without HAND **(C)**. Pie chart denotes the number of RNA transcripts consistently detected in both the comparing groups (blue), or detected only in comparing HAND positive group (red) or detected only in those without HAND (green) or not detected in both the

comparing groups (yellow). The volcano plot represents the fold change in expression of RNA transcripts in HAND positive group over no HAND group for only those genes that are consistently detected in both the comparing groups. Dotted vertical line (red) corresponds to a 2 fold cut-off and the dotted horizontal line (green) corresponds to $p=0.05$. **(D)** The Venn diagram displays the number and overlap of significantly differentially expressed gene transcripts ($q<0.05$, FDR adjusted) among the MND and HAD relative those without HAND. Red upward arrow indicates the increase and the green downward arrow indicates the decrease in number of the gene transcripts. Comparison of gene transcript expression unique for these HIV-1 seropositive individuals with or without HAND identified multiple upstream regulators including - **(E)** Cytokines and growth factors, **(F)** Factors involved in signal transduction – kinases, phosphatase, ligand associated nuclear receptors, membrane associated receptors, and **(G)** Transcription factors.

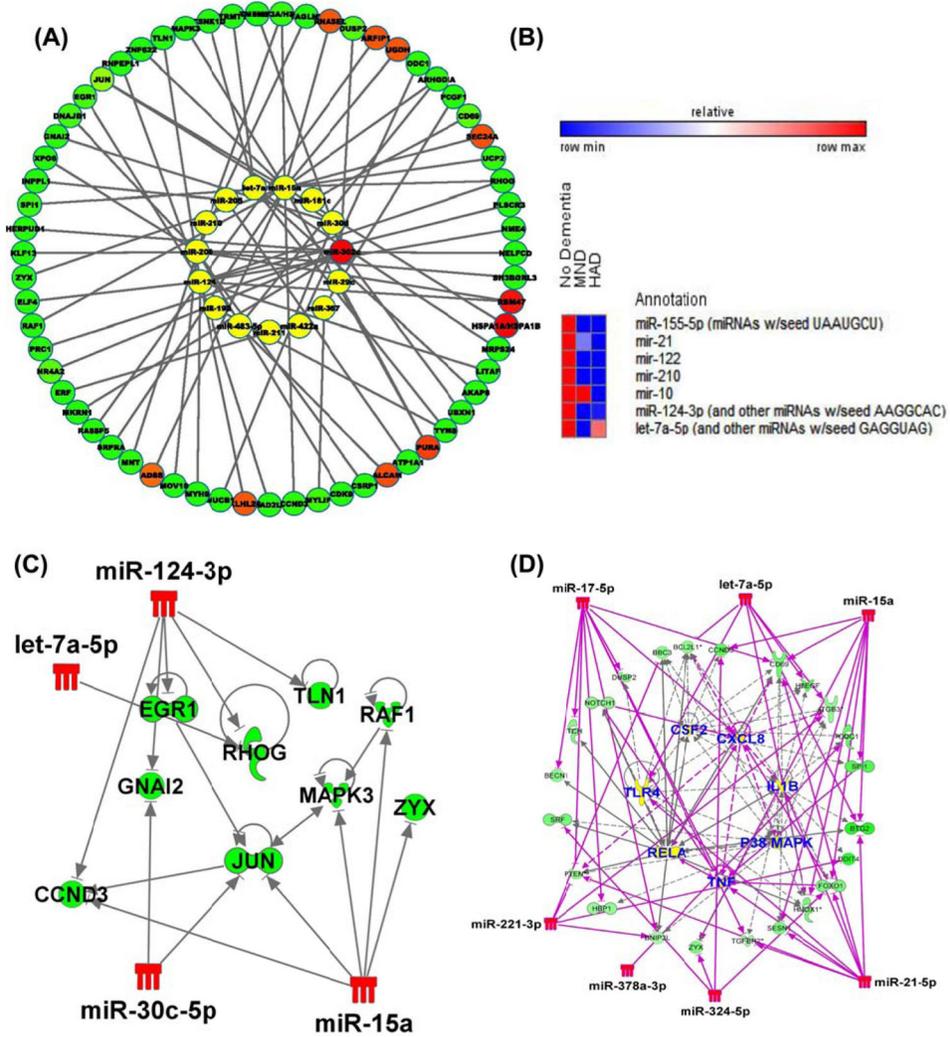


Figure 2. Network of miRNA-mRNA interaction and their associated functional role relevant in HAND pathogenesis

