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ISSN

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Publication Date

DOI

Peer reviewed

Select host restriction factors are associated with HIV persistence during antiretroviral therapy

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Objective: The eradication of HIV necessitates elimination of the HIV latent reservoir. Identifying host determinants governing latency and reservoir size in the setting of antiretroviral therapy (ART) is an important step in developing strategies to cure HIV infection. We sought to determine the impact of cell-intrinsic immunity on the HIV latent reservoir.

Design: We investigated the relevance of a comprehensive panel of established anti-HIV-1 host restriction factors to multiple established virologic and immunologic measures of viral persistence in HIV-1-infected, ART-suppressed individuals.

Methods: We measured the mRNA expression of 42 anti-HIV-1 host restriction factors, levels of cell-associated HIV-1 RNA, levels of total *pol* and 2-long terminal repeat (2-LTR) circle HIV-1 DNA and immunophenotypes of $CD4^+$ T cells in 72 HIV-1-infected individuals on suppressive ART (23 individuals initiated ART less than 1 year post-infection, and 49 individuals initiated ART greater than 1 year post-infection). Correlations were analysed using nonparametric tests.

Results: The enhanced expression of a few select host restriction factors, p21, schlafen 11 and PAF1, was strongly associated with reduced $CD4^+$ T-cell associated HIV RNA during ART ($P < 0.001$). In addition, our data suggested that ART perturbs the regulatory relationship between $CD4^+$ T-cell activation and restriction factor expression. Lastly, cell-intrinsic immune responses were significantly enhanced in individuals who initiated ART during early versus chronic infection and may contribute to the reduced reservoir size observed in these individuals.

Conclusion: Intrinsic immune responses modulate HIV persistence during suppressive ART and may be manipulated to enhance the efficacy of ART and promote viral eradication through reversal of latency *in vivo*.

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Introduction

Antiretroviral therapy (ART) has demonstrated efficacy and durability in suppressing HIV replication in infected individuals. However, ART does not achieve viral eradication due to the persistence of latently infected long-lived cells [1,2]. The elimination of the latent reservoir is critical to achieving HIV eradication. Identifying host determinants governing latency, viral production and reservoir size *in vivo* is an important step in developing effective strategies to clear the latent reservoir and cure HIV infection.

Data are limited describing the role of the host molecular and immunologic environment in defining HIV reservoir size. Recent reports involving *ex vivo* and *in vitro* approaches suggest that CD8^{+} cytotoxic T cells (CTLs) may mediate clearance and affect reservoir size [3,4]. Cell surface expression of CD38 and HLA-DR immune activation markers and programmed cell death protein 1 (PD-1) on CD4^{+} T cells is associated with levels of cell-associated HIV DNA and RNA during ART [5]. Circulating levels of anti-HIV-1 antibodies also reflect the degree of HIV persistence and may decay to undetectable levels when eradication has been achieved [6,7]. Cell surface expression levels of C-C chemokine receptor type 5 (CCR5), an entry coreceptor for HIV-1, are positively correlated with viral transcription during ART [8].

Several intrinsic immune genes have been discovered that restrict HIV replication in the absence of antiretroviral drugs. These factors include bone marrow stromal antigen 2 (BST-2)/tetherin [9,10], p21 [11], schlafen 11 (SLFN11) [12], SAM domain and HD domain-containing protein 1 (SAMHD1) [13], the RNA polymerase II associated factor 1 (PAF1) complex [14] and members of the tripartite motif (TRIM) [15–18] and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) families [19–27]. In addition to the robust literature describing the anti-HIV-1 effects of these factors *in vitro*, our group has recently demonstrated the relevance of restriction factors to the control of HIV in chronically infected individuals undergoing interferon-α/ribavirin therapy [28,29], and in HIV elite controllers, individuals who maintain undetectable viremia in the absence of ART [30]. Recent reports have demonstrated associations between the expression levels of cell-intrinsic antiviral factors and HIV reservoir size and transcriptional activity in elite controllers [31,32]. Genetic analyses of HIV proviral populations in interferon-α/ribavirin-treated and ART-suppressed individuals demonstrate that APOBEC3-mediated hypermutation plays a significant role in modulating the latent reservoir [29,33]. As yet, there are no data linking the expression of cell-intrinsic immune factors to the HIV latent reservoir during suppressive ART. In this study, we hypothesized that anti-HIV-1 host restriction factors play a role in defining HIV

reservoir size during ART, by suppressing viral expression and ongoing viral replication. To address this hypothesis, we examined relationships between multiple established measures of viral persistence during ART and gene expression levels of a comprehensive panel of anti-HIV-1 restriction factors.

