Early treated HIV-1 positive individuals demonstrate similar restriction factor expression profile as long-term non-progressors

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ABSTRACT

Background: A wide range of host restriction factors (RF) become upregulated upon HIV-1 infection to suppress viral infectivity and may aid viremic control in vivo. This cross-sectional study evaluated HIV-1 RFs and dependency factors in HIV infected individuals with progressive or non-progressive infection, as well as in early and late treated cohorts that exhibit different viro-immunological profiles due to differences in timing of treatment-initiation.

Methods: The expression profile of IFIT1, MX1, APOBEC3G, SAMHD1, BST2 (encoding TETHERIN), TRIM5, MX2, SLFN11, PAF1, PSIP1 (encoding LEDGF/p75), and NLRX1 was measured by qPCR in 104 HIV-1 positive individuals: seroconverters (SRCV; $n = 19$), long term non-progressors (LTNP; $n = 17$), viremic progressors (VP; $n = 12$), patients treated during seroconversion (Early treated; $n = 24$) or chronic infection (Late treated; $n = 32$), and non-infected controls.

Findings: Expression levels of early treated HIV-1 positive individuals were significantly upregulated in comparison to late treated patients (IFIT1: $p = 0.0003$; MX1: $p = 0.008$; APOBEC3G: $p = 0.002$; SAMHD1: $p = 0.0008$; SLFN11: $p < 0.0001$; BST2: $p < 0.0001$). Similarly, SLFN11, BST2, and SAMHD1 were highly expressed in LTNPs at comparable levels as in early treated HIV-1 positive individuals. Furthermore, SLFN11 and SAMHD1 expression negatively correlated with total and integrated HIV-1 DNA levels.

Interpretation: Early treatment initiation maintains initial RF elevation even after a decade of ART. Elevated expression of SLFN11, BST2, and SAMHD1 in LTNP and early treated subjects implies that these RFs may be associated with spontaneous virological control.

1. Introduction

A variety of pattern-recognition receptors are triggered by infection with human immunodeficiency virus-1 (HIV-1), and rapidly activate innate immune responses, including type I interferon (IFN) production. Subsequently, a set of antiviral response genes, collectively known as interferon-stimulated genes (ISGs), are induced [1]. Specific ISGs subclasses, the so-called restriction factors (RF), limit retroviral replication at distinct stages of the viral life cycle, and may contribute to HIV-1 control in vivo. The best characterized RFs include bone marrow stromal cell antigen 2 (BST2)/tetherin,

apolipoprotein B mRNA editing enzyme catalytic subunit 3G (APOBEC3G), SAM domain and HD domain containing protein 1 (SAMHD1) and tripartite motif containing 5 (TRIM5). RFs exhibit their anti-viral effects at different time points of the viral replication cycle: SAMHD1 depletes the dNTP pool interfering with reverse transcription, TRIM5 restricts HIV-1 early after infection by targeting the viral capsid, APOBEC3G is known to cause hypermutation of viral sequences, and tetherin restricts HIV-1 replication by inhibiting viral particle release [1,2]. Additional RFs have been described recently, such as MX2, SLFN11, PAF1, and SERINC3/5, which interfere with nuclear import, protein translation of viral RNAs, early events in replication cycle and fusion, respectively [1–5]. HIV-1 has developed various strategies to overcome the RF activity through a number of HIV-1 accessory proteins, such as the viral infectivity factor (Vif), viral protein U (Vpu), negative regulatory factor (Nef), and viral protein R (Vpr) [6].

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Research in context

Evidence before this study

The role of restriction factors in limiting HIV-1 replication has been intensively investigated *in vitro*, however, *in vivo* data is limited and often incomplete. Expression of some restriction and antiviral factors, *e.g. SLFN11*, *SAMHD1*, and *CDKN1A/ p21*, has been linked to non-progressive HIV-1 infection *in vivo*, in which HIV-1 positive individuals are able to maintain undetectable or low viral load levels without therapy initiation, the so-called elite controllers or long term non-progressors. Only few studies have examined restriction factor profiles in these specific HIV-1 patient cohorts and conflicting data about their contribution to the elite controller or long term non-progressor status exists. Therefore, additional research is necessary to understand how varied restriction factor expression patterns impact the clinical outcomes seen in different cohorts of HIV-1 positive individuals in order to better understand the potential contribution of these factors to a non-progressive phenotype.

Added value of this study

In the present study, we assessed expression levels of two antiviral factors, seven restriction factors and two dependency factors in a large number of patients ($n = 104$), who are grouped within five well-defined cohorts of HIV-1 positive individuals based on differential levels of virological control and treatment characteristics. These include seroconverters, viremic progressors, long term non-progressors, early treated individuals, who initiated therapy during seroconversion, and late treated HIV-1 positive individuals, treated during chronic infection. This is the first study to evaluate RF profile in such large and well-characterized cohorts. Our study clearly pointed to a signature of RF profile associated with non-progressive infection and showed that *SLFN11*, *BST2* and, to a lesser extent *SAMHD1*, were upregulated in long term non-progressors. Importantly, the same restriction factors were also significantly upregulated in early treated individuals suggesting that *SLFN11*, *BST2* and *SAMHD1* upregulation is specifically linked to cohorts characterized by a small reservoir size and favorable prognosis. Moreover, this is the first study to demonstrate a negative correlation between *SLFN11*, *SAMHD1* levels with total and integrated HIV-1 DNA, clearly implicating the involvement of the two RFs in restricting HIV-1 reservoir.

Implications of all the available evidence

Our study and previous data consistently show the impact of timing of treatment initiation on restriction factor expression profiles, suggesting that early therapeutic intervention prevents depletion of the early innate antiviral immune responses in peripheral blood mononuclear cells. Additionally, the potential contribution of restriction factors *SLFN11* and *SAMHD1* to a non-progressive HIV-1 infection *in vivo* as reported previously was confirmed in this analysis. Therefore, this study complements existing literature on the role of RFs in HIV reservoir restriction and provides an extensive and comprehensive overview of multiple antiviral factors and dependency factors profiles in HIV-infected patients with different virological and clinical status.

A recent report that evaluated the RF expression profile in viremic progressors (VP), ART-naïve HIV-1 positive individuals with high viral load (VL), late ART-treated HIV-1 positive individuals and ART-naïve individuals who spontaneously control HIV-1 viremia, the so-called elite-controllers (EC), has shown overall increased RF expression in VP as compared to uninfected individuals, ECs and late ART-treated HIV-1 positive individuals linking upregulated RF profile to high VL [7]. Interestingly, restriction factor Schlafen Family Member 11 (*SLFN11*), which is involved in the inhibition of viral protein synthesis, violated this typical RF profile and was shown to be upregulated in ECs, therefore suggesting a role in the suppression of HIV-1 replication *in vivo* [7]. Besides SLFN11, expression profiles of RF SAMHD1 and antiviral factor CDKN1A/p21, have been investigated in ECs and compared to VP and uninfected individuals. Both factors were shown to be upregulated in ECs, however, this data could not be confirmed in other cohorts [8–11]. In a longitudinal study, increased RF expression levels were observed during the natural course of HIV-1 infection [7,12], suggesting that it was linked to HIV-1 viral load (VL) levels. Other ISGs have been shown to follow a similar pattern indicating that VL and interferon production are important drivers of RF/ISG elevation [7,12,13]. Interestingly, RF PAF1, restricting HIV-1 during early events of infection, did not show this increased levels overtime and was stably expressed suggesting a restricting mechanism independent of VL and interferon induction. Additionally, timing of ART initiation has been suggested to impact host cell-intrinsic antiviral factors with increased RF expression in early *versus* late treated individuals [14].

