

Identification of Cigarette Smoke Condensate-Modulated Hiv-1-Associated Host Factors Using A Targeted Transcriptomic Approach

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Abstract

Tobacco use has been correlated with increased HIV-1 infection. In vitro characterization of alveolar macrophages obtained from uninfected cigarette smokers showed an increased susceptibility to HIV-1 infection when compared with cell from non-smokers. We showed that Cigarette Smoke Condensate (CSC) enhances HIV-1 infection in primary CD4+ cells. The underlying mechanisms of how CSC affects HIV-1 replication are unclear. The traditional approach that studies the specific cellular processes that are modulated by CSC and examines the resulting effects on viral replication has generated inconclusive results. Indeed, CSC has a pleiotropic effect on cell expression and transcriptomic studies have shown that CSC can modulate the expression of a large number of genes. This global transcriptomic approach generates a large number of unregulated and downregulated genes, prohibiting the specific functional analysis of genes that are related to viral replication. The lack of a reliable method of identifying the specific host factors that are recruited by HIV-1 poses a significant bottleneck to specific functional analysis of HIV-1-associated genes. In this study, we circumvent the abovementioned analysis bottleneck and focus our investigation on the genes that are involved in the HIV-1 replication by utilizing a host-HIV-1 interaction database to identify the CSC -modulated genes associated with HIV-1 replication. Using this targeted approach, we have successfully identified 30 host factors that are modulated by CSC and directly involved in HIV-1 replication. The current study not only allowed us to characterize the effect of CSC treatment on HIV-1-associated host factors but can also be applied to characterize substances of abuse that have the potential to modulate HIV-1-associated genes in the host cell. This study also establishes a new model for studying the host-pathogen interactions of other human viruses, such as the influenza virus and West Nile virus, both of which have readily available host-pathogen genomic/interactomic data.

Introduction

Cigarette smoking creates significant public health problems. It is responsible for most incidences of lung cancer and chronic obstructive pulmonary disease deaths in the United States and has also been shown to increase the incidence of hypertension, heart disease, asthma and respiratory infection [1]. Although the correlation between cigarette smoking and disease has been firmly established, its biological mechanisms are still not well understood. Composed of thousands of toxic and/or carcinogenic chemicals, cigarette smoke has been tied to numerous physiological changes [2]. Its immunomodulatory effects include an elevated peripheral white blood cell count, increased levels of proinflammatory cytokines [3],

lowered levels of serum immunoglobulins [4] and increased levels of auto-antibodies [5]. Cigarette smoke contains chemicals that form or are metabolized into DNA-damaging adducts [6,7], as well as oxidants that damage cellular organelles, lipids and proteins, thus contributing to inflammation [8]. Other constituents of tobacco smoke, such as heavy metals and benzoquinone [9], have been found to increase oxidative stress [10,11].

In addition to these deleterious effects, tobacco use has also been correlated with increased HIV infectivity rates. A study of alveolar macrophages sourced from uninfected smokers demonstrated an increased susceptibility to HIV infection when compared with those from non-smokers [12]. In another study, microglial cells treated with nicotine displayed increased HIV-1 expression, modified gene expression and constitutive expression of nicotinic acetylcholine receptor mRNA [13]. Demographical studies have also indicated

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a positive correlation with tobacco use and HIV acquisition. Cross-sectional surveys of pregnant women in Rwanda [14] and Haiti [15] which controlled for numerous risk factors found tobacco use increased the rate of HIV seroconversion. A cohort study of homosexual men adjusted for risk in the United States also found a positive association with tobacco smoke and HIV acquisition [16].

Expressional microarray studies examining the transcriptome of cigarette smoke-treated monocytes have demonstrated changes in expression of genes relating to the oxidative stress response [11], inflammation, inducible antioxidants and the cell-survival pathways involved in protein folding [17]. RNA-seq analysis of small airway epithelium brushings has indicated upregulation of genes related to immunity, transcription, signal transduction and protease/antiprotease balance [18]. In smokers with lung cancer, upregulation of genes relating to chemokine signaling, cytokine binding and cell adhesion molecules have also been demonstrated by RNA-seq [19].