(A) Results from miRNA and mRNA array were combined by IPA based miRNA target analysis and visualized by Cytoscape. Experimentally verified miRNAs (inner circles) that actively regulate mRNA (outer group of circles) based on previously published literature are included. Green and red in the mRNA or miRNAs sets represents up and down regulation, respectively, in HIV-1 seropositive individuals with HAD relative to HIV-1 seropositive individuals without HAND symptoms. (B) Comparison of gene transcript expression unique for HIV-1 seropositive individuals with or without HAND relative to HIV-1 seronegative individuals identified miRNAs as key regulators in different stages of HAND. (C) Network denoting specific miRNAs and their targets identified differentially regulated in HAD relative to HIV-1 seropositive subjects without HAND ($q < 0.05$, FDR adjusted) that are critical in chemokine signaling. (D) Network analysis of miRNA-mRNA pairs that are significantly dysregulated in HIV-1 seropositive subjects without HAND relative to HIV-1 seronegative subjects and their relation to downstream signaling molecules of multiple pro-

inflammatory pathways. Pathways related to CSF2, CXCL8, TLR4, IL1 β , RELA, TNF and p38 MAPK and only their associated miRNA-mRNA targets are shown.

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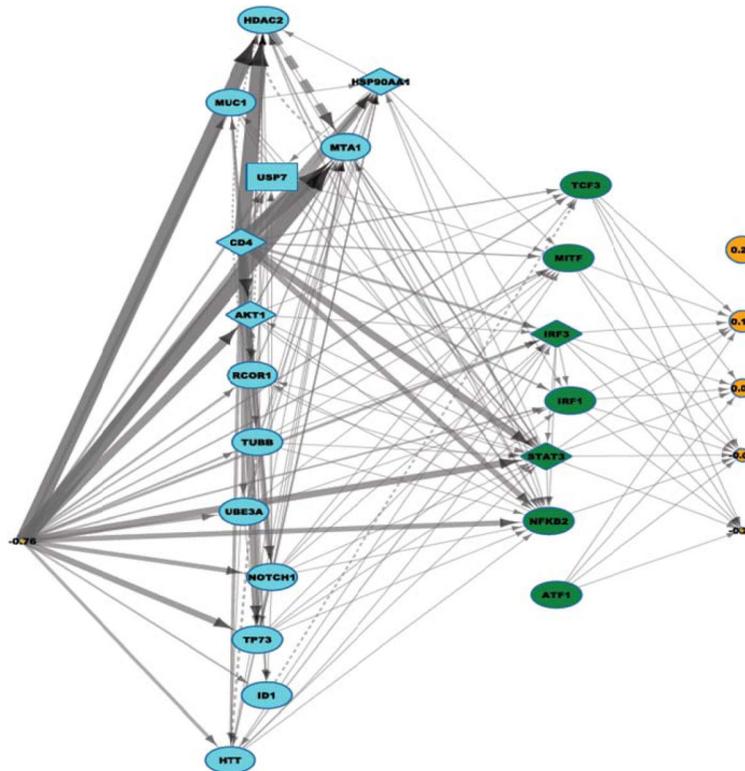


Figure 3. Dynamic signaling and regulatory network for HIV-1 viral proteins in HAND associated changes in PBMC

Blue nodes are intermediate signaling proteins and green nodes are the TFs that are predicted to directly up/down-regulate the differential expression of target genes (targets not shown in figure, but the average levels of the regulated targets for each TF is presented by the yellow nodes while the size of each of the yellow nodes indicates how many genes belong to the cluster represented by the node). The figure displays the top predicted proteins for HAD stage and also demonstrated is the relation to the HIV-1 proteins via the signaling proteins and differentially expressed genes in earlier phases (The red nodes are the HIV-1 proteins [sources], see Fig S4, <http://links.lww.com/QAD/B34>, for additional details). Note that some intermediate proteins may also be TFs. The functional role in the network figure is based on the location of the protein in the selected paths based on the IP.

