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Research Article

Transcriptome analysis of CD4$^+$ T cells from HIV-infected individuals receiving ART with LLV revealed novel transcription factors regulating HIV-1 promoter activity

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ABSTRACT

Some HIV-infected individuals receiving ART develop low-level viremia (LLV), with a plasma viral load of 50–1000 copies/mL. Persistent low-level viremia is associated with subsequent virologic failure. The peripheral blood CD4$^+$ T cell pool is a source of LLV. However, the intrinsic characteristics of CD4$^+$ T cells in LLV which may contribute to low-level viremia are largely unknown. We analyzed the transcriptome profiling of peripheral blood CD4$^+$ T cells from healthy controls (HC) and HIV-infected patients receiving ART with either virologic suppression (VS) or LLV. To identify pathways potentially responding to increasing viral loads from HC to VS and to LLV, KEGG pathways of differentially expressed genes (DEGs) were acquired by comparing VS with HC (VS-HC group) and LLV with VS (LLV-VS group), and overlapped pathways were analyzed. Characterization of DEGs in key overlapping pathways showed that CD4$^+$ T cells in LLV expressed higher levels of Th1 signature transcription factors (*TBX21*), toll-like receptors (*TLR-4*, *-6*, *-7* and *-8*), anti-HIV entry chemokines (*CCL3* and *CCL4*), and anti-IL-1β factors (*ILRN* and *IL1R2*) compared to VS. Our results also indicated activation of the NF-κB and TNF signaling pathways that could promote HIV-1 transcription. Finally, we evaluated the effects of 4 and 17 transcription factors that were upregulated in the VS-HC and LLV-VS groups, respectively, on HIV-1 promoter activity. Functional studies revealed that CXXC5 significantly increased, while SOX5 markedly suppressed HIV-1 transcription. In summary, we found that CD4$^+$ T cells in LLV displayed a distinct mRNA profiling compared to that in VS, which promoted HIV-1 replication and reactivation of viral latency and may eventually contribute to virologic failure in patients with persistent LLV. CXXC5 and SOX5 may serve as targets for the development of latency-reversing agents.

1. Introduction

Antiretroviral therapy (ART) in HIV-infected individuals aims to suppress the plasma viral load below the lower detection limit (20 copies/mL) of clinical assays. However, some patients undergoing ART experience intermittent or persistent low-level viremia (LLV), usually defined as a plasma viral load ranging from 50 to 1000 copies/mL (World Health Organization, 2016). The causes of LLV are under debate, as discordant findings have been reported regarding the association of drug resistance, low drug concentration, and suboptimal ART adherence with low-level viremia (Swenson et al., 2014, Konstantopoulos et al., 2015, Gonzalez-Serna et al., 2016, Palich et al., 2020). Persistent LLV is associated with virologic failure, while its impact on AIDS events and mortality remains uncertain (Crespo-Bermejo et al., 2021).

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A previous study suggested that plasma viruses of patients with LLV had two sources: ongoing viral replication and latent reservoir (Tobin et al., 2005). HIV-1 sequences recovered from the plasma of patients with LLV were identical to viral RNAs in resting $CD4^+$ T cells (Anderson et al., 2011). Therefore, the peripheral blood $CD4^+$ T cell pool is a source of LLV viral species, originating from either viral replication or a reactivated latent reservoir of $CD4^+$ T cells (Tobin et al., 2005, Anderson et al., 2011). HIV-1 replication and reactivation of viral latency are transcriptionally dependent on the activity of the viral 5′-LTR. HIV-1 5′-LTR contains a plethora of *cis*-elements recognized by activator and repressor transcription factors, including constitutively expressed factors, such as Sp1, and signal-induced factors, such as NF-κB and AP-1 (Dutilleul et al., 2020). Although HIV-1-infected individuals with 20–50 copies/mL plasma viral load, which is lower than the definition of LLV, had higher levels of activated $CD38^+$ $CD4^+$ T cells than patients with virologic suppression (VS) (Younas et al., 2021), it is not clear whether $CD4^+$ T cells in LLV individuals are extensively activated and whether this activation leads to alterations in gene expression and eventually regulates viral replication and reactivation of the latent reservoir.

A switch from cell-mediated Th1 to humoral Th2 dominant $CD4^+$ cell response may be associated with the progression to AIDS in HIV-infected individuals (Clerici and G. M. Shearer, 1993). This view arose from the observation that disease progression in HIV-infected patients was associated with a loss of IL-2 and IFN-γ production and increased IL-4 and IL-10 levels in $CD4^+$ T cells. HIV-1 specific Th1 response and IgG_2 antibodies have been suggested as an important biomarker of long-term nonprogerssion (Martinez et al., 2005). A previous study showed that a high number of IFN-γ-producing cells and elevated Th1 to Th2 cytokine ratio suppressed HIV-1 infection in the lungs (Rubbo et al., 2011). Till now, The Th1 and Th2 differentiation pattern of $CD4^+$ T cells in LLV is unknown.

Cytokine-cytokine receptor interaction plays an important role in HIV infection. CCL3 and CCL4, natural ligands of CCR5 (one of the two major co-receptors of HIV-1), belong to β-chemokines that can be secreted by specific subsets of $CD4^+$ T cells to protect themselves from HIV-1 infection (Casazza et al., 2009, Claireaux et al., 2022). Abortive HIV-1 infection causes depletion of the majority of "bystander" $CD4^+$ T cells (Doitsh et al., 2010). The death of bystander $CD4^+$ T cells was predominantly caused by caspase-1-mediated pyroptosis and these cells released bioactive IL-1β, leading to inflammation (Doitsh et al., 2014). While this CD4-T cell depletion and inflammation cycle cause death of more immune cells, it is unclear whether and how $CD4^+$ T cells may regulate IL-1β signaling to stop this process.

Toll-like receptors (TLRs) are membrane-bound sensors that recognize conserved microbial products, including viruses, bacteria, and fungi (Macedo et al., 2019). The translocation of microbes from the gastrointestinal tract is a major cause of systematic immune activation in patients with chronic HIV infection (Brenchley et al., 2006). Patients with LLV presented more frequent microbial translocation than patients with VS and healthy individuals (Reus et al., 2013). The human genome encodes ten TLRs expressed in a wide range of cell types, including $CD4^+$ T cells. Most studies have focused on the role of TLRs in monocytes, dendritic cells, or peripheral blood mononuclear cells in HIV-1 infection (Browne, 2020). Little is known about the effects of TLRs on $CD4^+$ T cell activation and viral replication during HIV-1 infection.

In this study, we compared the whole transcriptome of peripheral blood $CD4^+$ T cells from healthy controls (HC) and HIV-infected patients with VS and LLV to elucidate the intrinsic characteristics of $CD4^+$ T cells in LLV which may promote HIV-1 replication. Important KEGG pathways mapped by differentially expressed genes (DEGs) acquired by comparing LLV to VS and HC included Th1 and Th2 cell differentiation, the toll-like receptor signaling pathway, cytokine-cytokine receptor interaction, NF-κB and TNF signaling pathways. Our study also focused on effects of transcription factors (TFs) that were upregulated in LLV compared to VS and HC on HIV-1 promoter activity and identified two of these TFs that specifically regulated HIV-1 transcription.

2. Materials and methods

2.1. Study cohort

HIV-infected individuals with LLV (n = 20) or VS (n = 18), and HC (n=18) were recruited from Guangzhou Eighth People's Hospital, China in 2021. $CD4^+$ T cells of six LLV, five VS and five HC were used for RNA sequencing and the $CD4^+$ T cells from the rest members of these three groups were used for real-time PCR assay. HIV-infected subjects had received ART for at least four years, and their annual plasma viral load (VL) was detected with the COBAS fully automatic viral load analysis system (Roche) with a lower limit of 20 copies/mL. VS individuals with VL lower than 20 copies/mL for four consecutive years and patients with LLV with VL between 50 and 1000 copies/mL for at least two consecutive years were enrolled (Supplementary Table S1).