Materials and methods

Individuals and specimen processing

Twenty million cryopreserved peripheral blood mononuclear cells (PBMCs) were collected retrospectively from 72 ART-suppressed HIV-infected individuals enrolled in the SCOPE and Options cohorts within 1–2 years of ART initiation. Individual characteristics and treatment information are documented in detail in Supplementary Tables 1 and 2, http://links.lww.com/QAD/A631. Plasma RNA viral load and CD4^{+} T-cell counts were measured at all patient visits. Individuals were segregated into treatment timing groups on the basis of their estimated infection date and treatment start date: the early group initiated treatment less than 1 year (range = 0.11–0.58 years) after the estimated date of infection, while the chronic group initiated treatment more than 1 year after the estimated date of infection. All individuals provided written informed consent. This study was approved by the UCSF Committee on Human Research. Five hundred thousand cells were used for immunophenotyping by flow cytometry. CD4^{+} T cells were enriched from the remaining PBMCs (19.5 million cells) by negative selection using the EasySep Human CD4+ T Cell Enrichment Kit (Stemcell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's instructions. Genomic DNA and total RNA were extracted from enriched CD4^{+} T cells (1–2 million cells) using the Allprep DNA/RNA/miRNA Universal Kit (Qiagen, Frederick, Maryland, USA) with on-column DNase treatment (Qiagen RNase-Free DNase Set). HLA typing was performed using PCR sequence specific oligonucleotide probing as previously described [34]. CCR5 genotyping was performed using an endpoint PCR-based assay as previously described [8].

T-cell immunophenotyping

Markers of T-cell activation were measured using flow cytometry at the UCSF Core Immunology Laboratory, as previously described [35]. Briefly, cryopreserved PBMCs were quickly thawed, washed in warm FACS buffer (2 mmol/l EDTA, 0.5% BSA in ice-cold PBS) and surface stained for 30 min at 4°C. Cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, California, USA) and then stained with the following fluorescently conjugated mAbs: CD8-QDOT-605 (clone 3B5) and CD4-PE-Texas Red (clone S3.5) from Invitrogen; CD3-V450 (clone UCHT1), PD-1 Alexa Fluor-647 (clone EH12.1) and CD38-PE (clone

HB7), HLA-DR-FITC (clone L243), CD45RA-PE-Cy7 (clone L48) from BD Biosciences (San Jose, California, USA); and CCR7-APCeFluor780 (clone 3D12) from eBioscience (San Diego, California, USA). Stained cells were washed and resuspended in 0.5% formaldehyde. Rainbow beads (Spherotec, Lake Forest, Illinois, USA) were used to standardize instrument settings between runs. At least 250 000 lymphocytes were collected for each sample. Data were analysed using FlowJo software (version 9; Tree Star Inc., Ashland, Oregon, USA).

Gene expression profiling

Four hundred nanograms of RNA was transcribed into cDNA using random primers and the SuperScript VILO cDNA Synthesis Kit (Invitrogen), according to manufacturer's instructions. Quantitative real-time PCR utilized custom-made TaqMan low-density arrays (TLDAs) from Applied Biosystems (Foster City, California, USA) following the manufacturer's instructions. Thermal cycling was performed using an Applied Biosystems ViiA 7 Real-Time PCR System. Up to 450 ng cDNA in 200 μl of Applied Biosystems TaqMan Universal PCR Master Mix with UNG was loaded onto the designated ports of the TLDA plates. Data were analysed using the Applied Biosystems ViiA 7 software. A panel of six housekeeping genes was included in the TLDA plates (GAPDH, 18S, ACTB, PPIA, RPLP0 and UBC). *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was identified as the most stably expressed gene from those six housekeeping genes among all samples using the GeNorm algorithm [36]. Therefore, raw cycle threshold numbers of amplified gene products were normalized to the housekeeping gene, *GAPDH*, to control for cDNA input amounts. Fold induction was determined using the comparative Ct method [36].

Cell-associated total HIV RNA quantification by quantitative PCR

Cell-associated HIV RNA was quantified using a Nanodrop (ND-1000) spectrophotometer and normalized to cell equivalents by quantitative PCR (qPCR) using human genomic GAPDH expression. Cellular total HIV RNA levels were quantified with a qPCR TaqMan assay using long terminal repeat (LTR)-specific primers F522−43 (5′ GCC TCA ATA AAG CTT GCC TTG A 3′; HXB2 522−543) and R626−43 (5′ GGG CGC CAC TGC TAG AGA 3′; 626−643) coupled with a FAM-BQ probe (5′ CCA GAG TCA CAC AAC AGA CGG GCA CA 3′) on a StepOne Plus Real-time PCR System (Applied Biosystems) [37]. Cell-associated total HIV RNA copy numbers were determined in a reaction volume of 20 μl with 10 μl of 2× TaqMan RNA to Ct 1 Step kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), 4 pmol of each primer, 4 pmol of probe, 0.5 μl reverse transcriptase and 5 μl of RNA. Cycling conditions were 50°C for 2 min, 95°C for 10 min, then 60 cycles of 95°C for 15 s and 59°C

for 1 min. About 250−500 ng of total cellular RNA per sample were characterized in triplicate reaction wells, and copy numbers were determined by extrapolation against a seven-point standard curve (1−10 000 copies) performed in triplicate.