Despite these studies, the underlying mechanisms of how cigarette smoke affects HIV-1 replication are unclear. Moreover, the relationship between the host factors that are affected by cigarette smoke and those that interact with HIV-1 proteins is unknown. Expressional microarray and RNA-Seq characterize changes in the transcriptome and thus generate data containing a large number of upregulated and downregulated genes that are irrelevant to HIV-1 replication. However, the lack of a reliable method of identifying the specific host factors that are recruited by HIV-1 poses a significant bottleneck to specific functional analysis of HIV-1-associated genes. Nonetheless, recent developments of a host-HIV-1 interaction database have identified specific host gene networks that are utilized by HIV-1 and can solve this problem by identifying the cellular factors and processes that are specific for viral replication [20,21].

In this study, we proposed to circumvent the abovementioned analysis bottleneck and focus our investigation on the genes that are involved in the HIV-1 replication by utilizing a host-HIV-1 interaction database to identify the CSC-modulated genes associated with HIV-1 replication. Specifically, we will utilize the NIAID HIV-1-Human Protein Interaction Database, which is a well-curated public database that contains approximately 2500 genes that have been experimentally determined to interact with HIV-1 [20,21]. Therefore, creatively combining these new tools will allow us to identify (for the first time) the cellular factors that modulate HIV-1 replication upon CSC stimulation. To this end, we developed a RNA-Seq workflow to characterize global expressional changes of Cigarette Smoke Condensate (CSC)-treated human Peripheral Blood Mononuclear Cells (PBMC). A semantic approach was employed to identify the host factors modulated by CSC and involved in HIV-1 replication. This targeted approach was successful in generating a pipeline of candidate CSC-modulated HIV-1-associated cellular factors for specific genetic and functional studies examining the effect of CSC on HIV-1 replication. This study also establishes a new model for studying the host-pathogen interactions of other human viruses, such as the influenza virus and West Nile virus, both of which have

readily available host-pathogen genomics/interactomics data [22,23].

Materials and Methods

Cell culture

Human PBMCs from a healthy donor were obtained from the Comprehensive NeuroAIDS Center at Temple University. The cells were activated with PHA (phytohemagglutinin 2 µg/ml) for two days in complete RPMI-1640 medium (supplemented with 10% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin). PHA-containing medium was removed after activation, and the cells were resuspended in complete RPMI-1640 with IL-2 (10 U/ml).

Treatment of PBMCs with CSC and HIV-1 infection

CSC was purchased from Murty Pharmaceuticals Inc. (Lexington, KY) and prepared by the smoking of University of Kentucky 3R4F research cigarettes using a Federal Trade Commission smoking machine. The condensate was collected on glass fiber filters and extracted with DMSO to yield a 4% solution. The PBMCs were treated with CSC (0.004% or 0.04%) or vehicle (DMSO) prior to and after infection with NL4-3, an X4-tropic HIV-1 strain. At the peak of virus production, quantitative PCR (qPCR) using gag-specific primers was used to evaluate proviral integration in the host genomic DNA in duplicate for all infections and normalized using the housekeeping gene porphobilinogen deaminase (PBGD). The amount of integrated provirus corresponds to the efficiency of successful virus replication. By comparison with a vehicle-treated control, the fold change in replication efficiency was calculated.

RNA isolation and RNA-Seq

Ten million PBMCs were treated with CSC (0.004% or 0.04%) for 6 hours before RNA isolation. Two biological replicates were performed for each CSC concentration. DMSO-treated cells were used as the baseline for expression comparison. The total RNA was isolated from the treated cells using an RNeasy Mini kit (Qiagen) with on-column DNase treatment to eliminate any DNA carryover in the downstream RNA-Seq process. The quality and quantity of the resulting RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) prior to the RNA-seq library construction using the TruSeq RNA sample preparation kit according to the manufacturer's recommendations (Illumina). Briefly, mRNA was purified from the total RNA using poly-A selection to effectively remove ribosomal RNA and contamination with small RNAs. The mRNA was then chemically fragmented and converted into single-stranded cDNA using random hexamer priming, and a second strand was generated to create a double-stranded cDNA. Adapters containing the full complement of the sequencing primer hybridization sites for multiplexed reads were added to the DNA to generate the RNA-Seq library. The RNA-Seq library was sequenced on an Illumina HiSeq platform by Genewiz Inc. (South Plainfield, NJ). To avoid bias or incomplete sampling of the mRNA species, two technical

replicates were conducted for each sample. The RNA-Seq data were submitted to the NCBI Sequence Read Archive under accession number SRA053625.