Virological control, both spontaneous and after treatment discontinuation, has been associated with a low viral reservoir as measured by HIV-1 DNA [15,16]. Host RF expression may play an important role in determining the size of the HIV-1 reservoir size as described in recent studies showing associations between RF levels and the reservoir size in ECs and long-term non-progressors (LTNPs) [17,18]. Additionally, a negative correlation between these factors and HIV-1 transcriptional activity (cell-associated HIV-1 RNA) in ART-suppressed individuals has been described, therefore suggesting that they may contribute to the control of viral transcription and replication during treatment [14,19].

Having the opportunity to access a large cohort of HIV-1 positive individuals with unique clinical phenotypes such as ART-naïve seroconverters (SRCV), ART-naïve viremic progressors (VP), ART-naïve LTNPs, early, and late ART-treated HIV-1 infected individuals, we have comprehensively investigated the role of antiviral factors, restriction factors and dependency factors, with an emphasis on their relationship with virological and immunological parameters and timing of treatment initiation. We specifically selected *APOBEC3G*, *SAMHD1*, *BST2*, *TRIM5* and *MX2* because these RFs have been extensively investigated *in vitro*, however comprehensive *in vivo* data is still lacking. *SLFN11* and *PAF1*, linked to EC status *in vivo* [7] and stable expression profile [12], respectively, were included to validate these findings in well-characterized cohorts of HIV positive individuals. Antiviral factors *MX1* and *IFIT1*, well-characterized ISGs without direct HIV-1 specificity, were selected to monitor immune activation and their relationship to RFs expression. Additionally, two HIV-1 dependency factors, *PSIP1* and *NLRX1*, involved in integration [20] and repression of IFN response [21], were included as they were expected to be linked to HIV-1 progression. Given that LTNPs represent an ideal model for spontaneous viral control [22], we aimed to clarify whether their RF expression patterns differed from other HIV-1 positive individuals in order to better understand the potential contribution of these factors to a non-progressive phenotype. Moreover, because some early ART-treated individuals have shown long-term control of viral replication after stopping ART, decreased virological burden in terms of HIV-1 DNA [23,24] and enhanced immune restoration as measured by CD4/CD8 T cell ratio, we have compared their RF levels with other groups of HIV-1 positive individuals to assess the impact of early treatment intervention on
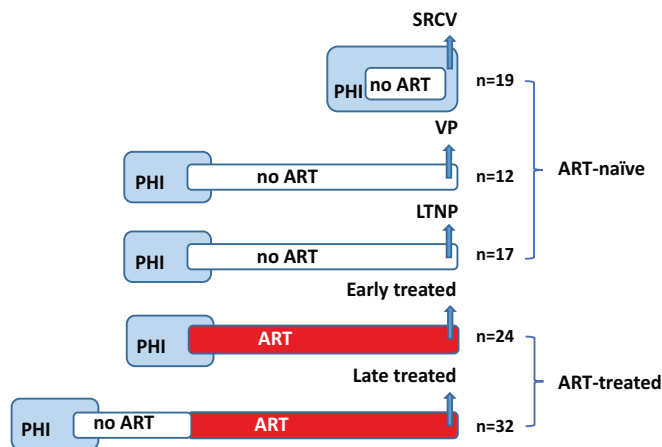
Fig. 1. Patient cohorts. Blue arrows represent time of sampling. Viral load levels of ART-naïve HIV-1 positive individuals at time of sampling are depicted in Supplementary Fig. 1. *PHI: primary HIV-1 infection; ART: antiretroviral therapy; SRCV: seroconverters; VP: viremic progressors; LTNP: long-term non-progressors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

innate antiviral responses and whether these were comparable to LTNPs.

2. Materials and methods

2.1. Patient cohorts

HIV-1 positive subjects were recruited between 2014 and 2017 from two clinical centers, the Ian Charleson Day Centre, Royal Free Hospital, London, United Kingdom and the AIDS Reference Center, Ghent University Hospital, Ghent, Belgium. Peripheral blood mononuclear cells (PBMCs) were obtained from 104 HIV-1 positive individuals at one time-point from 5 cohorts based on their disease status: 1) ART-naïve SRCV ($n = 19$), who were sampled during the acute phase of infection; 2) ART-naïve LTNPs ($n = 17$) who maintained HIV-1 viremia ≤1000 copies/ ml and CD4+ T cells >500 cells/mm^3 over >7 years post-infection and 3) long-term-ART treated individuals who had initiated ART during seroconversion (early treated; $n = 24$) or 4) the chronic phase of infection (late treated; $n = 32$), as previously described [23], and 5) ART-naïve VP ($n = 12$) with long-term VL > 3000 HIV-1 copies/ml. Uninfected healthy controls ($n = 14$) were sampled from the Red Cross Flanders (Belgium) as a control group. Appropriate sample

size was computed as described by Cohen in R package pwr [25]. The power calculation for an expected difference of 50% in expression levels resulted in a minimum cohort size of 12 patients; given 80% power, 0·05 significance level and standard variation of gene expression levels in patient cohorts.

The Ethical Committees of Ghent University Hospital and of the Royal Free Hospital approved this study (reference numbers: B670201317826/B670201733030 and 13/LO/0729). All study subjects gave written informed consent. The subject cohorts are described in Fig. 1 and details on study design and inclusion criteria have been described previously [23]. Baseline characteristics and clinical parameters (duration of ART, VL zenith, CD4 T cell count at sampling, CD4 nadir and CD4/CD8 T cell ratio at sampling) are summarized in Table 1.

2.2. Peripheral blood mononuclear cells (PBMCs)

Blood was collected from all participants ($n = 118$) at a single time point. PBMCs were isolated from fresh whole blood using density gradient centrifugation with Lymphoprep (ELITech Group, Belgium) and stored in freezing media (Fetal Calf Serum (FCS) + 10% dimethyl sulfoxide (DMSO)).

2.3. Primers

Primers for reference genes and *MX1* were found in RTPrimerDB (RRID: SCR_007106), a freely accessible database for real-time PCR primers and probes [26]. *APOBEC3G* and *SAMHD1* assays were commercially available (Bio-Rad, Belgium), *SLFN11* and *IFIT1* assays were described in literature [27,28], and primers for other restriction and dependency factors were designed manually using PrimerXL, a pipeline based on Primer3 with *in silico* assay validation [29,30]. To check *in silico* target specificity, primers were blasted. Primers used are listed in Supplementary Table 1 (Table S1) and were ordered at Integrated DNA Technologies (IDT, Belgium).

2.4. Quantitative real-time PCR (qPCR)

RNA was extracted from 10^7 PBMCs per sample (RNA innuprep mini kit, Analytik, Germany) and eluted into 30 μl nuclease-free water. Genomic DNA (gDNA) was removed in a column-based manner. RNA concentration was determined using the Qubit Fluorometer (Invitrogen, CA, USA) and one microgram of total RNA was reverse transcribed into cDNA using the qScript cDNA SuperMix (Quantabio, MA, USA) according to the manufacturer's protocol. cDNA was diluted to a concentration of 10 ng/μl and 20 ng was used in a 10 μl real-time PCR

Table 1
Clinical characteristics and virological markers of patient cohorts.