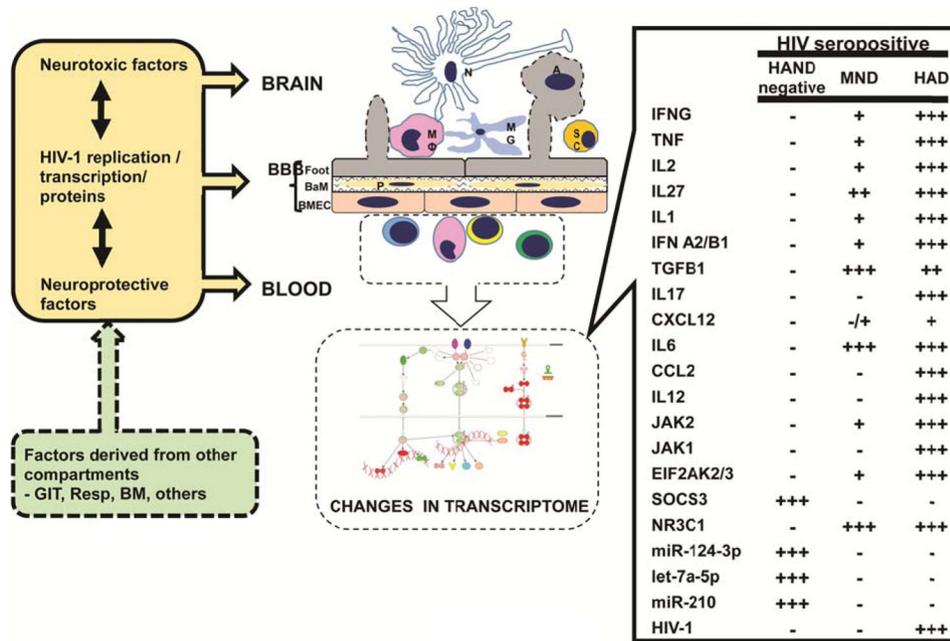


Figure 4. Transcriptome changes in PBMC related to HAND pathogenesis and their key regulators

Schematic representation of the relation of transcriptome changes in PBMC and in brain cells induced by neurotoxic, neuroprotective, viral factors and other factors derived from other compartments such as gastrointestinal (GIT) system, respiratory tract, bone marrow (BM) and others. Peripheral blood mononuclear cells in the brain microvasculature are exposure to diverse factors that play critical role in HAND pathogenesis. Evaluation of global transcriptome changes in all the populations of PBMCs, enriches transcriptome changes that occur across diverse cell types, including those transcriptome changes which are overlapping with diverse cells of the central nervous system compartment. Systematic analysis of these transcriptome changes in different stages of HAND identified key regulators associated with different stages of HAND. (-, denotes negative effect, +/-, denotes no effect, +, ++, and +++, denotes mild, moderate and severe positive effects, respectively). N-neuron, A-as strocyte, MG-microglia, MΦ-macrophage, SC- neuronal support cells, BMEC-brain microcapillary endothelial cells, BaM-basement membrane, Foot- foot of astrocyte, P-pericytes, BBB-blood brain barrier.

Table 1

Clinical status, CD4 counts, viral load of subjects used in the study.

Clinical Status	Avg. CD4 count	Range of CD4 count	Avg. Viral Load	Range of Viral Load
HIV-1 seronegative subjects (N=38)	876	385 – 2194	N/A	N/A
HIV-1 seropositive subjects - no HAND (N=16)	404	100 – 912	82892	40 – 289528
HIV-1 seropositive subjects - MND (N=8)	161	22 – 451	220796	11595 – 681894
HIV-1 seropositive subjects - HAD (N=16)	345	65 – 636	185904	15744 – 791689

The neurocognitive status of the subjects was identified based on well established clinical neuropsychological testing conducted as part of MACS visit. CD4 count and viral load corresponds to the values at the time of sample collection.