Results and Discussion

CSC enhances HIV-1 replication in PBMCs

To investigate the effect of CSC treatment on HIV-1 replication, we infected PBMCs with HIV-1 in the presence of 0.004% or 0.04% CSC. These concentrations were used in the experiments because they have minimal toxicity to the cells. Treatment of PBMCs with 0.4% CSC reduced cell viability and was not included in the characterization. HIV-1 replication was assessed by qPCR-based detection of the gag gene normalized to PBGD. PBMCs infected with HIV-1 following treatment with 0.004% or 0.04% CSC demonstrated a 9.8-fold and 7.8-fold increase in gag copy number, respectively (Figure 1). The 2-fold difference between the two treatment groups were not statistically significant thus the difference in viral replication between 0.004% and 0.04% CSC treatments may be due to experimental variations. These results indicate that CSC treatment of HIV-1 target cells increases viral replication by an unknown mechanism.

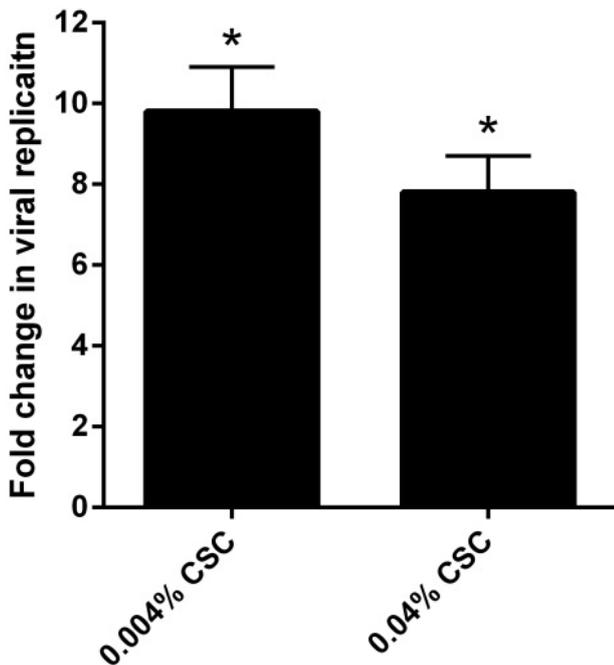


Figure 1: The effects of CSC treatment on HIV-1 replication in PBMCs. Fold change in viral replication was calculated by comparing the replication efficiency of HIV-1 in CSC-treatment groups to that of DMSO-treated cells. Mean fold change is depicted with standard deviation. *, $p < 0.05$, Student's *t* test ($n=2$)

Analysis of global gene expression data

CSC has been shown to have pleiotropic effects on cell expression. A previous microarray study of cigarette smoke-treated monocytes indicated numerous changes in the expression of genes involved in cell survival and inflammation [17]. RNA-Seq-based transcriptomic studies of airway epithelial cells

originating from smokers demonstrated modulation of genes relating to signal transduction and transcription [18]. CSC treatment of HIV-1 target cells is expected to result in a large number of upregulated and downregulated genes. Importantly, alterations in gene expression can potentially modulate HIV-1 replication. We investigated the transcriptomic changes in these cells resulting from CSC treatment to identify the cellular pathways associated with both CSC and HIV-1 replication. To this end, RNA-Seq has generated more than 40 million reads for each sample. The RNA-Seq data were assembled using QSeq (DNASTAR), as guided by the UCSC hg19 reference genome. The transcript levels were normalized for RNA length and the total number of reads using reads per kilobase of exon model per million mapped reads to accurately reflect molar concentration in the initial samples.