## Cell-associated total HIV DNA quantification by droplet digital PCR assay

Cell-associated HIV *pol* and 2-LTR circle DNA were quantified by droplet digital PCR as previously described [38]. In brief, droplets for the PCR were generated with a Bio-Rad QX-100 emulsification device. Amplicons were HXB2 position 2536−2662 for HIV pol and HXB2 position 9585−51 for 2-LTR circle DNA. HIV DNA levels were normalized to cell number using a separate droplet digital PCR assay for RPP30 DNA. About 1 μg of total cellular DNA per sample was characterized in triplicate droplet digital PCR reactions.

Statistical analysis and data visualization

Spearman's rank correlation coefficient and two-tailed Mann−Whitney *U* tests were performed using GraphPad Prism (version 6.0c; GraphPad Inc., San Diego, California, USA). Bonferroni correction was performed to adjust for multiple comparisons. The heat map was generated using standardized *Z*-scores, and the clustering dendrogram depicting relatedness between gene expression profiles was generated using hierarchical clustering with complete linkage (created using the R statistical package).

Results

Levels of cell-associated HIV *pol* and 2-LTR circle DNA and HIV RNA were measured in negatively selected peripheral blood CD4$^+$ T cells from 72 HIV-1-infected individuals on suppressive ART. Multiparametric flow cytometry was used to assess the percentage of activation markers, including HLA-DR, CD38 and PD-1, on CD4$^+$ T cells. We observed considerable variability in these immunologic and virologic parameters (Fig. 1). The median and interquartile range (IQR) for each virologic parameter were as follows (copies per million cells): cell-associated HIV RNA: 1217.7 (357.9−3265.7); cell-associated HIV *pol* DNA: 419.1 (152.6−1039.9); cell-associated HIV 2-LTR circle DNA: 42.5 (17.3−71.8); HIV transcriptional ratio (ratio of cell-associated HIV RNA to cell-associated HIV *pol* DNA): 2.6 (1.4−5.2). The median and IQR for each immunologic parameter were as follows: percentage of CD4$^+$ T cells coexpressing the HLA-DR and CD38 activation markers: 4.8 (3.6−7.1); percentage of CD4$^+$ T cells expressing the PD-1 exhaustion marker: 14.9 (12.2−21.2). The observed variability in virologic and immunologic parameters could not be attributed to established host genetic predictors of viral control in the setting of
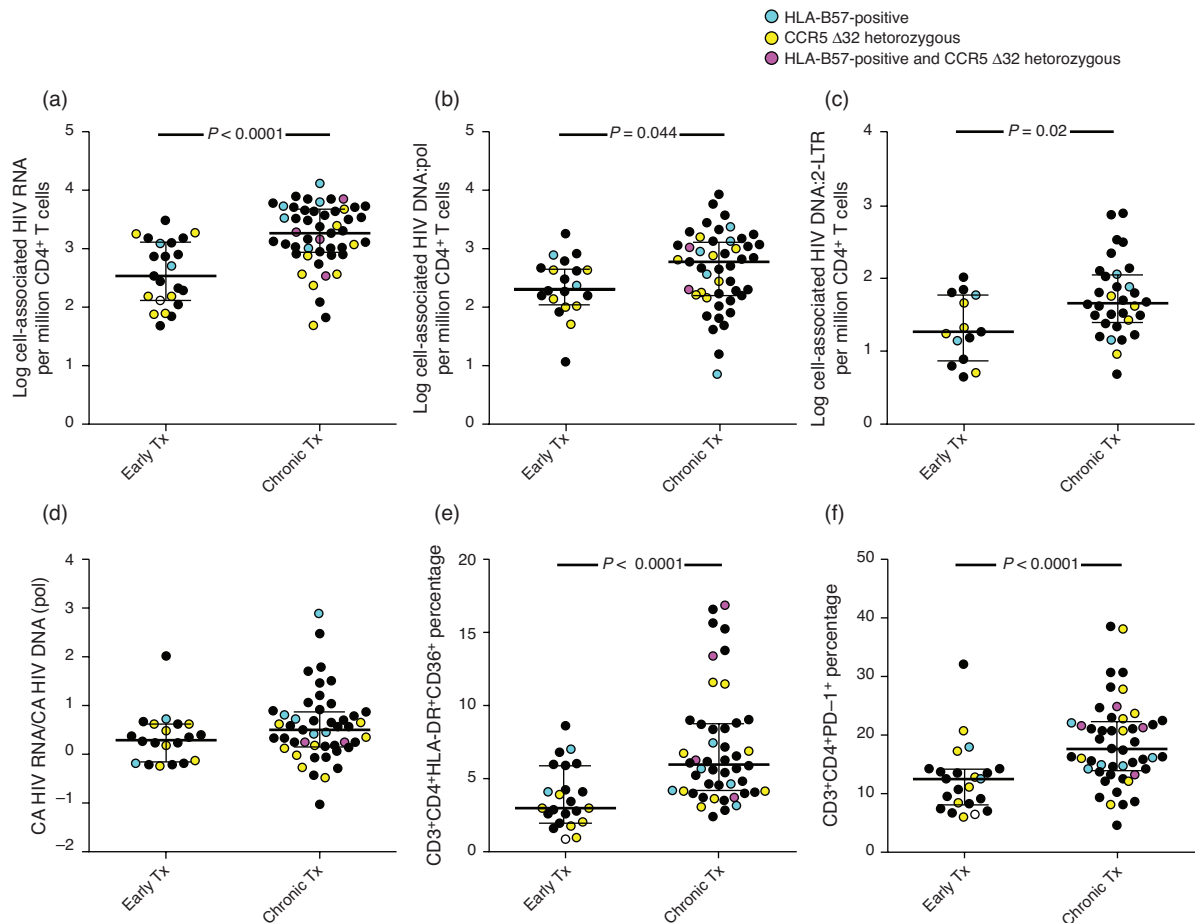
Fig. 1. Variability of HIV reservoir measurements in relation to antiretroviral therapy initiation timing and host genotype. Multiple virologic and immunologic measures of the HIV reservoir are displayed for 72 individuals on suppressive ART. Early Tx and Chronic Tx labels represent individuals who initiated ART <1 year after infection and >1 year after infection, respectively. (a−c) Frequencies of HIV nucleic acids in peripheral CD4$^+$ T cells. (d) Transcriptional ratio (ratio of cell-associated HIV RNA to cell-associated HIV *pol* DNA). (e,f) Cell surface expression of activation and exhaustion markers. Individuals who were heterozygous for the CCR5 delta-32 mutation are highlighted in yellow, individuals carrying the HLA-B57 allele are highlighted in cyan, and individuals with both of these genetic features are highlighted in pink. Lines and error bars represent median and interquartile range. *P* values were obtained using Mann–Whitney tests. CA, cell-associated.