2.2. $CD4^+$ T cell isolation

A total of 10–20 mL of peripheral blood was collected from both HC and HIV-infected individuals. Whole blood was balanced with one volume of phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque PLUS (Cytiva, Marlborough, USA). Thereafter PBMCs were washed twice with PBS, and $CD4^+$ T cells were negatively isolated with the Human CD4 T Lymphocyte Enrichment Set-DM (BD Biosciences, San Jose, USA).

2.3. RNA sequencing and data analysis

Total RNA from $CD4^+$ T cells (2×10^6) was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). RNA integrity was assessed using a Bioanalyzer 200 system (Agilent). One microgram of total RNA was used as the input for cDNA preparation. Sequencing libraries were then prepared using an Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Beverly, USA), and the quality of amplified libraries was analyzed using an Agilent Bioanalyzer 2100. The cDNA libraries were clustered using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, USA). After clustering, libraries were sequenced using an Illumina NovaSeq 6000, producing 150 bp paired-end reads.

Raw reads were processed with Perl, and clean reads were obtained by removing reads containing adapters or poly-N and low-quality reads from raw reads. The clean reads were aligned to the GRCh38 reference genome using Hisat2 v2.0.5. Read numbers for each gene were obtained using featureCounts v1.5.0-p3, and FPKM was calculated. Differential gene expression analysis was conducted using the DESeq2 R package (version 1.16.1). Statistical enrichment of the KEGG pathways of differentially expressed genes was performed using the clusterProfiler R package.

2.4. Reverse transcription and real-time PCR

One microgram of RNA was digested with RQ1 RNase-Free DNase (Promega, Madison, USA) according to manufacturer's instructions. The RNA was then reverse transcribed with PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan) according to manufacturer's instructions. Real-time PCR was performed using FastStart Universal SYBR Green Master (Rox) (Merck Millipore, Billerica, USA) on a Bio-Rad CFX96 real time-PCR detection system or a Thermo ABI QuantStudio DX Real-Time PCR. The real-time PCR condition was 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative mRNA level of gene was calculated by ΔΔCt with GAPDH as reference gene. Primers for real-time PCR were listed in Supplementary Table S2.

2.5. Cell culture

TZM-bl (NIH AIDS Reagent Program) and HEK293T cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Waltham, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, USA) at 37 °C with 5% CO_2. Cells were tested negative for mycoplasma contamination.

2.6. Plasmid construction

The HIV 5′-LTR promoter luciferase reporter was constructed by cloning the first 640 base pairs of pNL4-3 5′-LTR into pGL3 vectors. Mutations in the key *cis*-elements of the viral 5′-LTR were generated by site-directed mutagenesis. Coding sequences of 21 transcription factors were recovered from cDNA of HEK293T cells, TZM-bl cells, or PBMCs and cloned into the pcDNA3.1 vector. The sequences of all the plasmid constructs were confirmed by DNA sequencing.

2.7. Transfection

For siRNA transfection, TZM-bl cells were plated in 96 well plates 24 h prior to transfection. Three siRNAs (RiboBio, Guangzhou, China) targeting each transcription factor were mixed and transfected with 0.5 μL RNAiMAX (Invitrogen, Carlsbad, USA), resulting in a final siRNA concentration of 100 nmol/L (siRNA-targeting sequences were listed in Supplementary Table S3). After 12 h, 5 ng of the Tat construct was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Forty-eight hours after siRNA transfection, cells were washed twice with PBS and lysed with passive lysis buffer (Promega, Madison, USA), followed by luciferase activity measurement using a dual-luciferase assay kit (Promega, Madison, USA). For the overexpression assay, HEK293T cells were plated in 48 well plates and rested overnight. Three hundred nanograms of pcDNA3.1 constructs, 50 ng pGL3-HIV 5′-LTR, and 5 ng empty vector or Tat plasmid were co-transfected with 0.5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After 48 h, the cells were collected, and luciferase activity was measured.

2.8. Statistical analysis

Statistical analysis was performed using GraghPad Prism 6. Shapiro-Wilk normality test was used to examine whether data values were normally distributed. Unpaired *t*-test was used to analyze difference between groups with a normal distribution. Mann-Whitney test was used for calculating difference between groups that were not normally distributed. Data were considered significant at $P < 0.05$.

3. Results

3.1. Differentially expressed genes in HC, VS, and LLV

We collected peripheral blood $CD4^+$ T cells from five HC, five VS, and six LLV patients for RNA sequencing; the clinical characteristics of the participants are presented in Supplementary Table S1. Individuals with VS had a plasma viral load <20 copies/mL for at least four consecutive annual assessments, whereas the LLV persons developed a 50–1000 copies/mL viral load for at least two consecutive years. With a cutoff of adjusted $P < 0.05$ and fold change > 2, we identified 129, 1298, and 716 upregulated genes, and 7, 92, and 73 downregulated genes by comparing VS with HC (VS-HC), LLV with HC (LLV-HC), and LLV with VS (LLV-VS), respectively (Supplementary Table S4, Fig. 1A and B). DEGs in the LLV-HC group showed a greater magnitude of fold change than those in the VS-HC and LLV-VS groups, while DEGs in the VS-HC group exhibited the lowest absolute fold change (Fig. 1C). A total of 619 upregulated and 37 downregulated genes were shared between the LLV-HC (47.7% and 40.2% of the total number of upregulated and downregulated genes, respectively) and LLV-VS groups (86.5% and 50.7% of the total number of upregulated and downregulated genes, respectively) (Fig. 1D). Together, these results suggest that gene expression patterns of $CD4^+$ T cells in LLV were substantially different from those in VS and HC, which might be either a cause or a result of LLV.

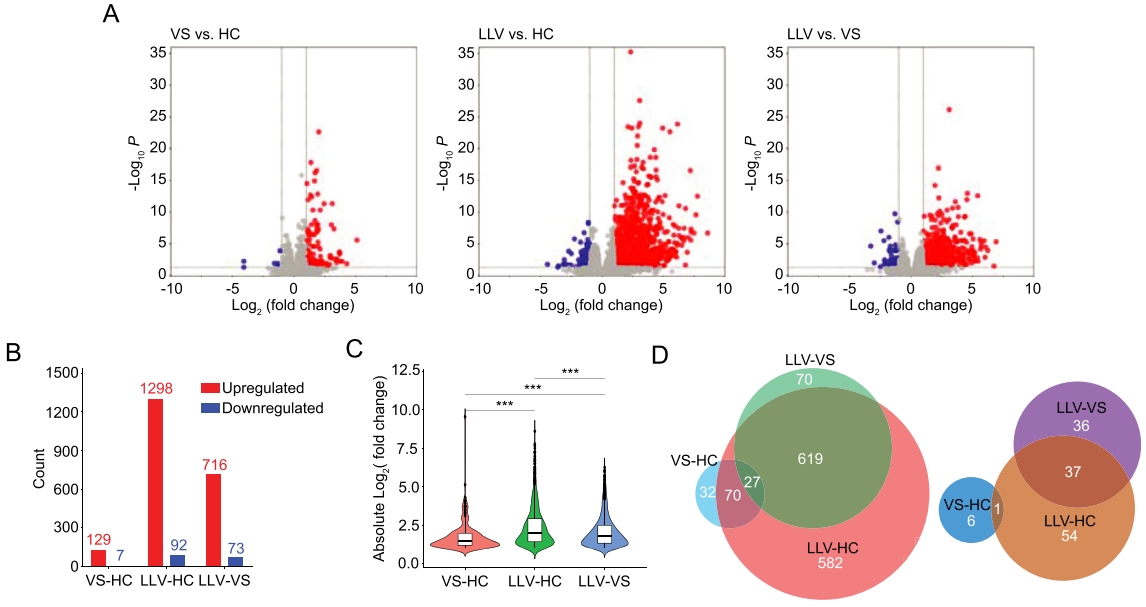

Fig. 1. Characteristics of differentially expressed genes in HC, VS, and LLV individuals. **A** Volcano plot identifying DEGs by comparing VS with HC (left), LLV with HC (middle), and LLV with VS (right) based on adjusted *P* value < 0.05, and fold change > 2. Significantly upregulated genes are shown in red, and significantly downregulated genes are shown in blue. **B** Number of upregulated and downregulated genes in the VS-HC, LLV-HC, and LLV-VS groups. **C** Violin plot showing absolute $\log_2$ (fold change) values of DEGs in the VS-HC, LLV-HC, and LLV-VS groups. **D** Euler diagram illustrating the overlap of upregulated (left) and downregulated (right) DEGs between the VS-HC, LLV-HC, and LLV-VS groups. ***, $P < 0.001$.