Differentially expressed genes after CSC treatment were determined by pairwise comparison of treatment group with the DMSO control group on a gene-by-gene basis. In the pairwise comparison, we applied Benjamini and Hochberg's procedure using a false discovery rate of 0.05 to determine the adjusted *p* value. The transcripts were considered differentially expressed if the expression values differed by 2-fold and had an adjusted *p* value less than 0.05. The lists of genes with significant differences in expression after the 0.004% and 0.04% CSC treatments are shown in Supplemental Table 1 and Supplemental Table 2, respectively. These genes were cataloged by gene ontology by which genes are systematized in a species-independent manner by the biological processes that they are involved in based on biological processes, molecular function and cellular localization. In PMBCs treated with 0.004% CSC, there were 2412 genes exhibiting a significant change in expression of 2-fold or larger, with 693 being upregulated and 1719 being downregulated (Figure 2). PMBCs treated with 0.04% CSC demonstrated significant differential expression in 1403 genes, of which 412 were upregulated and 991 downregulated.

Figure 2: Modulation of host factors by CSC treatments. The expressions of 2412 and 1403 genes were significantly modulated after treatments with 0.004% and 0.04% CSC, respectively. Red represents upregulated genes and blue represents downregulated genes.

Identification of CSC-modulated HIV-1-associated genes

Because RNA-Seq characterizes changes in the global transcriptome [24], we expected to identify many genes that were irrelevant to HIV-1 replication. This "big data" problem posed a significant bottleneck to our analysis and has also been a problem in previous studies that employed microarrays [17-

19] because of the lack of a reliable method for identifying the specific host factors that are recruited by HIV-1. Here, we developed a targeted workflow to circumvent this bottleneck and focus our investigation on the genes involved in HIV-1 replication. A panel of 2553 focused genes was algorithmically generated based on the curated biological knowledge database from the NIAID HIV-1 human protein interaction database and used to investigate the abovementioned CSC-modulated genes to identify those that engaged in HIV-1 replication. Of the 2412 genes that were differentially expressed upon 0.004% CSC treatment, we identified 31 upregulated and 136 downregulated HIV-1-associated genes (Figure 3a and Supplemental Table 3). In the 0.04% CSC treatment group comprising 1403 genes, 20 upregulated genes and 104 downregulated genes known to interact with HIV-1 were found (Figure 3b and Supplemental Table 4).

Figure. 3 Identification of HIV-1-associated host factors modulated by CSC treatments. Host factors significantly modulated by (a) 0.004% and (b) 0.04% CSC were overlaid with genes identified in the NIAID HIV-1 human protein interaction database.

Based on the information from the HIV-1 human protein interaction database, we then conducted a comprehensive and exhaustive literature review on these 291 differentially expressed HIV-1-associated genes and found that 257 of the genes are modulated as a result of HIV-1 infection and do not have a direct interaction with the viral proteins. To test the hypothesis that CSC is able to modulate the host factors involved with HIV-1 during replication, we focused our study on the host factors that have a direct interaction with HIV-1 proteins. To this end we identified a total of 18 host factors in the 0.004% CSC treatment (Table 1) and 22 host factors in the 0.04% treatment that serve as potential modulators of HIV-1 infection (Table 2). There were six genes that appeared in both treatment groups. Interestingly, except for CEBPB, all of the differentially expressed HIV-1-associated host factors were downregulated by CSC treatment. These results demonstrated that the expressions of the host factors involved in HIV-1 replication were overwhelmingly suppressed by CSC treatment.

Host factors		HIV-1 genes
ADCY1*	adenylate cyclase 1	env, tat, nef
AKT3	v-akt murine thymoma viral oncogene homolog 3	env, tat
ATM	ataxia telangiectasia mutated	pol, rev
CBX5	chromobox homolog 5	vpr
CCR5*	chemokine (C-C motif) receptor 5	env
CEBPB	CCAAT/enhancer binding protein	tat
EP300	E1A binding protein p300	tat, vpr
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	env, tat
PIK3CB	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	env, tat
PIK3R1*	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	nef, tat
POU2F1	POU class 2 homeobox 1	tat
PRKAR2A*	protein kinase, cAMP-dependent, regulatory, type II, alpha	gag, vpr, nef
PRKAR2B*	protein kinase, cAMP-dependent, regulatory, type II, beta	gag, vpr, nef
PRKD1	protein kinase D1	tat
PTPRC	protein tyrosine phosphatase, receptor type, C	env
REL*	v-rel reticuloendotheliosis viral oncogene homolog	tat
SP1	Sp1 transcription factor	tat, vpr
SP3	Sp3 transcription factor	tat

*, asterisk represents host factor identified in both 0.004% and 0.04% CSC
Table 1: HIV-1 associated host factors after 0.004% CSC treatment.