	SRCV*	VP*	LTNP*	Early treated*	Late treated*
Clinical characteristics	$n = 19$	$n = 12$	$n = 17$	$n = 24$	$n = 32$
Age (yrs)	35 (29–44)	43 (34–46)	49 (38–51)	44 (41–49)	48 (45–53)
Total cART (yrs)	0	0	0	10.7 (8.4–11.99)	9.8 (6.1–14.7)
Total VL suppression (yrs)	0	0	0	10.3 (8.1–11.3)	6.5 (5.0–10.4)
log VL zenith (copies/ml)	6.3 (5.3–6.5)	4.3 (4.1–4.7)	2.5 (2.2–2.8)	5.5 (5.0–5.9)	4.9 (4.2–5.5)
CD4 nadir (cells/μl)	475 (311–592)	544 (431–626)	624 (562–693)	391 (280–485)	155 (51–266)
CD4 at collection (cells/μl)	475 (311–607)	601 (562–643)	793 (685–1010)	714 (645–994)	625 (484–889)
CD4/CD8	0.54 (0.4–0.7)	0.51 (0.4–0.7)	0.91 (0.8–1.5)	1.10 (0.7–1.3)	0.74 (0.6–0.9)
Viral reservoir markers					
total HIV-1 DNA (c/M PBMC)	1846 (624–4966)	385 (297–1320)	48 (20.2–56.5)	90 (34–124)	137 (56.1–219.2)
Integrated HIV-1 DNA (c/M PBMC)	165.7 (113.3–628.1)	75.5 (48.5–246.9)	2.82 (0–15.84)	18.7 (9.6–58.2)	58.7 (31.5–91.8)
CA HIV-1 usRNA (c/M PBMC)	15.5 (0.9–100.6)	29 (9.8–402.5)	0.4 (0.3–3.5)	1.5 (0.3–3.7)	6.1 (0–10.1)
2-LTR circles (c/M PBMC)	5.2 (3.9–14.8)	1.9 (0.9–3.0)	0.8 (0.6–2.7)	1.8 (0–3.4)	1.3 (0–2.2)

* Values are reported as median (IQR); SRCV: seroconverters; VP: viremic progressors; LTNP: long-term non-progressors; PBMCs: peripheral blood mononuclear cells; CA: cell-associated; usRNA: unspliced RNA; ART: antiretroviral therapy.
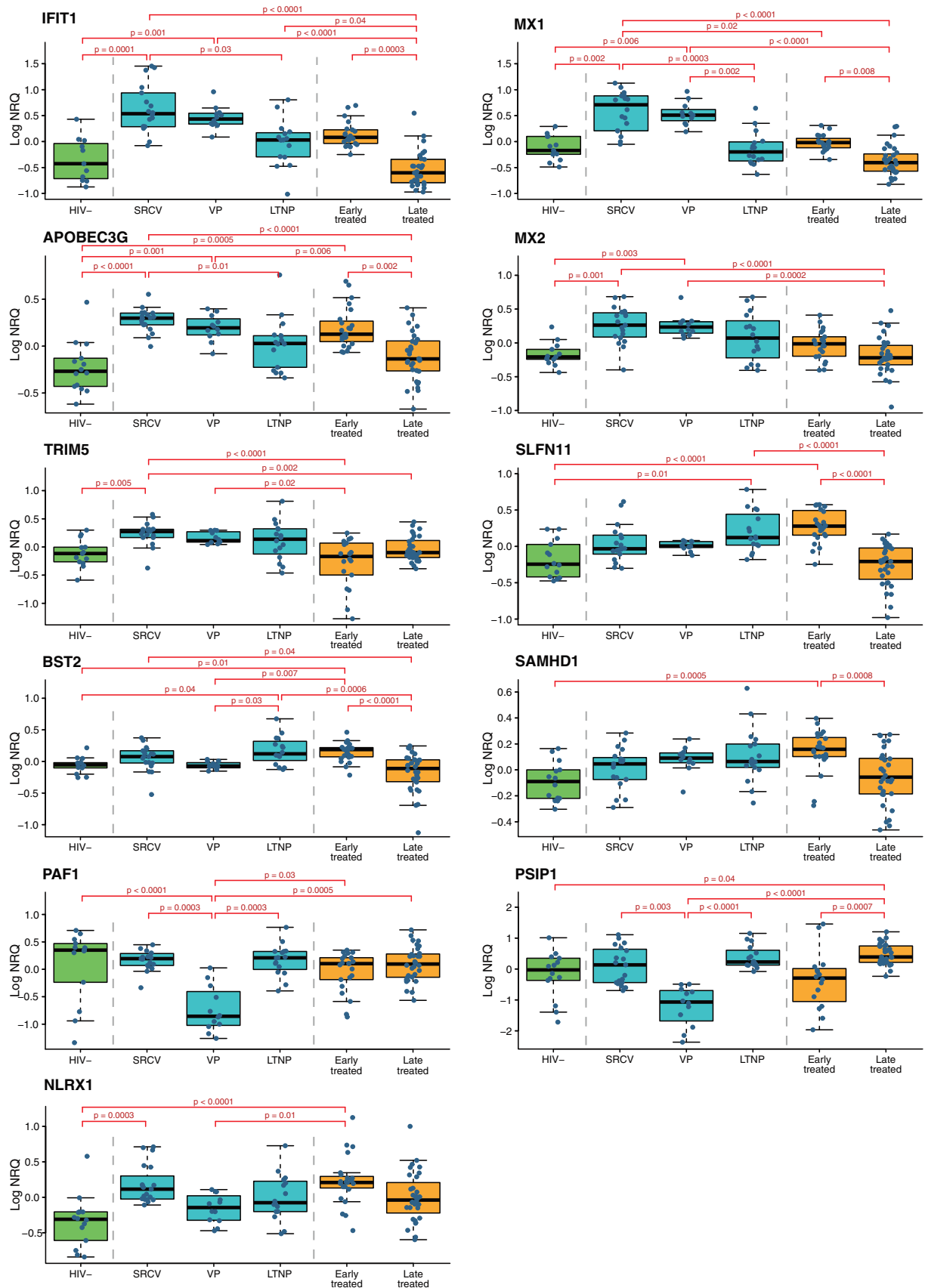
Fig. 2. Antiviral factor, HIV-1 specific restriction factor and dependency factor expression profile in different cohorts of HIV-1 positive individuals. Boxplots showing normalized relative quantity (NRQ) levels for *IFIT1, MX1, APOBEC3G, MX2, TRIM5, SLFN11, BST2, SAMHD1, PAF1, PSIP1* and *NLRX1* in uninfected, ART-naïve (SRCV, VP and LTNP) and ART-treated (Early and Late) HIV-1 positive individuals. Kruskal-Wallis analysis was performed and significant p-values ($p < 0.05$) are marked in red.*NRQ: Normalized Relative Quantity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reaction with SYBR Green (LightCycler480 SYBR Green I Master, Roche Applied Science, Belgium) for restriction factors and dependency factors. A second cDNA dilution (2.5 ng/µl) was generated for reference gene measurement as lower input is sufficient for their adequate analysis. Per reaction, 5 µl SYBR Green Master Mix, 2.5 µl H_2O and 250 nM of each primer were added and every reaction was performed in duplicate. Cycling conditions on LightCycler 480 (Roche Applied Science, Germany) were 95 °C for 5 min, 45 amplification cycles of 95 °C for 10s, 58 °C for 30s and 72 °C for 30s. A melting curve from 60 °C to 95 °C was run and melting curve analysis was performed with LightCycler480 software to determine assay specificity. Samples with aberrant melting curves were excluded from the analysis. The stability of 8 reference genes: actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA-box binding protein (*TBP*), beta-2-microglobulin (*B2M*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*), hydroxymethylbilane synthase (*HMBS*) and ubiquitin C (*UBC*) was determined on a subset of samples and the three most stable reference genes, *ACTB*, *GAPDH* and *YWHAZ*, were selected with the GeNorm algorithm for normalization [31]. PCR efficiency of each primer pair was calculated using a pooled sample cDNA standard curve and applying formula: $10^{-1/slope}$. Gene expression analysis was performed with qbasePLUS software from Biogazelle [32] allowing to perform automated normalization, PCR efficiency calculation and adequate inter-run calibration. Normalization factors are calculated within this software based on the geometric mean of relative quantities (RQ) of the selected reference genes. Relative gene expression values of RF, dependency factors and ISGs are expressed as normalized relative quantities (NRQ) [33].