untreated infection such as the HLA–B57 allele or the CCR5 delta–32 mutation (Fig. 1) (Supplementary Table 3, http://links.lww.com/QAD/A631). The only significant correlation observed between any parameter and either of these host genetic polymorphisms was a negative correlation between CD4$^+$ T-cell-associated HIV RNA and the presence of the CCR5 delta–32 mutation, as previously reported by our group [8]. The CCR5 delta–32 mutation accounted for 13.6% of the variance in cell-associated RNA, suggesting that other factors contribute appreciably to this variable.

Of the 72 individuals in our study, 23 individuals initiated ART during acute/early infection (<1 year, range 0.11–0.58 years) and 49 individuals initiated ART during chronic infection (>1 year). The early and chronic treatment initiation groups were similar in terms of age, sex, ethnicity and treatment duration (Supplementary

Table 1, http://links.lww.com/QAD/A631). No difference was observed in pretreatment viral load between groups (*P* = 0.478) (Supplementary Figure 1, http://links.lww.com/QAD/A631). Individuals who initiated ART during early infection exhibited lower cell-associated HIV RNA (*P* < 0.0001), lower HIV *pol* DNA (*P* = 0.04), lower HIV 2-LTR circle DNA (0.02) and lower expression of activation and exhaustion markers (*P* < 0.0001) than individuals who initiated ART during chronic infection (Fig. 1).

To test the hypothesis that cell–intrinsic immunity affects HIV reservoir size during suppressive ART, we examined relationships between measures of the latent reservoir and gene expression levels of 42 anti-HIV-1 host restriction factors (Fig. 2). After correction for multiple comparisons using the stringent Bonferroni test (considering each of the 42 restriction factors as distinct
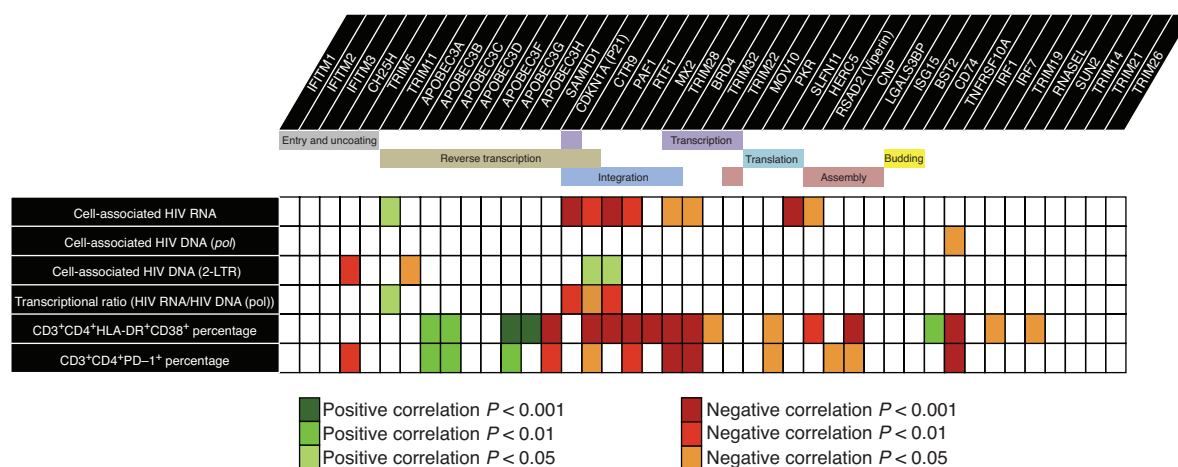
Fig. 2. Heat map describing statistical associations between HIV reservoir size and host restriction factor expression in antiretroviral therapy suppressed individuals. Restriction factors are arranged by targeted viral life cycle stage (when known), displayed under factor names. The Bonferroni-corrected *P* value threshold was *P* < 0.0012; correlations highlighted in dark green and dark red were significant after correction. Correlations were evaluated using Spearman's rank correlation coefficient tests.