Host factors		HIV-1 genes
ADCY1*	adenylate cyclase 1	env, tat, nef
BCL2	B-cell CLL/lymphoma 2	pol
CCNT1	cyclin T1	gag, tat
CCR5*	chemokine (C-C motif) receptor 5	env
ICAM1	intercellular adhesion molecule 1	env, gag
ITGB3	integrin, beta 3	tat
MME	membrane metallo-endopeptidase	tat
NCOA3	nuclear receptor coactivator 3	tat
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	tat
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	nef, tat
PIK3R1*	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	nef, tat
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	gag, vpr, nef
PRKAR2A*	protein kinase, cAMP-dependent, regulatory, type II, alpha	gag, vpr, nef
PRKAR2B*	protein kinase, cAMP-dependent, regulatory, type II, beta	gag, vpr, nef
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	vpu
REL*	v-rel reticuloendotheliosis viral oncogene homolog	tat
RPA4	replication protein A4	pol

SDC2	syndecan 2	env, tat, gag
SDC4	syndecan 4	env, tat, gag
SERPINA1	serpin peptidase inhibitor, clade A	env, gag
TCERG1	transcription elongation regulator 1	tat
TGM2	transglutaminase 2	env, rev

*, asterisk represents host factor identified in both 0.004% and 0.04% CSC treatments.

Table 2: HIV-1 associated host factors after 0.04% CSC treatment.

Ingenuity Pathway Analysis (IPA) of CSC-modulated HIV-1-associated genes

To identify specific biological processes that are affected by CSC treatment and involved in HIV-1 replication, the integrated gene network of the differentially expressed genes was analyzed using IPA (Ingenuity Systems). IPA is based on well characterized metabolic and cell signaling pathways described in the published literature [25-27]. One highly significant network (significant score >50) was identified in each of the treatment groups. In the 0.004% CSC treatment group, the highest scored network consisted of viral proteins closely linked to each other through key host transcriptional factors (Figure. 4). These transcriptional factors are responsible for regulating cell death and survival. In the 0.04% CSC treatment group, the highest scored network consisted of a smaller transcriptional network connected by actin to a satellite network responsible for cell adhesion and integrity (Figure. 5).

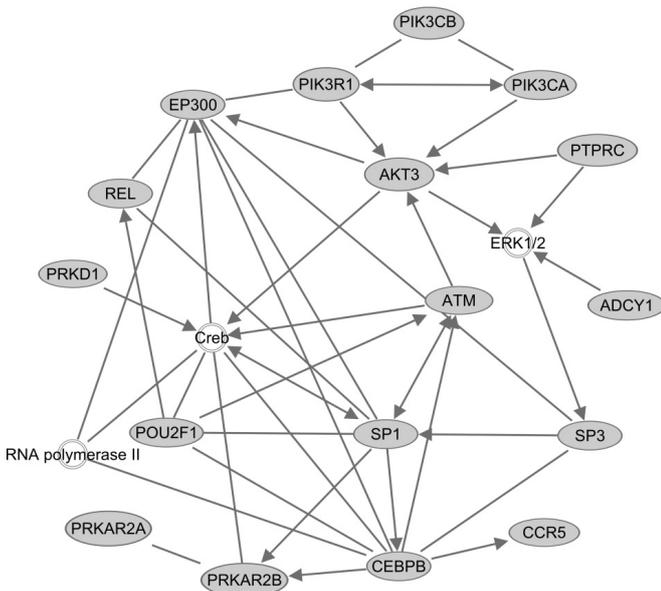


Figure 4: Network analysis of HIV-1 associated host factors modulated by 0.004% CSC treatment. Seventeen of the 18 host factors (grey oval) in Table 1 belong to a highly scored network. Among the host factors, CBX5 is not present in this network. Key cellular proteins (white circle) related to the host factors are shown.