2.5. Quantification of the HIV-1 reservoir, ongoing replication and transcription

Total HIV-1 DNA, integrated HIV-1 DNA, circular 2-long terminal repeats (2-LTR) and CA HIV-1 usRNA analyses were performed on 82 samples included in this study from a previous analysis [23] and the subsequently remaining 22 samples were analyzed with the same technology listed in Supplementary Table 2 (Table S2).

2.6. Total and integrated HIV-1 DNA

Briefly, total genomic DNA was extracted from 10^7 PBMCs using DNeasy Blood & Tissue Kit (Qiagen, The Netherlands). Restriction digestion and ddPCR reactions were performed as described previously [34,35]. For normalization, reference gene ribonuclease P/MRP subunit p30 (RPP30) was used and total HIV-1 DNA quantification was completed with ddpcRquant, an in-house developed software [36].

To quantify integrated HIV-1 DNA, the repetitive sampling Alu-HIV PCR method was applied [37]. Alu-HIV PCR is a nested PCR, including a first round with an HIV-1 specific reverse primer in the HIV-1 *gag* region and a human Alu-specific forward primer. This reaction is performed in 40 replicates. Additionally, 20 replicates for background quantification using only the HIV-1 *gag* primer were included. This method was performed as described previously [37]. In contrary to previous published results [23], the correction factor of 0.1 for integrated HIV-1 DNA values was not applied as recently described [38].

2.7. Episomal HIV-1 2-LTR circles

2-LTR quantification by ddPCR was performed as previously described [23,34]. Briefly, plasmid DNA (pDNA) was isolated using the QIAprep Spin Miniprep kit (Qiagen) from 10^7 PBMCs and pDNA was eluted into 25 µl to increase DNA concentration. pSIF1-H1-Puro non-HIV plasmid (System Biosciences, CA, USA) with determined amount of copies was spiked to the samples as an internal control for copy number normalization as previously described [34]. The internal reference plasmid contained a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and WPRE primers and probe were used for quantification [39]. Primers used are listed in Supplementary Table 2 (Table S2) [39,40].

2.8. Cell-associated HIV-1 usRNA

One microgram of total RNA was reverse-transcribed into cDNA using the qScript cDNA SuperMix (Quantabio). ddPCR quantification of CA HIV-1 usRNA was performed as described previously [41,42]. Normalization of cDNA input was done with the abovementioned normalization strategy in the qPCR section. ddPCR concentration values for CA HIV-1 usRNA were calculated using Quantasoft and adequate threshold determination was performed with ddpcRquant [36]. For normalization, raw CA HIV-1 usRNA concentration values were divided by the corresponding normalization factors [41].

2.9. Statistical analysis

Non-parametric Kruskal-Wallis with post-hoc Nemenyi statistical analysis was performed. Tukey correction was used for multiple comparisons. Spearman correlation analysis was performed to define significant correlations between RF, dependency factors, ISGs and immunological and virological reservoir parameters. After initial univariate analyses, multivariate principal component analysis (PCA) analysis was performed. We used for statistical analyses and graphing R software with the following packages: PMCMR, Hmisc, graphics, ggplot2, and corrplot.

3. Results

3.1. Study participants

Fourteen HIV negative and 104 HIV-1 positive individuals were enrolled into this cross-sectional study [23]. HIV-1 positive individuals comprised 3 cohorts of ART-naïve and 2 cohorts of ART-treated individuals (Fig. 1). ART-naïve individuals included: 1) SRCV ($n = 19$), patients diagnosed during acute seroconversion characterized by high VL, 2) VP ($n = 12$), patients diagnosed during chronic infection, 3) LTNPs ($n = 17$), individuals capable of maintaining high CD4 count and undetectable or low VL without ART initiation (Supplementary Fig. 1). ART-aviremic HIV-1 positive individuals included individuals that initiated treatment: 4) during seroconversion (Early treated, $n = 24$), or 5) during chronic infection (Late treated, $n = 32$) (Fig. 1). Their clinical and laboratory characteristics are described in Table 1.

### 3.2. SLFN11, BST2 and SAMHD1 are associated with a LTNP phenotype

LTNPs control HIV-1 replication in the absence of ART and display a low viral reservoir [15,23]. Hence, these individuals are frequently studied as a model of functional cure, as defined by long-term control of HIV-1 replication in the absence of ART or as in post-treatment controllers after ART discontinuation [43,44]. Mechanisms that drive this type of virological control are not fully elucidated. Host RF besides the previously described *SLFN11*[7] could play a significant role in HIV-1 suppression in these groups. To comprehensively address the role of multiple antiviral factors and dependency factors in VL control, we have analyzed the expression profile of *IFIT1*, *MX1*, *APOBEC3G*, *SAMHD1*, *BST2*, *TRIM5*, *SLFN11*, *MX2*, *PAF1*, *PSIP1*, and *NLRX1* in HIV-1 positive individuals with various degrees of virological control, such as viremic or aviremic subjects that are either spontaneous controllers or ART-mediated controllers.