statistical experiments), cell-associated HIV RNA was negatively correlated with the expression of three host restriction factors: p21 (*P* < 0.0001, corrected *P* = 0.002), SLFN11 (*P* < 0.0005, corrected *P* = 0.02) and PAF1 (*P* < 0.001, corrected *P* = 0.035) (Fig. 2, Fig. 3, Supplementary Table 4, http://links.lww.com/QAD/A631). To test whether CD4$^+$ T-cell composition played a role in the observed relationships, we examined the correlations between the expression of p21, SLFN11 and PAF1 and the percentage of naive, central memory, effector memory and terminal differentiated effector memory (TEMRA) CD4$^+$ T cells. p21 and SLFN11 expression did not correlate with percentage of any of the four CD4$^+$ T-cell subsets, whereas PAF1 expression correlated positively with the percentage of naive CD4$^+$ T cells (*P* = 0.005), and negatively with the percentage of effector memory CD4$^+$ T cells (*P* < 0.0001) (Supplementary Figure 2, http://links.lww.com/QAD/A631).

No correlation was observed between pretreatment viral load and levels of cell–associated HIV RNA during ART (*P* = 0.177) (Supplementary Figure 3, http://links.lww.com/QAD/A631).

There were no significant associations observed between levels of cell-associated HIV *pol* or 2-LTR circle DNA and restriction factor expression after Bonferroni correction (Fig. 2). Although not quite statistically significant after correction, HIV transcriptional activity, defined as the ratio of HIV RNA to *pol* DNA [39], correlated negatively with the expression of both p21 (*P* = 0.002, corrected *P* = 0.088) and PAF1 (*P* = 0.001, corrected *P* = 0.055) (Fig. 2, Supplementary Table 4, http://links.lww.com/QAD/A631).

The expression of several restriction factors exhibited significant correlations with percentage of CD4$^+$ T cells
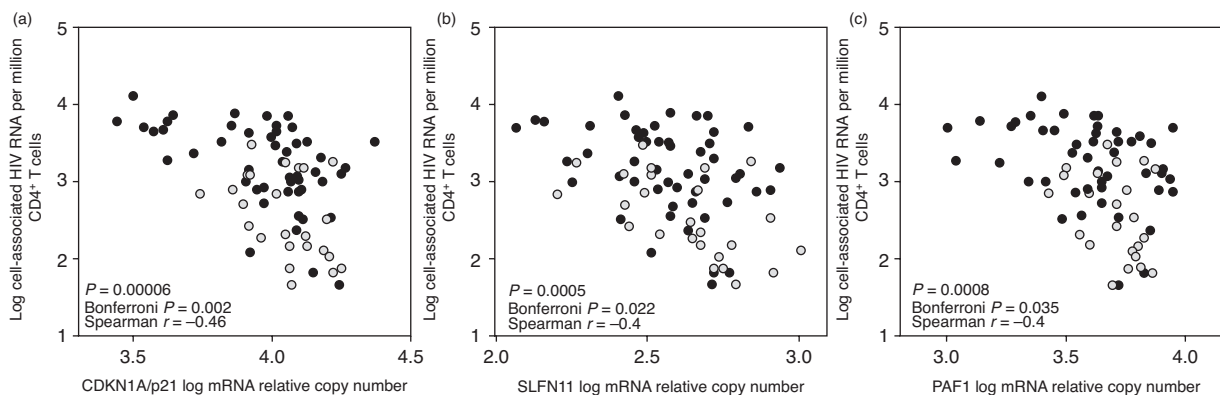

Fig. 3. Correlations between cell-associated HIV RNA and expression of the p21, SLFN11 and PAF1 host restriction factors in antiretroviral therapy suppressed individuals. Both raw and Bonferroni-corrected *P* values are displayed for correlations between CD4$^+$ T-cell-associated HIV RNA and (a) p21 mRNA expression, (b) SLFN11 mRNA expression and (c) PAF1 mRNA expression. Individuals who initiated ART during early and chronic infection are labelled with silver and black dots, respectively.