Several host factors were present in the networks of both treatments, including a group of protein kinases represented by PRKAR2A and PRKAR2B. These kinases have a well-established role in phosphorylating HIV-1 Gag, Vpr and Nef. This phosphorylation is essential for various stages of viral replication [28]. Both kinases phosphorylate Nef, enabling HIV-1 to replicate in resting cells [29]. The phosphorylation of Vpr

activates the viral protein, leading to cell cycle arrest and cell death [30]. CSC treatment significantly downregulated these kinases, which may lead to reduced viral replication, suggesting that the proteins may not play a role in the elevated HIV-1 replication in CSC-treated PBMCs.

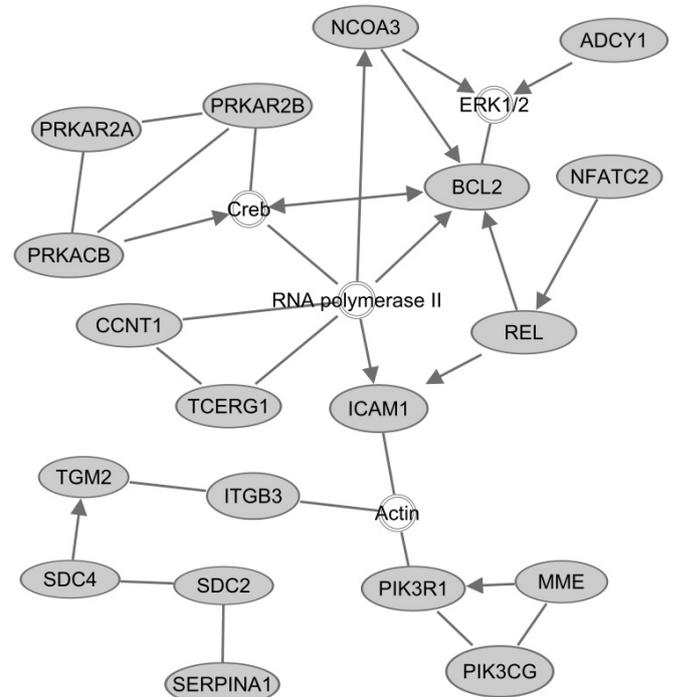


Figure 5: Network analysis of HIV-1 associated host factors modulated by 0.04% CSC treatment. Nineteen of the 22 host factors (grey oval) in Table 2 belong to a highly scored network. Among the host factors, CCR5, PRKDC and RPA4 are not present in this network. Key cellular proteins (white circle) related to the host factors are shown.

Downregulation of Tat-associated host factors after CSC-treatment

Tat-associated host factors constituted a large fraction of CSC-regulated genes in our study. The Tat protein promotes viral replication through the phosphorylation of cellular factors that mediate transcription [31]. Tat acts by binding to the trans-activating response element on the HIV-1 genomic RNA and recruiting cellular transcriptional factors to increase the production of full-length viral RNA. The Tat-associated host factors that are present in the networks of both treatments are REL and PIK3R1 (Figure 4 and 5). REL is a strong transactivator of HIV-1 gene expression and functions in concert with Vpr and EP300 to activate transcription [32,33]. PIK3R1 participates in the Akt pathway and is responsible for Tat-induced LTR transactivation involving the TAR element [34].

Tat-associated transcriptional factors continued to play an important role in our findings during the examination of the interactions that may occur between CSC and HIV-1 proteins (Figure 4 and 5). For example in Figure. 4, when SP1 or SP3 interacts with Tat, they can enhance or repress, respectively, the basal expression from the HIV-1 LTR promoter [35-37]. SP1 is also able to interact with Vpr in a complex involving p53 that also serves to transactivate the LTR promoter [38]. PRKD1, a member of the protein kinase C family, plays an essential role in Tat-mediated transactivation and participates in the transi-

tion from latent to active viral replication [39]. A transcription activator, POU2F1, has been shown to repress transcription of the provirus through high-affinity binding of the Tat and LTR promoter [40]. In the satellite transcriptional network depicted in Figure. 5, several Tat-associated host factors were also identified. NCOA3 have been reported to be a transcriptional co-activator of Tat [41]. NCOA3 can interact with the HIV-1 LTR and has been suggested to be involved in the transcriptional reactivation of the HIV-1 promoter from latency [42]. TCERG1 associates with Tat and the cellular RNA polymerase II to play an essential role in Tat transactivation [43].