IFIT1 and *MX1* are well-characterized ISGs but are not RFs directly acting on HIV-1 life cycle and their expression profile was assessed in this study as a marker of interferon exposure. Differences between expression profiles of *IFIT1*, *MX1* and HIV-1-specific RFs could reveal whether and to what extend specific HIV-1 RF responses are driven by
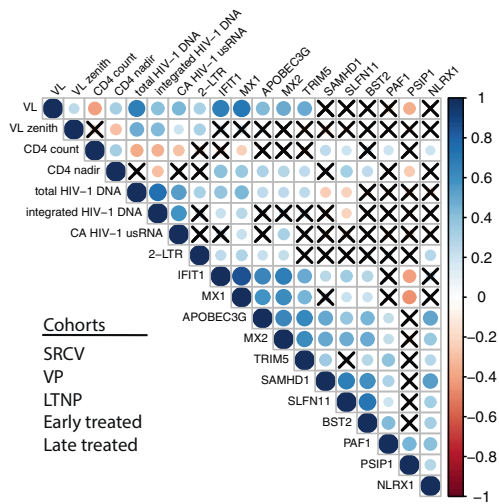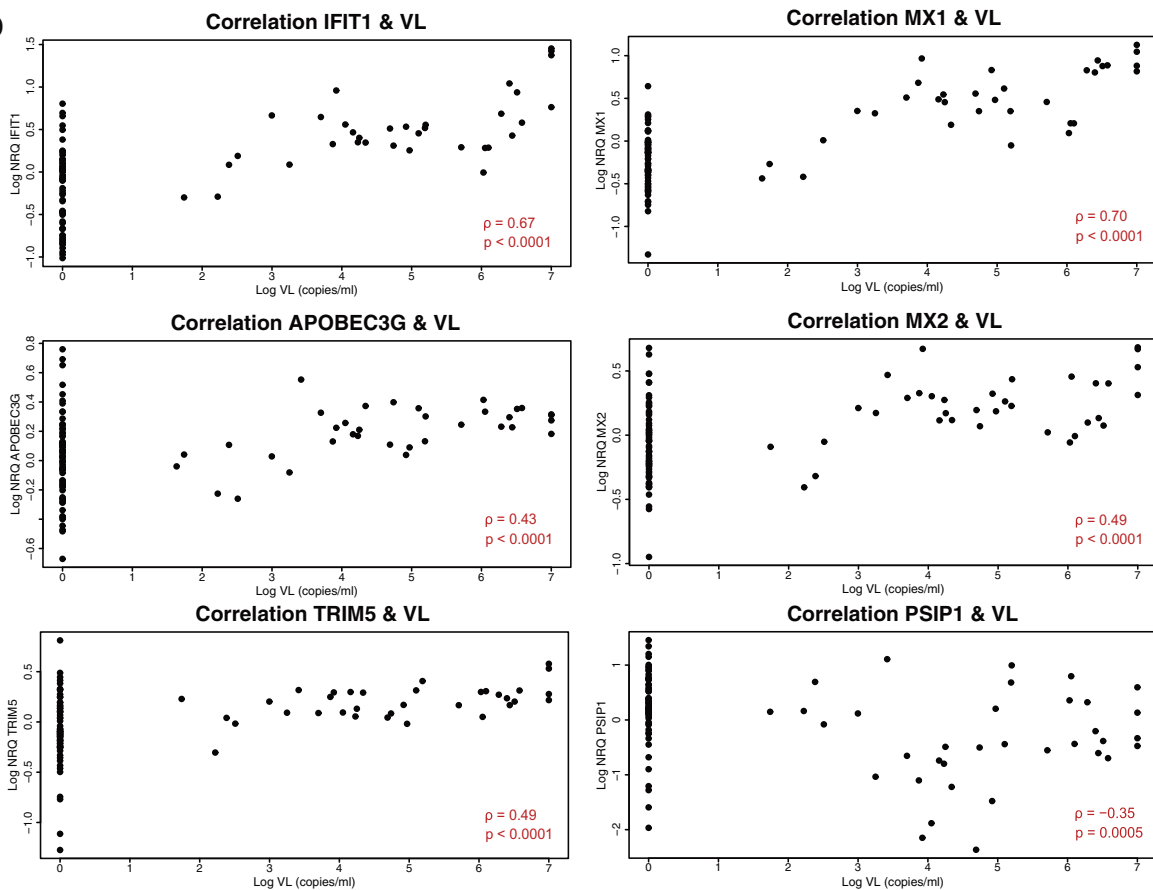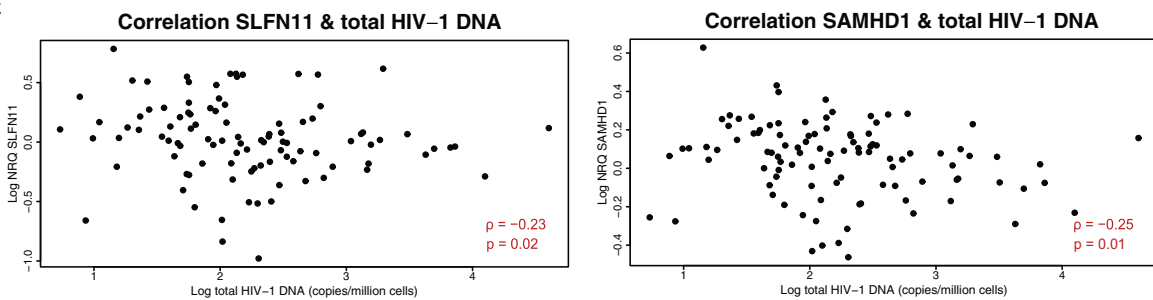
interferon exposure. Univariate analysis showed significant upregulation of *IFIT1* and *MX1* in SRCV and VP, both cohorts characterized by high viremia, as compared to LTNPs and HIV negative individuals (Fig. 2; Supplementary Fig. 1). Furthermore, similar expression profiles were present for several but not all RF directly acting on HIV-1 lifecycle such as *APOBEC3G, MX2* and *TRIM5*. These RF displayed also elevated levels in SRCV ($p < 0.0001$, $p = 0.001$ and p = 0.005, respectively) and VP (p = 0.001 and p = 0.003, respectively) as compared to HIV negative individuals (Fig. 2). This expression profile was not seen for *SLFN11, BST2, SAMHD1* and *PAF1* and dependency factors *PSIP1* and *NLRX1,* implying that VL levels may selectively drive the expression of only some RF and ISGs.

In LTNPs, *SLFN11* and *BST2* levels were increased significantly as compared to HIV negative individuals ($p = 0.01$ and $p = 0.04$, respectively, Fig. 2). *SLFN11* and *BST2* displayed also slightly elevated expression levels in LTNPs in comparison to SRCV and VP, although not reaching statistical significance in most of the cases. Similarly, *SAMHD1* expression was boosted in some LTNPs, but overall did not demonstrate significant upregulation ($p = 0.09$ compared to HIV negative individuals). Moreover, both, *SLFN11* and *BST2*, also demonstrated significantly elevated levels in LTNP as compared to late treated individuals ($p < 0.0001$ and $p = 0.0006$; Fig. 2) pointing to the association of elevated *SLFN11* and *BST2* with spontaneously induced non-progressive phenotype. Taken together, overall increased levels of *SLFN11* and *BST2* in LTNPs suggest their potential contribution in viral load control.

Beyond the RFs that correlate with VL or those linked to LTNP phenotype, our data demonstrate that additional dependency factors such as *PSIP1* and *NLRX1* may be associated with lack of virological control. *PSIP1* encodes an integrase dependency factor (LEDGF/p75) [45,46] and is not linked to VL or IFN response. In this study, it was found to be stably expressed in HIV negative individuals, SRCV and LTNPs, although significantly downregulated in VP as compared to SRCV and LTNPs ($p = 0.003$ and $p < 0.0001$, respectively) (Fig. 2). Surprisingly, *PAF1,* a RF manifesting its antiviral activity early in the replication cycle [47], showed a similar expression profile with comparable levels in the different cohorts, except for VP in whom it was downregulated, suggesting the association of *PSIP1* and *PAF1*, and viremic progression (Fig. 2). NLRX1 sequesters the DNA sensing molecule STING, stimulator of interferon genes, thus inhibiting IFN response. Therefore, we hypothesized that *NLRX1* could also be associated to viremic progression. Surprisingly, *NLRX1* was not differentially expressed in VP, although upregulation in SRCV and early treated individuals was detected as compared to uninfected individuals ($p = 0.0003$ and $p < 0.0001$, respectively). Early treated HIV positive individuals also demonstrated a trend for *NLRX1* upregulation as compared to late treated individuals, however not significantly, suggesting a different IFN regulation mechanism in both ART-treated cohorts.