3.2. Key DEGs in overlapped KEGG pathways between VS-HC and LLV-VS groups

To characterize pathways potentially responding to increasing plasma viral load (HC to VS and to LLV), we compared overlapped DEG-mapped KEGG pathways of the VS-HC group with those of the LLV-VS group. Forty-one KEGG pathways were enriched in both these groups (Supplementary Table S5, Fig. 2A). Among these, we focused on three pathways that were the most related to the functions of $CD4^+$ T cells: Th1 and Th2 cell differentiation, toll-like receptor signaling, and cytokine-cytokine receptor interaction.

Regarding the genes involved in Th1 and Th2 cell differentiation, *IFNG* and *IL12A* were significantly upregulated in patients with VS compared to that in HC (Fig. 2B). IL-12α heterodimerizes with IL-12β to form IL-12, mediating Th1 development and promoting IFN-γ production in Th1 cells (Heufler et al., 1996). *IFNG* and *IL12A* were not significantly upregulated in LLV compared to that in VS (Fig. 2B). *TBX21*, *NOTCH3,* and *IL12RB2* levels were significantly higher in LLV than in VS (Fig. 2B). TBX21 is a lineage-defining transcription factor involved in Th1 development (Szabo et al., 2000). The NOTCH3-Delta1 interaction transduces signals promoting $CD4^+$ T cells toward the Th1 phenotype (Maekawa et al., 2003). IL12RB2, upregulated by IFN-γ in $CD4^+$ T cells, is a subunit of the IL12 receptor (Presky et al., 1996). Our data suggest that in LLV individuals, at least from an autocrine perspective, $CD4^+$ T cells differentiate towards a Th1-like phenotype not only by upregulating Th1 lineage-defining TBX21 but also by increasing Th1-related cytokine and receptor genes such as *INFG*, *IL12A*, *IL12B2,* and *NOTCH3*.

In the TLR signaling pathway, seven genes, including genes encoding proteins involved in downstream transcriptional regulation (*JUN*, *FOS,* and *NFKBIA*) and pro-inflammatory chemokines and cytokines (*CXCL8*, *TNF*, *CCL4,* and *IL12A*) (Fig. 2C), were markedly upregulated in VS compared with HC. The levels of *CXCL8*, *TNF,* and *CCL4* were also increased in LLV individuals. *TLR8*, *TLR4*, *TLR2*, *TLR6,* and *TLR7* levels were all significantly increased in LLV compared with VS (Fig. 2C). TLR2 and TLR6 are plasma membrane TLRs that form heterodimers and together recognize diacylated lipopeptides of gram-positive bacteria (Kang et al., 2009). In addition, the TLR2/6 dimer recognizes HIV-1 p24, leading to phosphorylation of IκBα, increased CXCL8 production, and CCR5 expression in primary T cells *in vitro* (Henrick et al., 2015). TLR4 recognizes lipopolysaccharides (LPS) from gram-positive bacteria (Poltorak et al., 1998). TLR7 and TLR8 are localized to endosomes and lysosomes and sense single-stranded RNA (ssRNA), including HIV-1 (Heil et al., 2004). Engagement of these TLRs with their ligands recruits adapter factors to activate downstream signaling pathways including NF-κB, AP-1 and interferon regulatory factors (Macedo et al., 2019).

Regarding cytokine-cytokine receptor interaction, 7 and 36 genes were significantly changed in the VS-HC and LLV-VS groups, respectively. There were five overlapping genes (Fig. 2D). *CCL4* was upregulated in the VS-HC and LLV-VS groups, and *CCL3* was upregulated in LLV versus VS (Fig. 2D). CCL3 and CCL4 are anti-HIV-1-entry chemokines (Casazza et al., 2009, Claireaux et al., 2022). Four IL-1-related genes (*IL1A*, *IL1B*, *IL1RN*, and *IL1R2*) were upregulated in LLV compared with VS (Fig. 2D). IL-1α and IL-1β are pro-inflammatory cytokines. IL-1α enhances HIV-1 replication (Mclaren et al., 2015). IL-1β is released by $CD4^+$