expressing markers of T-cell activation (CD38, HLA-DR) and exhaustion (PD-1) during ART (Fig. 2, Supplementary Table 4, http://links.lww.com/QAD/A631). In particular, the expression of eight host restriction factors correlated negatively with HLA-DR, CD38 coexpression on CD4$^+$ T cells after Bonferroni correction: bromodomain containing 4 (BRD4) ($P < 0.0001$, corrected $P < 0.004$), tumor necrosis factor receptor superfamily member 10A (TNFRSF10A) ($P < 0.0001$, corrected $P < 0.004$), TRIM28 ($P < 0.0001$, corrected $P < 0.004$), CTR9 ($P = 0.0001$, corrected $P = 0.004$), 2',3'-cyclic-nucleotide 3'-phosphodiesterase ($P = 0.0002$, corrected $P = 0.008$), RTF1 ($P = 0.0003$, corrected $P = 0.01$), MX2 ($P = 0.0004$, corrected $P = 0.017$) and SAMHD1 ($P = 0.0004$, corrected $P = 0.017$). Expression of two factors correlated positively with the coexpression of these activation markers: APOBEC3G ($P < 0.0001$, corrected $P < 0.004$) and APOBEC3H ($P = 0.0007$, corrected $P = 0.03$). The expression of three factors correlated negatively with the percentage of CD4$^+$ T cells expressing the PD-1 exhaustion marker: BRD4 ($P = 0.0002$, corrected $P = 0.008$), TNFRSF10A ($P = 0.0004$, corrected $P = 0.016$) and TRIM28 ($P = 0.0004$, corrected $P < 0.016$) (Fig. 2). Comprehensive gene-by-gene statistics are presented in Supplementary Table 4, http://links.lww.com/QAD/A631.

We next examined the relationship between restriction factor expression patterns and ART initiation timing. Early ART initiators exhibited significantly elevated restriction factor expression as compared with individuals who initiated ART during chronic infection (Figs. 4 and 5). A total of 16 individual restriction factors exhibited higher expression levels in individuals who initiated ART during early infection (Figs. 4 and 5), while no factors exhibited the opposite pattern.