3.3. Restriction factor levels remain increased in early treated HIV-1 patients after a decade of ART

Current WHO guidelines recommend immediate treatment initiation after the acquisition of HIV-1 infection. Our previous study, performed in the same HIV-1 cohorts, demonstrated a beneficial impact of early treatment a decade after ART initiation on immunovirological markers, such as CD4/CD8 ratio, total and integrated HIV-1 DNA and

CA HIV-1 usRNA [23]. We hypothesized that the timing of treatment initiation could also affect RF levels and therefore allow us to distinguish between early and late treated individuals, and to allow us for direct comparison of RF profile in ART-suppressed controllers and in ART-naïve spontaneous controllers (LTNP). Generally, similar expression profiles are seen for antiviral factors linked to VL (*IFIT1, MX1, APOBEC3G, MX2* and *TRIM5*) in uninfected individuals and late treated HIV positive individuals. Thus, late treated individuals do not demonstrate any increase or decrease in RF profile as compared to uninfected subjects indicating that when ART is initiated during chronic infection, expression levels of antiviral factors are normalized to levels observed in HIV negative individuals. SRCV and VP demonstrated a highly significant upregulation for *IFIT1, MX1, APOBEC3G* and *MX2* as compared to late but not early treated individuals ((SRCV: $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively) and (VP: $p < 0.0001$, $p < 0.0001$, $p = 0.006$, $p = 0.0002$, respectively; Fig. 2)). Similarly, *TRIM5* upregulation was detected in SRCV as compared to late and not early treated HIV positive individuals (p = 0.002), suggesting differences in RF profiles imposed by time of ART initiation. Interestingly, early treated HIV-1 positive individuals show increased expression levels for *IFIT1, MX1, SLFN11, BST2, SAMHD1,* and *APOBEC3G* compared to late treated individuals ($p = 0.0003$, $p = 0.008$, $p < 0.0001$, $p < 0.0001$, $p = 0.0008$, $p = 0.002$, respectively), even after a decade of ART (Fig. 2). In addition, *SLFN11, BST2, SAMHD1* and *APOBEC3G*, but not *IFIT1* and *MX1*, demonstrated higher expression levels in early treated individuals as compared to uninfected individuals ($p < 0.0001$, $p = 0.01$, $p = 0.0005$, and $p = 0.0005$). Furthermore, *MX2* expression exhibits the same trend, although no significant upregulation in early treated individuals could be detected.

Overall, this data suggests that RFs linked to HIV-1 control in this study, namely *SLFN11, BST2* and *SAMHD1*, show similar upregulation in early treated HIV-1 positive individuals as in LTNPs. Unlike in LTNPs, *IFIT1, MX1* and *APOBEC3G*, antiviral factors driven by VL, also showed significant upregulation in early treated individuals (Fig. 2) suggesting that initiation of ART during acute infection affects expression of both antiviral factors driven by VL or linked to non-progression. Altogether, this data points to an impact of early treatment in maintaining innate antiviral responses over the long-term.

3.4. IFIT1, MX1, APOBEC3G, MX2 and TRIM5 expression is associated with high viral load

Initial univariate analysis revealed that *IFIT1, MX1, APOBEC3G, MX2* and *TRIM5* demonstrated increased expression levels in cohorts of HIV positive individuals characterized by high VL. To further explore this relationship, a spearman correlation analysis of combined data of all cohorts (SRCV, VP, LTNP, Early treated and Late treated) was performed and detected positive correlations between *IFIT1, MX1, APOBEC3G, MX2*, and *TRIM5,* and VL (Fig. 3a and b), suggesting that indeed increased VL induces antiviral factors *IFIT1* and *MX1* and several RFs directly acting on HIV-1 lifecycle. On the contrary, *SLFN11* and *SAMHD1*, RFs linked to LTNP status exhibited a negative correlation with viral reservoir marker, total HIV-1 DNA ($p = 0.02$ and $p = 0.01$, respectively, Fig. 3c). For *SLFN11*, a significant negative correlation with integrated HIV-1 DNA was also observed (Fig. 3a). Thus, these data suggest that *SLFN11* and *SAMHD1* could contribute to reduction of viral reservoir size in HIV positive individuals.