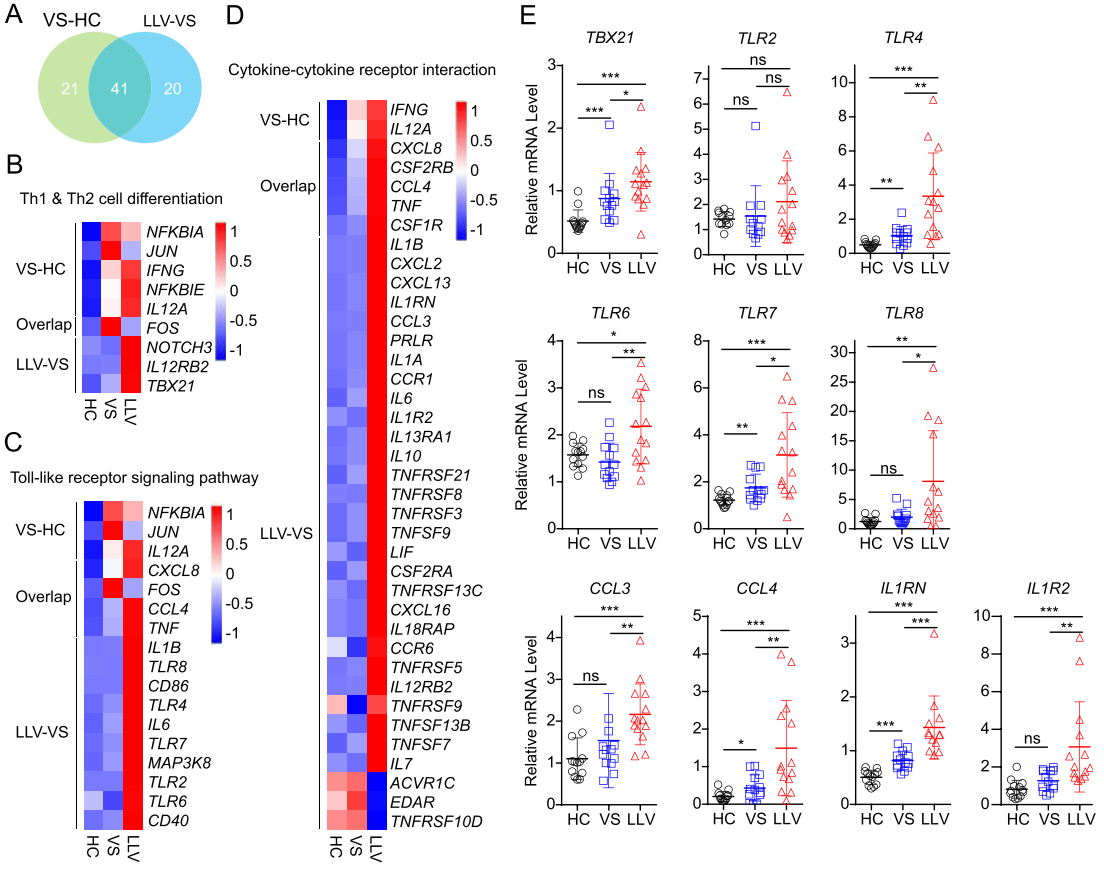

Fig. 2. Key KEGG pathways shared by VS-HC and LLV-VS groups. **A** Overlap of KEGG pathways between VS-HC and LLV-VS groups. **B-D** Relative expression levels of DEGs in Th1 and Th2 cell differentiation (**B**), toll-like receptor signaling pathway (**C**), and cytokine-cytokine receptor interaction (**D**) in $CD4^+$ T cells of HC, VS, and LLV individuals. Unless otherwise specified, "VS-HC", "LLV-VS", and "Overlap" indicate genes significantly changed in only the VS-HC group, LLV-VS group, or both groups, respectively, in all figures. **E** Quantification of relative mRNA levels of *TBX21*, *TLR2*, *TLR4*, *TLR6*, *TLR7*, *TLR8*, *CCL3*, *CCL4*, *IL1-RN* and *IL1R2* by real-time PCR in a larger population. HC (n = 13), VS (n = 13) and LLV (n = 14). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

T cells abortively infected with HIV-1 and links pyroptosis-induced depletion of CD4$^+$ T cells and inflammation (Doitsh et al., 2010, Doitsh et al., 2014). IL1RN inhibits IL-1α and IL-1β signaling by competitively interacting with receptor IL-1R1 (Eisenberg et al., 1990, Hannum et al., 1990). IL1R2 is a decoy receptor of IL-1α and IL-1β and competes for their binding to IL-1R1, thereby nullifying signaling (Giri et al., 1994). Increased expression levels of *ILRN* and *IL1R2* might be a negative feedback mechanism for the upregulation of *IL1A* and *IL1B*.

We next analyzed the expression levels of *TBX21*, *TLR-2*, *-4*, *-6*, *-7* and *-8*, *CCL3*, *CCL4*, *IL1RN* and *IL1R2* in a larger cohort by real-time PCR. The data showed that most of these genes were upregulated in LLV individuals compared to VS or HC, except TLR-2 that was not differentially expressed between these three groups (Fig. 2E). Taken together, these results showed that upregulated expression of Th1-lineage transcription factor (*TBX21*), toll-like receptors (*TLR-4*, *-6*, *-7* and *-8*) and important host factors in HIV infection (*CCL3, CCL4, IL1R2* and *IL1RN*) distinguished CD4$^+$ T cells of LLV from those of VS.

3.3. Evidence of activation of NF-κB and TNF signaling pathways in VS and LLV

Next we studied the NF-κB and TNF signaling pathways, which were both shared by the VS-HC and LLV-VS groups and are involved in HIV-1 transcription. TNF signaling triggers the activation of NF-κB, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Pasquereau et al., 2017). AP-1, downstream component of JNK, and some members of the CREB/ATF family, downstream components of MAPK and ERK, specifically bind to respective *cis*-elements of HIV-1 5′-LTR and enhance HIV-1 promoter activity (Rabbi et al., 1997, Yang et al., 1999, Yeung et al., 2009, Caselli et al., 2012, Banerjee et al., 2017). Based on the transcriptome analysis in our study, *TNF* was significantly increased in VS compared with that in HC and might activate NF-κB. *NFKBIA* and *TNFAIP3*, encoding inhibitory factors of NF-κB, were also markedly upregulated in VS compared with that in HC, implying negative feedback of NF-κB activation (Fig. 3A) (Browne, 2020, Van Den Eeckhout et al., 2020). *TNF, IL1B,* and *TLR4* were upregulated in LLV compared to VS. These factors can activate NF-κB (Fig. 3A). In addition to increased *TNF*, genes encoding AP-1 subunits, including *JUN*, *JUNB,* and *FOS*, were markedly upregulated in VS compared with that in HC (Fig. 3B). Our results suggested that upregulated *TNF*, *IL1B* and *TLR4* in LLV may activate NF-κB and TNF signaling pathway.

3.4. Effects of differentially expressed transcription factors on HIV-1 5′-LTR promoter activity

To better understand the role of transcription factors in HIV-1 transcription, we focused on the differentially expressed genes encoding TFs in the VS-HC and LLV-VS groups. Twenty-one TF genes were differentially expressed in the VS-HC group and were all upregulated, while fifty-nine transcription factor genes were differentially expressed (48 upregulated and 11 downregulated) in the LLV-VS group (Supplementary Table S6). Networks of upregulated TFs in these two groups were generated based on betweenness score, which indicates the centrality of a gene in a network (Yu et al., 2007). JUN and FOS had the highest betweenness scores in the VS-HC group, indicating that AP-1, consists of JUN and FOS, may play a leading role in these TFs (Fig. 3C). ATF3, which targets SWI/SNF in the HIV-1 promoter to stimulate transcription, also

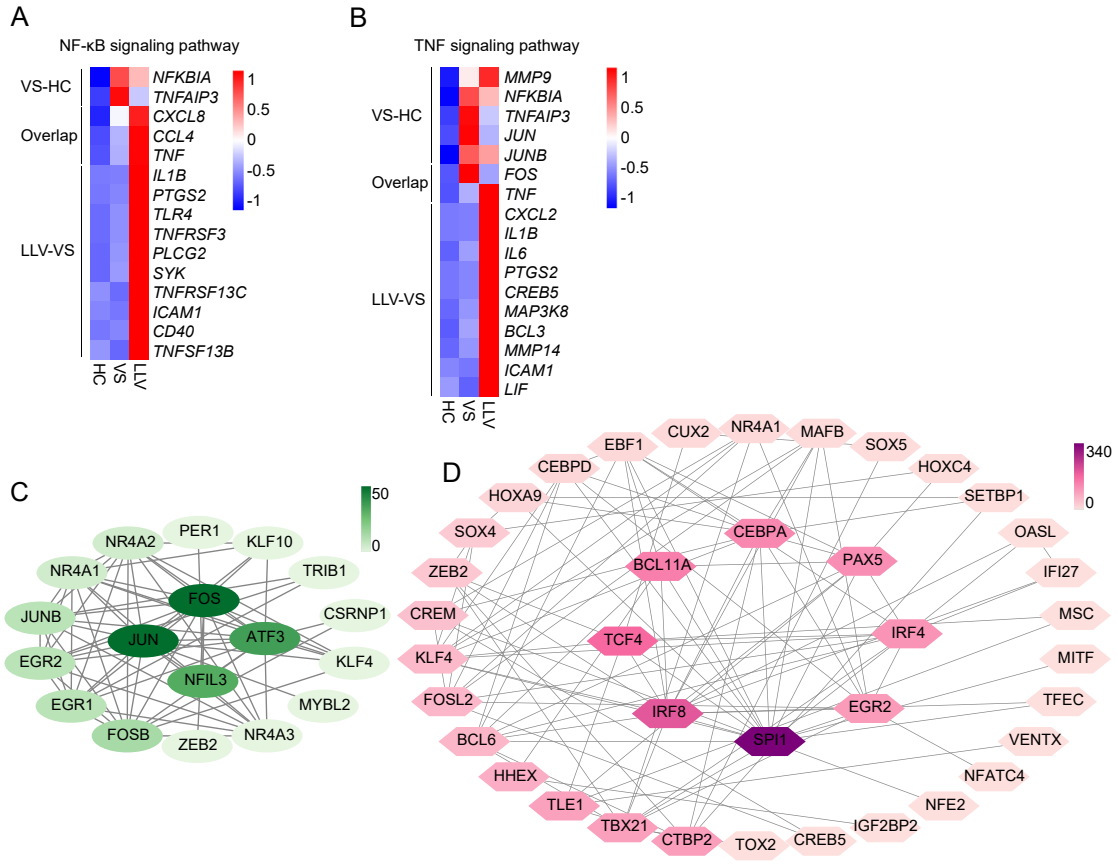

Fig. 3. Differentially expressed transcription factors in VS-HC and LLV-VS groups. **A**–**B** Relative expression levels of DEGs in NF-κB (A) and TNF signaling pathway (B) in HC, VS, and LLV individuals. **C**–**D** Betweenness-based networks formed by differentially expressed transcription factors in VS-HC (**C**) and LLV-VS groups (**D**). Betweenness scores are mapped by color intensity.