Discussion

Characterization of the interplay between cell-intrinsic antiviral responses and HIV persistence may lead to novel approaches to reduce the size of the viral reservoir and suppress persistent inflammation during ART. We hypothesized that the expression profile of host restriction factors plays an important role in determining HIV reservoir size during suppressive therapy. To address this hypothesis, we performed a comprehensive assessment of 42 established anti-HIV-1 host restriction factors and examined their relationships with immunologic and virologic measurements of the HIV reservoir in CD4$^+$ T cells from ART-suppressed individuals. Our data
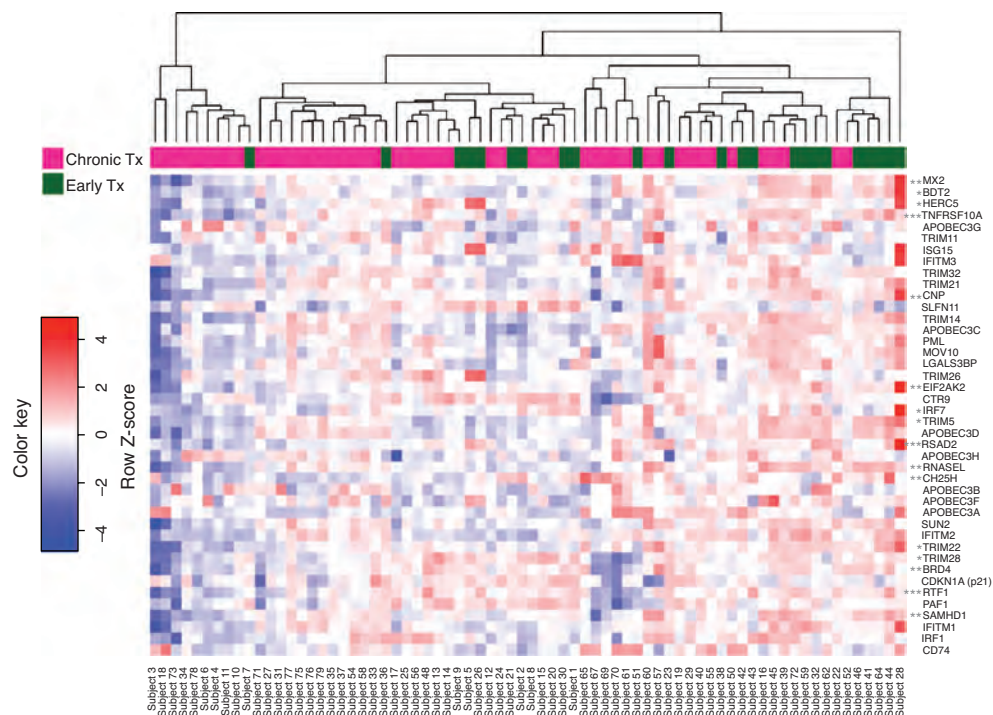
Fig. 4. Heat map and dendrogram depicting host restriction factor gene expression patterns in 72 HIV-infected individuals on suppressive antiretroviral therapy. Heat colours show standardized Z-scores across samples; red indicates upregulated expression, and blue indicates downregulated expression. The dendrogram depicting relatedness between individual gene expression profiles was generated using hierarchical clustering with complete linkage. Asterisks indicate statistically significant elevation of gene expression in Early Tx group versus Chronic Tx group as follows: *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ (no genes exhibited elevated expression in the Chronic Tx group). All statistical comparisons were performed using a Mann–Whitney test.
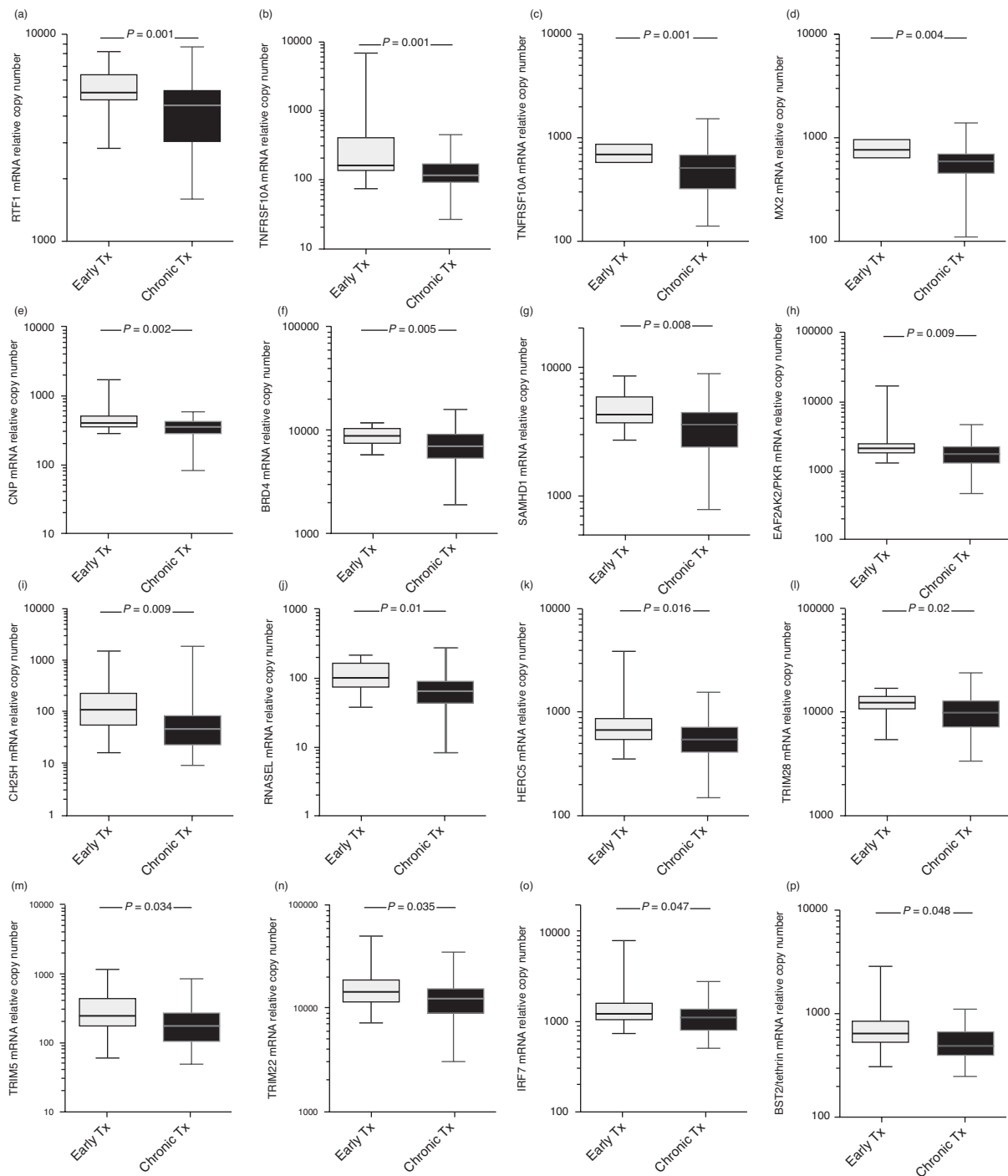
Fig. 5. Elevated host restriction factor expression in individuals who initiated antiretroviral therapy during early infection. Early Tx and Chronic Tx labels represent individuals who initiated ART <1 year after infection and >1 year after infection, respectively. Whisker bars represent minimum to maximum range. All statistical comparisons were performed using a Mann–Whitney test.

revealed that a few select host restriction factors, p21, SLFN11 and PAF1, exhibit highly significant negative correlations with levels of cell-associated HIV RNA, and may therefore contribute to the control of viral expression and ongoing replication during ART. Curiously, these factors are not induced by interferon-α treatment *in vivo*, in contrast to the majority of recognized anti-HIV-1

restriction factors [28]. This regulatory feature may be pertinent to the development of interventions to attenuate HIV-associated inflammation and immune activation through the blockade of type I interferon signalling [40], as interferon-α blockade may not compromise the viral control mediated by these particular factors. The molecular biology and cellular roles of the

p21 and SLFN11 factors have been extensively characterized, providing key insights into the mechanisms underlying our observations. It is provocative that these particular restriction factors exert their antiviral activity indirectly by depleting critical cellular resources supporting viral replication, in contrast to factors such as APOBEC3G, BST2-tetherin and TRIM5α that attack HIV directly.