Fig. 3. Correlation analysis of antiviral, HIV-1 specific restriction and dependency factor expression levels with virological and viral reservoir markers in HIV-1 positive individuals. (a) Correlation plot illustrating relations between antiviral, HIV-1 specific RF and dependency factor expression levels, and different viral reservoir markers in HIV-1 positive individuals (combined data of SRCV, VP, LTNP, Early and Late treated individuals). Spearman correlation analysis was performed. Positive and negative correlations are depicted in blue and red, respectively. Non-significant p-values ($p > 0.05$) are indicated with an X. (b) Spearman correlation plots for antiviral and HIV-1 specific RF expression with viral load in HIV-1 positive individuals (combined data of SRCV, VP, LTNP, Early and Late treated individuals). (c) Spearman correlation plots for *SLFN11/SAMHD1* with total HIV-1 DNA in HIV-1 positive individuals (combined data of SRCV, VP, LTNP, Early and Late treated individuals). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
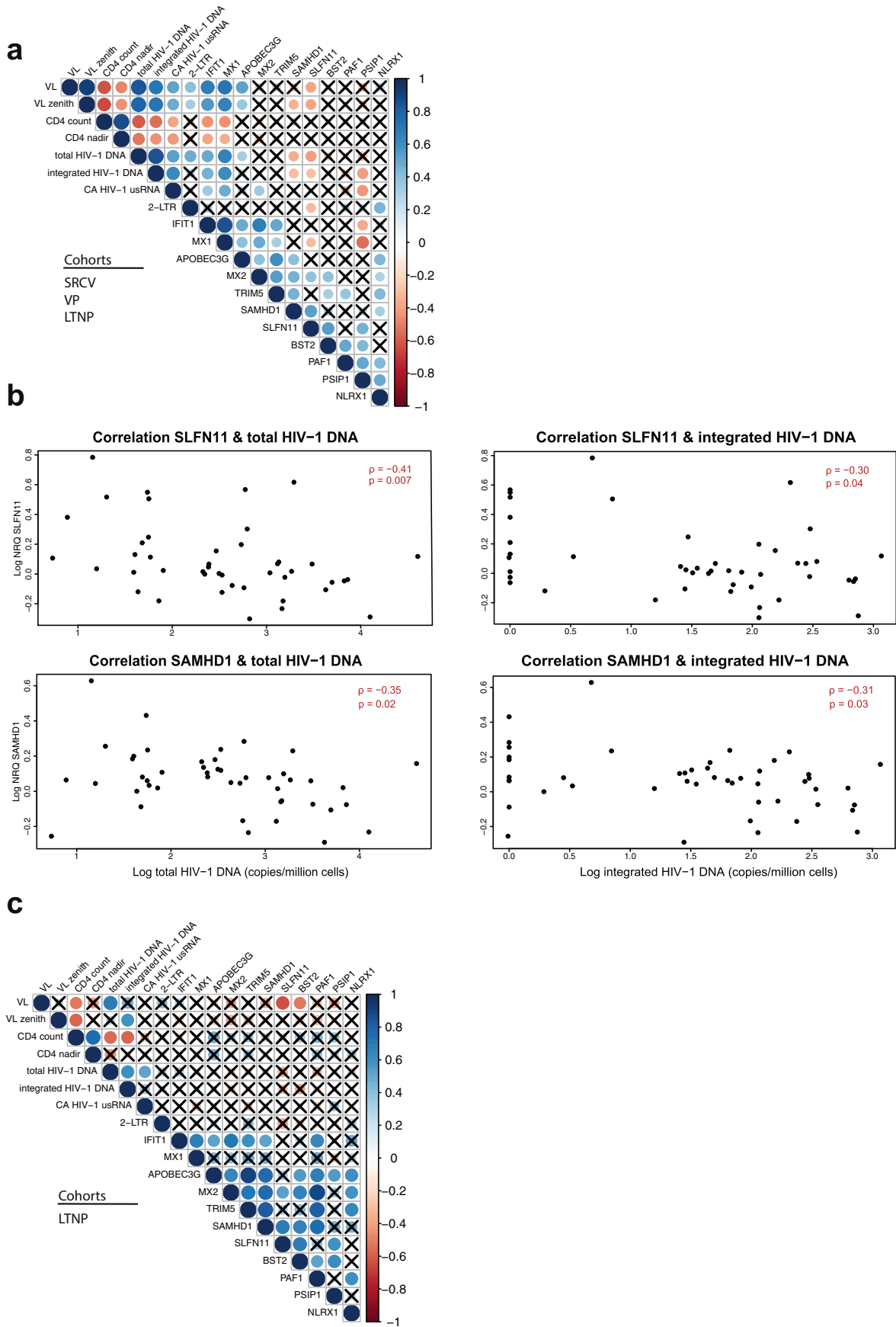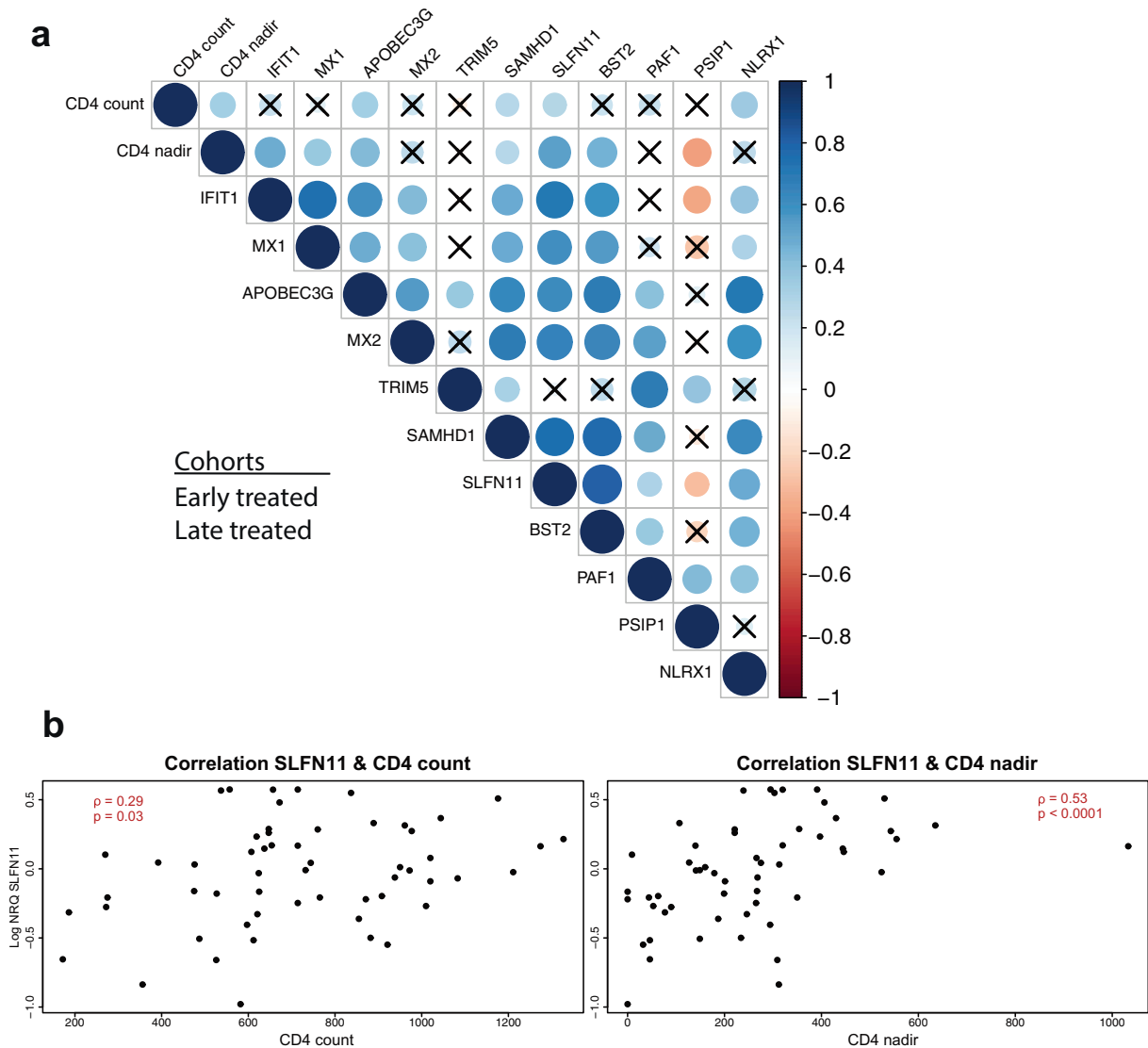
Fig. 5. Antiviral, HIV-1 specific restriction and dependency factors associated with CD4 count in ART-treated HIV-1 positive individuals. (a) Spearman correlation plot illustrating correlations between CD4 count/nadir, antiviral, RF and dependency factors in both early and late ART-treated HIV-1 positive individuals. (b) Spearman correlation plots for *SLFN11* and CD4 count/nadir.

3.5. SLFN11 and SAMHD1 expression is linked to a smaller viral reservoir in ART-naïve patients

Similarly as in all cohorts combined, negative correlations were found in ART-naïve HIV-1 positive individuals, comprising SRCV, VP and LTNPs between *SLFN11*, *SAMHD1*, and HIV-1 persistence markers. Both, *SLFN11* and *SAMHD1* were negatively correlated with VL zenith, total and integrated HIV-1 DNA (*SLFN11*: $\rho = -0.35$, $\rho = -0.41$ and -0.30, resp.; *SAMHD1*: $\rho = -0.31$, $\rho = -0.35$ and -0.31, respectively), showing that higher *SLFN11*/*SAMHD1* expression was associated with lower VL and smaller viral reservoir in ART-naïve HIV-1 positive individuals (Fig. 4a and b). Additionally, a negative correlation was found for *SLFN11* and *BST2*, with VL specifically in LTNP ($\rho = -0.63$ and $\rho = -0.55$, resp. Fig. 4c and Supplementary Fig. 2). These data

suggest that *SLFN11*, *SAMHD1* and *BST2* could play a role in reducing viral load and therefore contribute to virological control.

3.6. The CD4 T cell count is positively correlated with restriction factor levels in patients on ART

The CD4 T cell count/nadir is a prognostic marker for HIV-1 disease progression, with high CD4 count/nadir linked to a favorable disease outcome. The CD4 T cell count is routinely monitored for ART efficacy [48] and has shown enhanced recovery in early *versus* late treated individuals [49]. We were therefore interested to determine whether RF levels in ART-naïve or -treated individuals were linked to the CD4 T cell count/nadir and whether it might be used as a marker of disease progression. Interestingly, the CD4 T cell count/nadir

Fig. 4. Correlation analysis of antiviral, HIV-1 specific restriction and dependency factor expression levels with virological and viral reservoir markers in ART-naïve HIV-1 positive individuals. (a) Correlation plot illustrating antiviral, HIV-1 specific RF and dependency factor expression levels with virological and viral reservoir markers using combined data of ART-naïve HIV-1 positive individuals comprising SRCV, VP and LTNP. (b) Spearman correlation analysis depicting negative correlations for *SLFN11*/*SAMHD1* with total/integrated HIV-1 DNA in ART-naïve HIV-1 positive individuals (SRCV, VP and LTNP) (c) Correlation plot illustrating antiviral, HIV-1 specific RF and dependency factor expression levels with virological and viral reservoir markers in LTNPs. Spearman correlation analysis was performed. Positive and negative correlations are depicted in blue and red, respectively. Non-significant p-values (p > 0·05) are indicated with an X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed a positive correlation with expression levels of *IFIT1*, *MX1*, *APOBEC3G*, *SAMHD1*, *SLFN11*, and *BST2* in individuals on ART (Fig. 5a and b). The same antiviral factors were also significantly upregulated in early *versus* late treated individuals and some of them (*SLFN11*, *BST2*, and *SAMHD1*) were strongly linked to spontaneous VL control (LTNPs). This data suggests that ART-treated individuals displaying increased RF levels are associated with better immune preservation.