had a high betweenness score (Henderson et al., 2004). The highest betweenness score among all upregulated transcription factors in the LLV-VS group was assigned to SPI1, which binds to the HIV-1 *pol* HSP7 region and promotes viral replication (Fig. 3D) (Goffin et al., 2005). IRF8 and IRF4, belonging to the interferon regulatory factor family, play central roles in the network (Fig. 3D). IRF8 binds HIV-1 5′-LTR and, depending on the microbial stimulation, suppresses viral transcription (Viglianti et al., 2021). TCF4, ranking third among all transcription factors in LLV-VS, inhibited HIV-1 5′-LTR promoter activity as a downstream effector of the β-catenin pathway (Barbian et al., 2022).

Then, we selected 4 and 17 top upregulated transcription factors that have not been examined for their function in HIV-1 infection from the VS-HC and LLV-VS groups, respectively, to evaluate their effects on HIV-1 5′-LTR promoter activity (Fig. 4A). The expression levels of these TFs were firstly validated by real-time PCR in a larger cohort. The relative mRNA levels of these TFs in HC, VS and LLV groups were consistent with the RNA sequencing results, except that NR4A2 was downregulated in LLV versus VS (Fig. 4B). To examine whether these candidate genes affected viral transcription at endogenous level, we designed siRNAs targeting the 21 genes and validated their efficiency (Supplementary Fig. S1). siRNAs targeting each of the genes on the separated coding region were mixed and transfected into TZM-bl cells, which harbors one copy of a truncated HIV-1 genome with a firefly luciferase gene under the control of HIV-1 5′-LTR promoter. With a cutoff fold change ≥ 2, knockdown of NR4A1, MAFB, VENTX, SOX5, IFI27, HHEX, ETV7, TFEC, and YBX3 significantly enhanced HIV-1 gene expression in both absence and presence of Tat (Fig. 5A). Depletion of NR4A2, and EBF1 markedly increased HIV-1 promoter activity only in the presence of Tat (Fig. 5A). Downregulation of CXXC5 significantly decreased viral promoter transcription in both absence and presence of Tat (Fig. 5A). We then evaluated the function of the candidate transcription factors using an overexpression assay. The candidate genes were cloned into an overexpression vector pcDNA3.1, and each vector was co-transfected with a construct expressing firefly luciferase driven by HIV-1 5′-LTR. Overexpression of NR4A2, VENTX, SOX5, and IFI27 significantly inhibited, whereas CXXC5 markedly enhanced, viral transcription at the basal level (Fig. 5B). In the presence of Tat, co-expression of VENTX, SOX5, IFI27, and YBX3 significantly decreased viral promoter activity, whereas CXXC5 increased viral transcription (Fig. 5B). To clarify the specific effects of candidate transcription factors on HIV-1 5′-LTR promoter activity, we examined their effects on Simian Virus 40 (SV40) and cytomegalovirus immediate early (CMV-IE) promoters. Overexpression of IFI27, VENTX, and YBX3 showed inhibitory effects on the activities of the SV40 and CMV-IE promoters (Fig. 5C). NR4A2 decreased SV40 promoter activity but increased that of the CMV-IE promoter (Fig. 5C). SOX5 had a less negative effect on SV40 and CMV-IE promoters than HIV-1 5′-LTR (Fig. 5C). Instead of enhancing HIV-1 5′-LTR transcription, CXXC5 inhibited the activities of the SV40 and CMV-IE promoters (Fig. 5C). Collectively, our data showed that CXXC5 and SOX5 exerted coordinated effects on HIV-1 5′-LTR promoter activity in both knockdown and overexpression assays, respectively; both transcription factors showed HIV-1 5′-LTR-specific activities.

To date, neither CXXC5 nor SOX5 has been implicated in HIV-1 5′-LTR promoter activity. To map elements of HIV-1 5′-LTR possibly targeted by CXXC5 and SOX5, we studied their functions on 5′-LTR reporter constructs with loss-of-function mutations in NF-IL6-, USF-, TCF-1α-, NF-κB/NFAT-, AP-2-, and Sp1-binding sites (Fig. 6A) (Bosque and V. Planelles, 2009). At the basal level or with Tat induction, NF-κB/NFAT-binding site mutations markedly reduced the positive impact of CXXC5 on HIV 5′-LTR activity. Mutations in TCF-1α- and Sp1-binding sites showed a similar but not significant effect at the basal level (Fig. 6B). These results suggest that the CXXC5-mediated enhancement of HIV-1 5′-LTR activity is dependent on NF-κB/NFAT binding sites. Mutations in Sp1-binding sites on LTR markedly enhanced

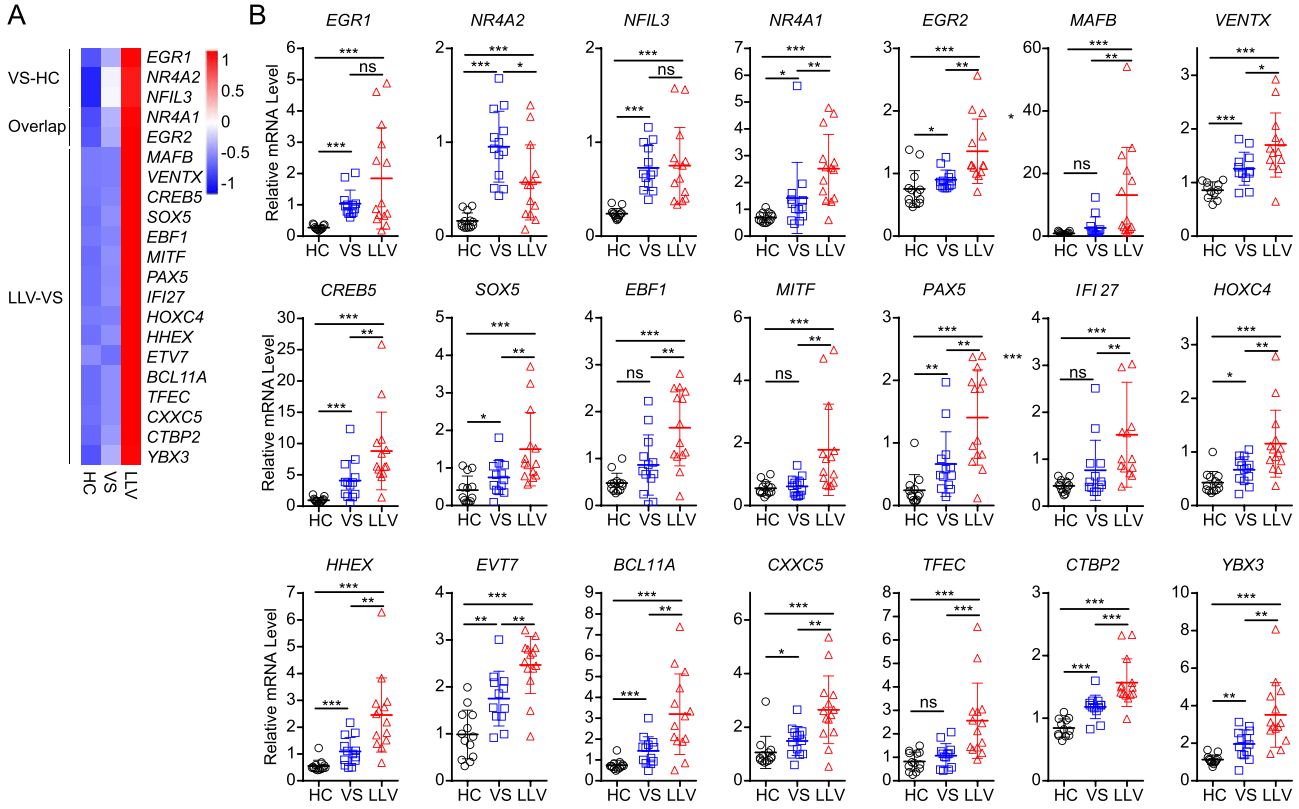

Fig. 4. Expression levels of candidate transcription factors upregulated in VS-HC and LLV-VS groups. **A** Relative expression levels of the 21 top upregulated TFs based on RNA sequencing analysis in HC (n = 5), VS (n = 5), and LLV (n = 6). **B** Quantification of relative mRNA levels of 21 candidate TFs by real-time PCR in a larger group. HC (n = 13), VS (n = 13) and LLV (n = 14). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