The p21 cyclin–dependent kinase (Cdk) inhibitor has been associated with the control or repression of HIV *in vitro* [41−44], and with the natural control of HIV *in vivo* [11]. p21 inhibits trans-activator protein (Tat)-mediated HIV transcription by suppressing the activity of the positive transcription elongation factor (P-TEFb) in the host cell [11,45−51]. Therefore, in the setting of complete ART-mediated virologic suppression, the inverse relationship between p21 expression and CD4^{+} T-cell-associated HIV RNA observed in our study may primarily reflect p21-enforced repression of proviral transcription. In addition to P-TEFb pathway-dependent effects, p21 may inhibit HIV reverse transcription by promoting the activity of another host restriction factor, SAMHD1 [42]. SAMHD1 interferes with HIV reverse transcription by reducing the intracellular deoxyribonucleoside triphosphate (dNTP) pool [52], and promoting degradation of viral genomic RNA through ribonuclease activity [53]. In direct relation to the biology of HIV in the setting of ART, recent publications have revealed that SAMHD1 increases viral sensitivity to nucleoside reverse transcriptase inhibitors (NRTIs) through depletion of cellular dNTP levels [54,55]. Therefore, our observed relationship between p21 and HIV reservoir size may be attributed to the beneficial effects of p21 expression on the capacity of ART to effectively and thoroughly suppress viral replication. This hypothesis is additionally bolstered by recent data demonstrating that p21 inhibits HIV reverse transcription by suppressing CDK2-dependent phosphorylation of reverse transcriptase [56].

Along with p21, elevated expression of SLFN11 has been associated with HIV elite controller status [30]. SLFN11 is a recently identified anti–HIV-1 restriction factor that potently suppresses HIV replication by codon usage based inhibition of HIV protein synthesis [12]. SLFN11 inhibits HIV protein translation, but the current concept of its antiviral activity does not include repression of HIV transcription. Therefore, SLFN11-mediated suppression of cell-associated HIV RNA during ART likely results from its capacity to block ongoing replication. The mechanism underlying PAF1 antiviral activity has not been characterized in great detail as yet, although transcriptional repression is a possible candidate considering its known role in transcriptional elongation and histone modification [14,57].

Our secondary objective was to assess the relationship between the expression of host restriction factors and cellular activation in the setting of ART. Cell-based measurements of viral persistence are consistently associated with markers of immune activation and the frequency of PD-1-expressing CD4^{+} T cells [5]. In contrast to previously published data on ART-untreated individuals revealing positive correlations between restriction factor expression, viral load and T-cell activation [30,58,59], the expression of several restriction factors during ART exhibited significant negative correlations with percentage of CD4^{+} T cells expressing markers of T-cell activation and exhaustion. Our data therefore suggest that ART may perturb the biology and regulation of restriction factors, which may have important implications for the development of HIV curative strategies. The efficacy of the 'shock and kill' HIV eradication framework depends critically on pervasive reactivation and production of latent viruses, as well as the complete inhibition of new infection events upon viral reactivation [60,61]. Restriction factors that attack HIV postintegration (e.g. BST-2 and SLFN11) may impede viral production and limit efficacy, although factors that attack HIV preintegration (e.g. APOBEC3 and SAMHD1) may enhance efficacy by blocking new infection events. Considering that many latency-reversing agents (LRAs) induce cellular activation [60,61], our data suggest that administration of these agents likely modulates the expression of multiple host restriction mechanisms, impacting their efficacy. For example, our data revealed that SAMHD1 expression may be reduced upon cellular activation during suppressive ART, and as mentioned previously, SAMHD1 increases NRTI efficacy [54,55]. LRA-mediated downregulation of SAMHD1 may therefore lead to incomplete ART-mediated suppression of viral replication, which may allow reseeding of the viral reservoir when latency is reversed therapeutically. LRAs may benefit from coadministration with small molecules or gene therapeutic approaches that tailor the activity of restriction mechanisms within infected and uninfected target cells. A limitation of our study is the lack of longitudinal analyses; characterization of pre-ART and post-ART samples from the same individuals would bolster our conclusions regarding the effects of ART on restriction factor expression and its relationship to cellular activation. The data presented here will benefit from investigation of ART effects in other cohorts, as well as detailed *in vitro* experiments to gauge the direct effects of LRAs on restriction factor expression.

In summary, a few select host restriction factors contribute to the control of HIV during suppressive ART. Our findings represent the first evidence of cell-intrinsic immune responses *in vivo* modulating HIV persistence in the setting of suppression. Although it is difficult to unequivocally demonstrate a causal relationship between restriction factor expression and the suppression of HIV transcription *in vivo* during ART, our findings and the robust literature on p21 and SLFN11

activity *in vitro* and *ex vivo* strongly suggest that these factors should be explored within the context of HIV latency reversal and eradication strategies. Lastly, the enhanced anti-HIV-1 intrinsic immune responses and decreased 2-LTR circle frequencies (possibly indicative of reduced viral replication) observed in individuals who initiated ART during early infection warrants additional exploration. This pattern suggests that cell-intrinsic immune mechanisms may play a role in the favourable disease outcomes associated with this group and provides another possible justification for universal early administration of ART.

Acknowledgements

Conflicts of interest

D.D.R. has consulted for Chimerix, BMS, Gilead, Gen-Probe, Heras, Merck, HIV Immunotherapeutics Institute, and Monogram. No competing financial interests exist for the other authors.

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