4. Discussion

An in-depth characterization of several antiviral and dependency factor profiles in cohorts of HIV-1 positive individuals with different levels of virological control and treatment characteristics could uncover their role in controlling HIV-1 replication *in vivo*, in particular when comparing viremic to spontaneously and ART-suppressed controllers.

Our study described a significant elevation of *IFIT1*, *MX1*, *APOBEC3G*, *MX2* and *TRIM5* expression, driven by HIV-1 viral load, in SRCV and VP. An earlier report has described such a modest positive correlation between expression of antiviral factors and VL [7]. Our data similarly suggest that viral load drives elevation of several antiviral factors and HIV-1 specific RF, confirming previously published results that VL is one of the major drivers of RF elevation *in vivo* [7,50].

Various mechanisms have been suggested as contributing to non-progressive infection *in vivo*, such as protective human leukocyte antigen (HLA) class I alleles including HLA-B*57, HLA-B*27 among others [51]. However, not all LTNPs carry these protective alleles, therefore additional immune responses associated with host antiviral factors could affect viral replication. In our cohort, only two LTNPs expressed HLA-B*57 or *27 alleles, while higher levels of *SFLN11* and *BST2* were linked to lower VL in the entire LTNP cohort. In previous reports, both *SLFN11* and *SAMHD1* have been suggested to contribute to viral control *in vivo* in ECs/LTNPs [7–9]. *SLFN11* restricts HIV-1 mRNA translation, probably by occupying tRNA, thereby limiting its availability for HIV-1 protein synthesis [52]. In contrast to our data, a previous study found comparable levels of *SLFN11* expression between ECs and HIV negative individuals [7]. An essential disparity between the present and previous studies [7] includes some of the characteristics of the HIV negative cohort, which were unexposed and highly HIV-exposed seronegative individuals, respectively. *SLFN11* levels were significantly more expressed in highly HIV-1 exposed individuals [7], suggesting that HIV-1 exposure without actual infection could increase *SLFN11* levels as a protective mechanism. Previously published data shows that CD4 T cells from ECs/LTNPs are more refractory to *ex vivo* HIV-1 infection in comparison to VP, therefore upregulation of *SLFN11* could also play a beneficial role in protecting cells from infection [11,53].

Both RFs, *SLFN11* and *SAMHD1*, showed negative correlations with VL and reservoir markers (total and integrated HIV-1 DNA). Both factors exhibited highest levels among LTNPs and early treated individuals, characterized by low viral load levels, suggesting that *SAMHD1* and *SLFN11* could contribute to the beneficial outcome in these individuals. *SAMHD1* interferes with HIV-1 replication by depleting the dNTP pool and thus hampers reverse transcription [2]. In terms of *SAMHD1*, conflicting data exists about its contribution to the EC or LTNP status. Some studies have described upregulated *SAMHD1* levels in EC/LTNPs in contrast to others [8,9]. Our data does not reveal significant *SAMHD1* elevation in LTNPs although a trend was noted. This finding combined with the negative correlation between VL and viral reservoir markers is however suggestive for *SAMHD1* contribution to viral control *in vivo*.

An important limitation of our study is the descriptive type of data from our patient cohorts and additional research is critical to confirm the potential roles of *SLFN11*, *BST2* and *SAMHD1* in viral control *in vivo*. Moreover, as some cohorts (VP and Early treated) were not sampled from both centers (London, UK and Ghent, Belgium), we cannot completely exclude a batch effect, however, Principal Component

Analysis (PCA) did not demonstrate substantial differences based on sampling center (London, UK or Ghent, Belgium) for virological/immunological characteristics within cohorts sampled at both centers (SRCV, LTNP and late treated HIV positive individuals) (data not shown). Furthermore, given that only restriction factor mRNA levels were analyzed, it is possible that protein levels do not reflect mRNA expression completely, although SLFN11 protein levels were found to mirror *SLFN11* mRNA levels [7].

HIV accessory proteins can counteract antiviral effects mediated by host RF, although mechanisms have not yet been identified for all of them. *Vif*, *Vpx* and *Vpu* are able to counteract *APOBEC3G*, *SAMHD1* and *BST2*, respectively. In contrast to *Vif* and *Vpu*, both encoded by HIV-1, *Vpx* is only encoded by HIV-2, thus HIV-1 cannot counteract *SAMHD1* activity [2]. This could be a possible mechanism explaining why *APOBEC3G*, efficiently counteracted by *Vif*, is not able to limit viral load levels in comparison to *SLFN11* and *SAMHD1* lacking an identified counteracting mechanism by HIV-1. However, caution should be taken in interpreting these results, as we cannot establish a causal relationship for *SLFN11* and *SAMHD1* in diminishing the viral reservoir size. Additional research focusing on functional mechanisms is therefore required.

Both, early and late ART-suppressed individuals are characterized by undetectable VL, thus, comparable RF levels would be expected if viral load levels were the major driver of IFN production and RF elevation. However, univariate analyses have revealed distinct expression profile for antiviral factors in early *versus* late treated HIV-1 positive individuals. Surprisingly, early treated subjects expressed higher *IFIT1*, *MX1*, *APOBEC3G*, *SLFN11*, *SAMHD1* and *BST2* levels as compared to the late treated ones. Some of them were also upregulated in LTNPs, revealing similarities between early treated patients and LTNPs, such as significantly upregulated *SLFN11*, *SAMHD1* and *BST2*. *IFIT1*, *MX1* and *APOBEC3G* levels were also significantly upregulated in early treated HIV-1 individuals suggesting that these responses remain intact in the long-term thanks to early treatment initiation. Our findings complement an earlier report in which increased expression of antiviral factors, including *SAMHD1* and *BST2*, has been linked to early treatment [14]. However, no significant expression elevation for *APOBEC3G* and *SLFN11* has been previously observed in early treated individuals. These dissimilarities may be related to the distinct cell type (CD4 T cells [14] *versus* PBMCs) or timing of sample collection used in the studies. We have collected blood after median of ten years on ART in comparison to 1–2 years post-ART initiation [14]. Overall, this data suggests that early treatment maintains initial RF elevation even after a decade of ART initiation.

In summary, our data show that *IFIT1*, *MX1*, *APOBEC3G*, *MX2* and *TRIM5* are linked with HIV-1 VL and thus are highly expressed in viremic HIV-1 positive individuals, characterized by high VL levels. Contrary, *SLFN11*, *BST2* and, to a lesser extent *SAMHD1*, are upregulated in aviremic individuals (LTNPs). Moreover, timing of treatment initiation was found to impact RF levels such that significantly elevated *APOBEC3G*, *BST2*, *SAMHD1* and *SLFN11* were present in early ART-treated subjects implying that early therapeutic intervention prevents depletion of innate antiviral responses which was observed in late treated individuals. We conclude that *SLFN11*, *BST2* and *SAMHD1* form a restriction factor signature associated with protection from disease progression. Induction of *SLFN11* and *SAMHD1* could limit viral load and viral reservoir size but direction of causality needs to be experimentally validated. Information gained will be useful for the design of novel antiviral therapeutics.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.02.006.

Declaration of interests

The authors declare that no competing interests exist.

Author contributions

CVH performed experiments, analyzed, interpreted and visualized data, and drafted the manuscript. WT performed experiments and interpreted data. EM and KV contributed to data collection. SR performed data analysis and interpretation. MS contributed to data interpretation and manuscript writing. LVDK, WDS and SK designed the study, supervised experiments and contributed to manuscript writing. All co-authors revised and edited the manuscript.

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