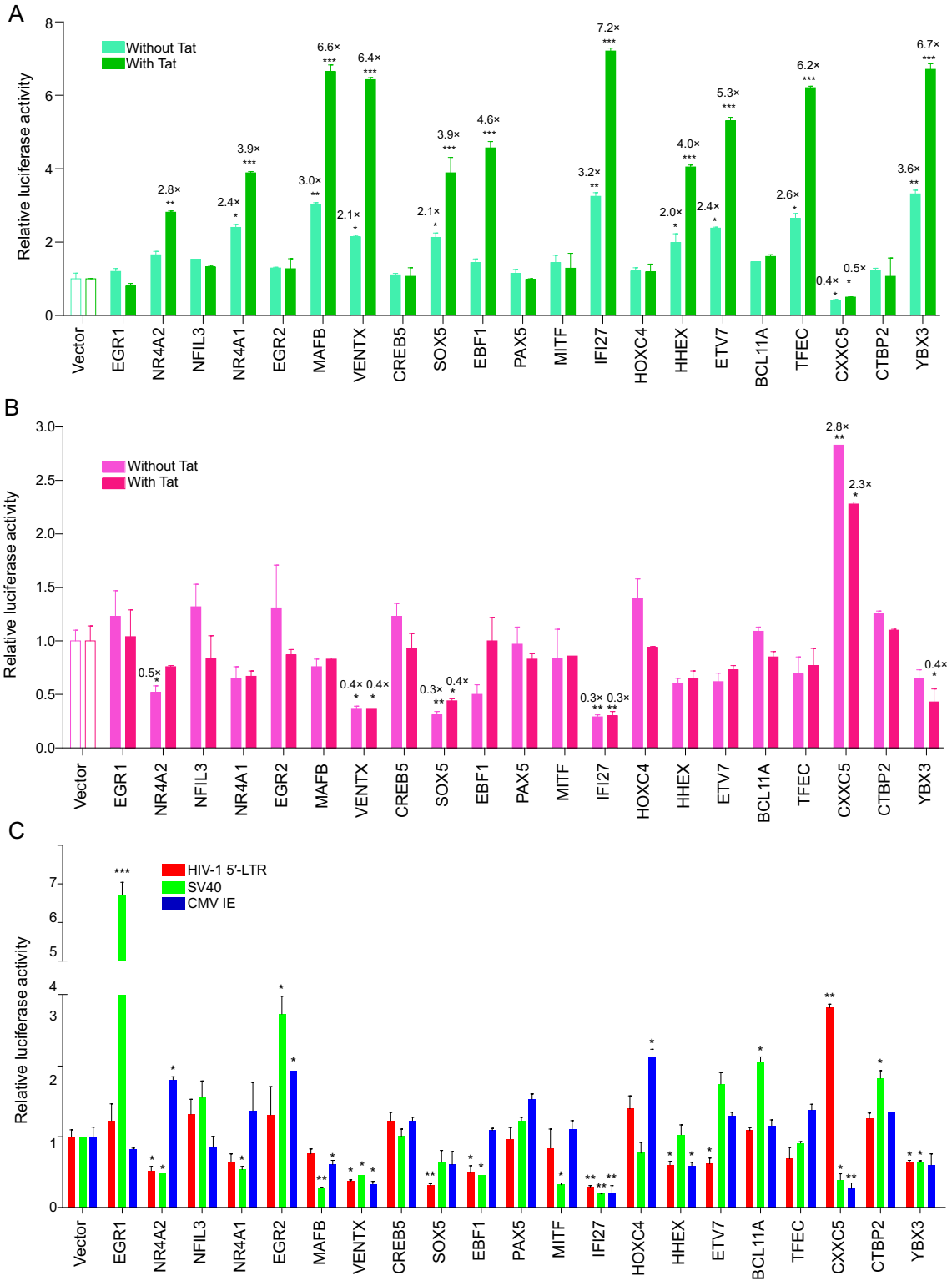

Fig. 5. Identification of candidate transcription factors (TFs) regulating HIV-1 promoter activity. **A** TZM-bl cells were transfected with a siRNA mixture targeting each of the candidate TFs in the absence or presence of Tat. Luciferase activities were determined 48 h later (mean ± SEM, n = 3). (**B**) Expression constructs of candidate transcription factors were co-transfected with the HIV-1 5′-LTR reporter vector with or without Tat in HEK293T cells. After 48 h, the luciferase activity was measured (mean ± SEM, n = 3). (**C**) Expression constructs of candidate genes were co-transfected with HIV-1 5′-LTR, SV40, or CMV-IE promoter reporter vectors in HEK293T cells. Luciferase activities were assessed 48 h after transfection (mean ± SEM, n = 3). Statistical significance was analyzed between luciferase activities of each reporter vector co-transfected with empty vector and vector encoding candidate transcription factors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inhibition induced by SOX5 with or without Tat, while mutations in TCF-1α- and NF-κB/NFAT-binding sites reduced the inhibitory effect of SOX5 slightly but not significantly in the presence of Tat (Fig. 6C). These data suggest that the inhibitory effect of SOX5 on HIV-1 5′-LTR could be antagonized by Sp1.

4. Discussion

The current study aimed to depict the distinct transcriptional characteristics of $CD4^+$ T cells in HIV-infected patients receiving ART with LLV compared to VS. We noticed a significant difference between the ART regimen used by LLV and VS recruited in this study (Supplementary Table S1), which may have possible impact on the transcriptome profiling of $CD4^+$ T cells. In the present cohort, the majority of LLV were treated with protease inhibitor [PIs; lopinavir in this study (LVP/r)] or integrase inhibitor [INSTIs; dolutegravir (DTG) and bictegravir (BIC) in this study], while the majority of VS were treated with non-nucleoside reverse transcriptase inhibitor [NNRTIs; efavirenz (EFV) in this study]. One important reason underlining this difference is that according to the Chinese guidelines for diagnosis and treatment of HIV/AID (Chinese Medical Association et al., 2021), ART regimen is recommended to switch to PIs or INSTIs when virologic failure occurs after 24-week treatment, and LLV individuals recruited in this study should have their ART regimen changed after years of low-level viremia. Indeed, LLV is associated with increased incidence of switch to second-line ART (Hermans et al., 2018). LVP/r has similar impact on immune function parameters of $CD4^+$ T cells as EVF(Torres et al., 2014), therefore, LVP/r may possibly not induce significant changes in RNA profiling of $CD4^+$ T cells compared to EFV. However, studies of DTG and BIC mainly focused on their side effects and roles in $CD4^+$ recovery (Kolakowska et al., 2019, Nickel et al., 2021), thus their impacts on $CD4^+$T cell status are yet to be determined.