In addition to its role as a powerful transactivator of HIV-1 gene expression, Tat also plays a role in the induction of cellular apoptosis. Through both intracellular and extracellular functions, Tat facilitates the upregulation of co-receptors, modulates immunoregulatory cytokines and exerts a direct cytopathic effect on lymphocytes and neurons [31]. Several factors downregulated in our study have also been associated with the induction of apoptosis during HIV-1 infection. We found that SDC2 and SDC4 (Figure 5), which are involved in cell binding and signaling and required for the uptake of Tat, are downregulated [44,45], suggesting an anti-apoptotic effect. The PTPRC, which normally serves as a regulator for T- and B-cell antigen receptor signaling, has been shown to regulate HIV-1 gp120-induced apoptosis via its association with CXCR4. The reduced expression of these pro-apoptotic cellular factors suggests that CSC modulation exhibits a protective effect on apoptosis and may reduce HIV-associated CD4+ cell depletion during infection, which thereby increases the number of virus-producing cells.

In addition to Tat, Vpr also plays a role in HIV-1 gene expression. Vpr binds to CCNT1 as part of a transcriptional complex involving Tat and CDK9 and enhances Tat transactivation of the viral LTR promoter [46,47]. In terms of posttranscriptional regulation, ATM kinase affects HIV-1 replication by stimulating the action of HIV-1 Rev, which is important in the nuclear export of viral RNA [48]. Whereas downregulated genes composed the more substantial portion of factors affecting HIV-1 replication in our study, one candidate, CEBPB, was upregulated and may enhance replication (Figure 4). CEBPB binds to and inhibits the antiviral cytidine deaminase APOBEC3G, which thereby facilitates reverse transcription of HIV-1. The protein renders CD4+ T cells highly permissive for HIV-1 replication [49]. The upregulation of CEBPB may play a role in enhancing HIV-1 replication in CSC-treated cells.

Potential host restriction factors modulated by CSC

Given the significant increase in HIV-1 replication after CSC treatment in PBMCs, it is possible that innate antiviral host factors were downregulated, which rendered the cells more permissive for infection. In the IPA analysis, several of these restriction factors were identified closely linked to each other in a satellite network and may be responsible for the increased HIV-1 replication (Figure 5). TGM2 is a multifunctional protein involved in a variety of cellular functions. In the context of HIV-1 infection, it specifically interacts with eukaryotic initia-

tion factor 5A and disrupts HIV replication through inhibition of Rev-mediated viral mRNA nuclear export [50]. Therefore, viral replication may be enhanced by the downregulation of TGM2. Moreover, SERPINA1 has potent antiviral activity and is produced when serpin interacts with cellular serine proteinases. The protein prevents HIV-1 maturation by inhibiting HIV-1 protease and blocks Gag processing [51]. It also limits membrane fusion and viral entry by inhibiting membrane-associated elastase, which in turn blocks Env processing [52]. MME was also identified in our analysis, forming a complex with neprilysin and interacting with Tat to prevent Tat dimerization, which is essential for function [53,54]. Specific genetic and functional characterizations are underway to reveal the potential roles of these restriction factors in modulating HIV-1 replication after CSC treatment.

The targeted transcriptome analysis described here allowed us to identify HIV-1-associated genes modulated by CSC treatment. The set of genes identified can be used for network analysis to reveal the pathways connecting the CSC-modulated genes, HIV-1 genes and HIV-1-associated host factors. This optimized framework not only allowed us to characterize the effect of CSC treatment on HIV-1-associated host factors but can also be applied to characterize substances of abuse that have the potential to modulate HIV-1-associated genes in the host cell. This study serves as an entrée for specific characterizations of the underlying mechanisms of CSC-mediated modulation of viral replication. We also anticipate that our study will provide a targeted transcriptomic workflow for the study of human viruses important to public health.

Disclosure

The authors declare that they have no competing interests.

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