HIV-1 controllers exhibit an enhanced antiviral state characterized by overexpression of p21 and MCPIP1

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Keywords: HIV-1 controllers, restriction factors, p21, MCPIP1, immune activation,

Words count abstract: 323

Words count text: 2.669

Figures: 3

Table: 1

Abstract

Several cellular proteins have been identified as antiviral restriction factors (RF), one of the arms of the innate immune response. Among the RF targeting the human immunodeficiency virus type 1 (HIV-1), the cyclin-dependent kinase inhibitor p21 and the monocyte chemotactic protein-induced protein 1 (MCPIP1) are recognized as multifunctional modulators of cell proliferation, immune homeostasis and innate response to viral infections. Recently, MCPIP1 overexpression was associated with an increase of both p21 transcripts and proteins levels in a model of renal carcinoma cells. Here, we explored the potential associations between MCPIP1 and p21 expression, as well as with cellular activation in individuals who naturally control HIV-1 replication (HIC), sustaining undetectable (elite controllers – EC) or low (viremic controllers – VC) viral loads. We found a selective upregulation of p21 and MCPIP1 mRNA levels in PBMC from HIC compared with both ART-suppressed and HIV–negative control groups ($P \leq 0.02$) and a strong positive correlation ($r \geq 0.57$; $P \leq 0.014$) between expressions of both RF independently of the VL, treatment condition and HIV status. The mRNA levels of p21, but not of MCPIP1, were positively correlated with activated $CD4^+$ T cells levels in HIC and EC ($r \geq 0.53$; $P \leq 0.017$). In relation to the monocyte activation, the mRNA levels of both p21 ($r = 0.74$; $P = 0.005$) and MCPIP1 ($r = 0.58$; $P = 0.040$) were positively correlated with plasmatic levels of sCD14 only in EC. Multivariate analysis confirmed the association among p21, MCPIP1 mRNA levels, and frequency of activated $CD4^+$ T cells. These data show for the first time the simultaneous overexpression of p21 and MCPIP1 host RF in the setting of natural suppression of HIV-1 replication *in vivo*. The positive correlation between p21 and MCPIP1 transcripts supports a common regulatory pathway connecting these RF and a possible synergistic effect on HIV-1 replication control. Pharmacological manipulation of these innate RF may open novel therapeutic perspectives to prevent HIV-1 replication and disease progression.

1 Introduction

Among the individuals infected by the human immunodeficiency virus type 1 (HIV-1), a rare group called HIV controllers (HIC) suppress viral replication in absence of antiretroviral therapy, maintaining RNA viral loads (VL) undetectable (< 50-80 copies/mL; elite controllers, EC) or at low levels (50-2,000 copies/mL; viremic controllers, VC). Natural control of HIV-1 replication is probably a multifactorial feature that involves different combinations of host and/or viral factors (1). Several host restriction factors (RF), which constitute components of the innate immune response, have been shown to limit HIV-1 replication *in vitro* (2). Importantly, a few RF are elevated in peripheral blood mononuclear cells (PBMC) of HIC compared to antiretroviral (ART)-suppressed and/or HIV-uninfected individuals (3–10), suggesting a role of host cell RF in the natural control of HIV-1 replication *in vivo*.

The cyclin-dependent kinase (CDK) inhibitor p21 is a multifunctional modulator of a broad range of biological processes like cell proliferation, that also indirectly limit the HIV-1 replication by blocking the biosynthesis of dNTPs required to viral reverse transcription and by inhibiting the CDK9 activity required by HIV-1 for viral mRNA transcription (6). The protein p21 has been shown to inhibit HIV-1 replication *in vitro* in various cellular systems (6,11–15) and is further expressed at high levels *ex vivo* in $CD4^+$ T cells from HICs (4,6,9,16). Another study revealed that p21 mRNA levels correlated with $CD4^+$ T cell activation in EC, but not in other HIV-infected groups, suggesting that the inducibility of this RF to immune activation is a singular characteristic of EC and may contribute to this special phenotype (3).

The monocyte chemotactic protein-induced protein 1 (MCPIP1) is a protein with a critical role in the regulation of the inflammatory response and immune homeostasis that also block HIV-1 replication by promoting the viral mRNA degradation through its RNase activity (17,18). MCPIP1 was pointed as an important RF of HIV-1 replication *in vitro* in quiescent $CD4^+$ T cells (17), but its role in controlling HIV-1 infection *in vivo* is undefined. Interestingly, a recent study revealed that MCPIP1 overexpression in renal carcinoma cells (Caki-1 cells) reduces the cellular growth by increasing the levels of p21 transcripts, along with other proteins involved in cell cycle progression/arrest (19). According to this study, MCPIP1 expression triggers activation of p21 by down-modulation of damage-specific DNA binding protein 1 (DDB1) and upregulation of chromatin licensing and DNA replication factor 1 (CDT1) (19).