Our results show that Th1 signature genes, including *TBX21*, *NOTCH3* and *IL12RB2*, were upregulated in $CD4^+$ T cells in LLV compared to VS (Fig. 2B, E). Although Th1 dominant immunity is an effective response to constrain HIV infection, patients with LLV experienced less controlled viral replication than those with VS. It was possible that these *TBX21*-upregulated Th1-like $CD4^+$ T cells in LLV were not fully functional. One evidence is that *IFNG*, of which the proximal promoter region is the direct target of TBX21, was not markedly increased in LLV versus VS (Fig. 2B) (Yang et al., 2020). NR4A1 is a mediator contributing to dysfunction of $CD4^+$ T cells (X. Liu et al., 2019). Both of our RNA sequencing and real-time PCR results showed significant higher expression of *NR4A1* in LLV compared to VS (Fig. 4A and B), suggesting that the Th1-like $CD4^+$ T cells in LLV may become dysfunctional. We also found that *IL10*, encoding a Th2 anti-inflammation cytokine, was increased in LLV versus VS (Fig. 2D). Unlike mice, human Th1 $CD4^+$ T cells can express IL10 by the cooperation of NFIL3 and TBX21 (Zhu et al., 2015, Rasquinha et al., 2021). As *NFIIL3* was upregulated in LLV compared to HC (Fig. 4A and B), $CD4^+$ T cells in LLV may increase IL10 production to suppress HIV-1-replication induced inflammation.

Expression of genes encoding ani-HIV entry chemokines, *CCL3 and CCL4,* and IL-1β inhibitory factors, *IL1RN* and *IL1R2*, were increased in patients with LLV compared to individuals with VS (Fig. 2D and E). $CD4^+$ T cells with protective autocrine of CCL4 against HIV-1 have *CCL4* mRNA upregulated (Casazza et al., 2009, Claireaux et al., 2022). The mRNA level of *CCL4* in LLV was about 2 and 6 times higher than that of VS and HC in real-time PCR results, respectively (Fig. 2E). These fold changes seem to be less significant than that of previous report (Casazza et al., 2009), thus whether increased mRNA of *CCL4* led to protective CCL4 production in LLV needs further functional validation. Increased expression of *IL1RN* and *IL1R2* in LLV can act as a double-edged sword. On one hand, upregulation of *IL1RN* and *IL1R2* may inhibit IL-1β signaling and therefore prevent the depletion of $CD4^+$ T cells. On the other hand, these survived $CD4^+$ T cells which may be latently infected by HIV-1 can contribute to size of HIV-1 latent reservoir.

Our study firstly focused on the well-demonstrated pathways regulating HIV-1 replication. NF-κB and TNF signaling pathways, both of which play important roles in HIV-1 5′-LTR transcription initiation, were enriched by DEGs in LLV-VS group (Fig. 3A and B). *TNF, IL1B and TLR4* was significantly higher in LLV compared to VS. Signaling of these factors led to activation of NF-κB and AP-1, contributing to HIV-1 transcription activation. In addition to *TLR4*, *TLR-6*, *-7,* and *-8* were also markedly upregulated in LLV versus VS (Fig. 2C, E). Possible microbial translocation in LLV may induce TLR4 and TLR6 signaling, leading to activation of NF-κB and AP-1 (Reus et al., 2013, Macedo et al., 2019). Moreover, engagement of TLR8, possibly by HIV-1 ssRNA, together with TCR signaling in $CD4^+$ T cells, may activate HIV-1 latency (Meas et al., 2020). Indeed, several TLR agonists, as the next generation of LRAs, have reached clinical trials aimed at not only reactivating viral latency but also enhancing the whole antiviral immune response (Macedo et al., 2019).

To identify differentially expressed transcription factors in VS-HC and LLV-VS groups that may regulate HIV transcription, we selected 21 candidate genes for functional studies (Fig. 4). We found that CXXC5 and SOX5 are novel transcription factors that potentially regulate HIV-1 5′-LTR promoter activity (Fig. 5). CXXC5 is a member of the CXXC domain-containing protein family. The CXXC domain binds to unmethylated CpG dinucleotides, and members of the CXXC-domain protein family play important roles in epigenetic regulation (Xiong et al., 2019). Twelve members of this family are found in mammals; of them, only CXXC5 is characterized by a short length and only one functional domain, a C-terminal CXXC domain (Xiong et al., 2019). Our functional studies showed that CXXC5 enhances HIV-1 5′-LTR promoter activity in either the absence or presence of Tat. In contrast, CXXC5 suppressed the transcriptional activity of the SV40 and CMV-IE promoters, suggesting that CXXC5 could play opposite roles in the replication of different viruses. Consistent with our findings, CXXC5 has been shown to promote or repress gene transcription depending on its associated partners. In plasmacytoid dendritic cells, CXXC5 promotes IRF7 transcription by recruiting Tet2, a methylcytosine dioxygenase that converts methylcytosine to 5-hydroxymethylcytosine, to mediate the demethylation of CpG islands in *IRF7* gene region, thereby contributing to TLR7/9-mediated antiviral response (Ma et al., 2017). In $CD8^+$ cells, CXXC5 associates with SUV39H1, a histone lysine methyltransferase, to induce H3K9me3 modifications in the *CD40L* promoter region and inhibit CD40L expression (Tsuchiya et al., 2016). We found that the positive effect of CXXC5 on HIV-1 transcription was dependent on the NF-κB-binding sites in the viral 5′-LTR (Fig. 6). Two possible mechanisms can explain this result: (a) CXXC5 binding site(s) overlapped with that of NF-κB, and consequently, mutations in the NF-κB-binding DNA sequence compromised the CXXC5-DNA interaction; and (b) NF-κB could promote CXXC5 interaction with HIV-1 5′-LTR, and the failure of NF-κB recruitment to LTR decreased binding of CXXC5 to viral DNA. For the second hypothesis, as NF-κB plays a vital role in the signal-dependent activation of HIV-1 transcription initiation, CXXC5 could be involved in related signaling pathways to promote viral replication. CXXC5 was upregulated in $CD4^+$ T cells in LLV individuals and, therefore, could be one of the host factors contributing to low-level viremia in these patients.

SOX5 belongs to the SoxD subfamily of the *SOX* gene family characterized by an evolutionarily conserved high-mobility-group box (HMG box) for DNA binding (Liang et al., 2020). Unlike other SOX subfamilies, SoxD proteins contain two coiled-coil domains, a leucine zipper, and a glutamine-rich motif in the N-terminus, thereby allowing SoxD members to form either homo- or heterodimers. This feature enables the DNA-binding flexibility of SoxD proteins. Our results showed that SOX5 suppressed HIV-1 5′-LTR promoter activity, and loss of Sp1 binding in LTR enhanced its inhibitory effect (Figs. 5 and 6). SOX5 inhibits the expression of epithelial-specific marker E-cadherin, promoting cancer epithelial-mesenchymal transition (Pei et al., 2014). However, whether SOX5 directly targets E-cadherin genomic elements to inhibit transcription remains unknown. SOX5 may indirectly repress gene transcription.

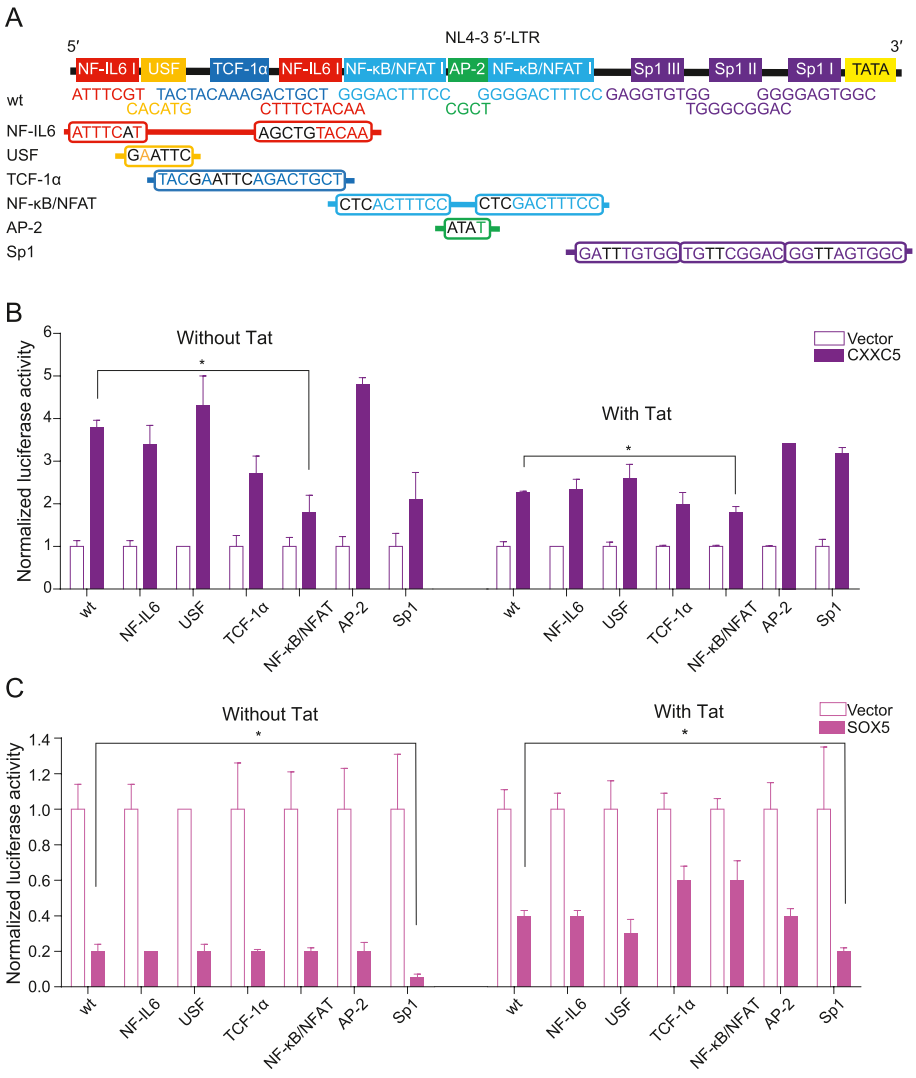

Fig. 6. Effects of CXXC5 and SOX5 on HIV-1 transcription were dependent on key *cis*-elements on viral 5′-LTR. **A** Schematic diagram of NL4-3 5′-LTR mutations in essential transcription factor-binding sites. **B** Empty or CXXC5 coding vectors were co-transfected with wild-type or mutant HIV-1 5′-LTR reporter vectors into HEK293T cells at the basal level (left) or with Tat induction (right). Forty-eight hours later, firefly luciferase activity was measured (mean ± SEM, n = 3). Luciferase activities of wild-type or mutant 5′-LTRs in CXXC5-coexpressing cells were first normalized to that of the empty vector, and then statistical significance between fold changes induced by CXXC5 in wild-type and mutant LTRs was analyzed. **C** Empty or SOX5 expression vectors were co-transfected with wild-type or mutant HIV-1 5′-LTR luciferase constructs in HEK293T cells with (left) or without Tat (right). Firefly luciferase activities were assessed 48 h after transfection (mean ± SEM, n = 3). Luciferase activities of wild-type or mutant 5′-LTRs in SOX5-overexpressing cells were normalized to that of the empty vector, and statistical significance between fold changes induced by SOX5 in wild-type and mutant LTRs was analyzed. *, $P < 0.05$.

EZH2 is the catalytic subunit of the polycomb repressive complex 2 that catalyzes the trimethylation of lysine 27 on histone H3, resulting in transcriptional silencing. SOX5 binds to the enhancer of EZH2 and transactivates its expression, possibly contributing to downstream transcription repression (Sun et al., 2019). Three tandem Sp1 binding sites located upstream of the TATA box in HIV-1 5′-LTR are required for the reassembly of the pre-initiation complex. Loss-of-function mutations in Sp1 binding sites on HIV-1 5′-LTR enhanced SOX5-mediated inhibition of viral gene expression, indicating that SOX5 might directly target the HIV-1 promoter to suppress its activity (Fig. 6). One mechanism explaining the interplay between SOX5 and Sp1 in HIV-1 promoter activity is that the binding of SOX5 to the 5′-LTR was weakened by Sp1 binding, and once Sp1 could not interact with viral 5′-LTR, SOX5 binding was strengthened, leading to stronger inhibition.

In this study, we identified several genes encoding transcription factors, which regulate HIV promoter activity, upregulated in LLV compared to VS. These TFs included SPI1 and CXXC5 that promote HIV-1 5′-LTR activity, as well as IRF8, TCF4 and SOX5, with inhibitory effect on viral transcription. We also provided evidence of activation of NF-κB and AP-1. Considering the expression levels and activation status of these transcription factors, the overall outcome of active HIV-1 replication and activation of viral latency in LLV may result from a dominant effect of HIV-1 transcription activators over repressors. Of all transcription factors binds to HIV-1 5′-LTR, only a few play essential roles in HIV transcription initiation, including TATA-box binding protein, Sp1, and NF-κB (Dutilleul et al., 2020). The HIV-1 5′-LTR enhancer region, containing at least two NF-κB-binding sites, is one of the strongest *cis*-regulatory elements. Activated NF-κB binds to the enhancer region and play a key role in viral transcription activation. In LLV, upregulation of *TNF*, *TLR4*, *TLR6* and *IL1B* potently led to downstream NF-κB and AP-1 activation. Consequently, activated NF-κB and AP-1, together with upregulated *SPI1* and *CXXC5*, dominated the inhibitory effects of *IRF8*, *TCF4* and *SOX5*, thus leading to active HIV-1 transcription. These features of transcription factors of $CD4^+$ T cells in HIV-infected individuals with persistent LLV,

together with drug resistance development and low drug concentration in tissues, may contribute to subsequent virologic failure.

5. Conclusions

For the first time, our study revealed novel transcriptome characteristics of peripheral blood $CD4^+$ T cells of HIV-infected patients with LLV compared to VS, including upregualtion of genes encoding Th1 lineage transcription factor, TLRs, anti-HIV entry chemokines, and IL-1β-inhibitory cytokine and receptors. We also provided evidence of activation of the NF-κB and TNF signaling pathways in $CD4^+$ T cells in LLV individuals, which potently contributed to HIV-1 replication and reactivation of latent reservoir. Of all the transcription factors significantly upregulated in LLV, CXXC5 and SOX5 specifically regulated HIV-1 5′-LTR promoter activity, which provides a new understanding of host factors modulating HIV-1 infection in $CD4^+$ T cells of LLV. CXXC5 and SOX5 may therefore be candidate targets for new anti-HIV treatments.

Data availability

All the data generated in the current study are included in the manuscript and in supplementary files.

Ethics statement

This study was approved by the Ethics Committee of Guangzhou Eighth People's Hospital (202033166), and all participants provided written informed consent.

Author contributions

Jingliang Chen: funding acquisition, investigation, writing-original draft. Yaozu He: investigation, formal analysis, data curation. Huolin Zhong: funding acquisition, investigation. Fengyu Hu: Conceptualization. Yonghong Li: resources, project administration. Yeyang Zhang: resources, methodology. Xia Zhang, Weiyin Lin, Quanmin Li, Feilong Xua and Shaozhen Chen: resources. Hui Zhang: supervision, writing-review and editing. Weiping Cai: supervision, writing-review and editing. Linghua Li: funding acquisition, supervision, writing-review and editing.

Conflict of interest

The authors declare no competing conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.03.001.

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