This evidence prompted us to ask whether the expression of MCPIP1 and p21 could be positively correlated in the setting of natural control of HIV-1 infection. To test this hypothesis, we quantified the *in vivo* expression of these and others antiviral cell host RF mRNAs in PBMC from HIC, ART-suppressed and HIV-uninfected individuals. We further explored the potential relationship between antiviral host RF expression and levels of systemic cellular activation in HIC.

2 Methods

2.1 Study Subjects

We analyzed a cohort of 21 HIC subjects followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI) in Rio de Janeiro, Brazil. All HIC maintained RNA VL of < 2,000 copies/ml without antiretroviral therapy for at least five years and were subdivided in two sub-groups: EC (n = 13) when most (≥ 70%) plasma VL determinations were below the detection limit (< 40-80 copies/ml

depending on the VL method used along patient follow-up), and VC (n = 8) when most (≥ 70%) VL determinations were between 41-2,000 copies/ml. Virological and immunological characteristics of these subjects were described in detail in previous studies (20,21). Two groups of ART-suppressed subjects (ART, n = 8) and healthy HIV-1-uninfected subjects (NEG, n = 10) were used as controls.

2.2 mRNA gene-expression analysis

Total RNA was extracted from 1 x 10^7 PBMC using RNeasy mini kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) in which buffer RLT was supplemented with β-mercaptoethanol and displaced on-column DNase treatment using a Qiagen RNase-Free DNase Set (Qiagen, Hilden, North Rhine-Westphalia, Germany) according to manufacturer's instruction. Total RNA yield and quality were determined using NanoDrop® 8000 spectrophotometer and an Agilent® 2100 Bioanalyzer. Only samples with an RNA integrity number (RIN) greater than 8.0 were used. Purified RNA (1 µg) was reverse-transcribed to cDNA using RT^2 First Strand Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). The cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix (Qiagen, Hilden, North Rhine-Westphalia, Germany) and the mixture was added into customized RT^2RNA PCR Array (Qiagen, Hilden, North Rhine-Westphalia, Germany) to measure the mRNA expression of cellular RF (MCPIP1, p21, SAMHD1, APOBEC3G, Tetherin, IFITM1, Mx1, Mx2, Schlafen 11 [SLFN11]and IFITM3) and three housekeeping genes (GAPDH, β-actin, and RNase-P), according to manufacturer's instructions. Values of the crossing point at the maximum of the second derivative of the four-parameters fitted sigmoid curve second derivative, Cp, was determined for each sample. The efficiency of each amplification reaction was calculated as the ratio between the fluorescence of the cycle of quantification and fluorescence of the cycle immediately preceding that. Genes used in the normalization among samples were selected by the geNorm method (22). Data were expressed as fold-changes in mRNA abundance calculated as the normalized gene expression in any test sample divided by the mean normalized gene expression in the control HIV-negative group.

2.3 $CD4^+$ T cell and monocyte activation analyses

We used data of $CD4^+$ T cell and monocyte activation obtained in a previous study conducted by our group including these patients (21), in which plasma levels of soluble CD14 (sCD14) were determined by ELISA-sCD14 Quantikine assay (R&D Systems Minneapolis, MN) according to the manufacturer's protocol and surface expression of comnined HLA-DR and CD38 on $CD4^+$ T cells was analyzed by flow cytometry.

2.4 Data analyses

The comparisons of mean log-fold changes in mRNA abundance were performed by either t-tests or one-way ANOVA nonparametric permutation tests (B = 1,000 permutations), followed by pair-wise comparisons with Holm-Bonferroni adjustment (23), for two or more groups respectively. Spearman coefficient was used for correlation analyses. A first-order log-Normal multiple regression analysis was fitted to model p21 gene expression as a function of MCPIP1 gene expression, $CD4^+$ T cell activation ($HLA\text{-}DR^+CD38^+$), and HIC groups (EC and VC). The threshold for statistical significance was set to $P < 0.05$. Data were analyzed with R software (version 3.5.2) (24).

3 Results

Twenty-nine HIV-1 positive (21 HIC and 8 ART-supressed), and 10 HIV-negative individuals were included in this cross-sectional study. Most HIV-positive (59%) and HIV-negative (60%) individuals were females and all individuals displayed $CD4^+$ T cells counts above 500 cells/µl (Table 1).

Analyses of host RF expression revealed a significant upregulation of p21 and MCPIP1 in HIC (Figure 1). The p21 mRNA was up-regulated in PBMCs from HIC compared to cells from both ART-suppressed (1.63-fold increase; P = 0.003) and HIV-negative (1.55-fold increase; P = 0.003) subjects (Figure 1A). Significant overexpression of the MCPIP1 mRNA in PBMC from HIC compared to ART-suppressed (1.68-fold increase; P = 0.003) and HIV-negative (1.37-fold increase; P = 0.02) individuals was also observed (Figure 1B). Expression of p21 mRNA was significantly ($P < 0.025$) elevated in PBMCs from both EC and VC compared to control groups (Figure 1C). Expression of MCPIP1 mRNA was also elevated in EC and VC subsets compared with ART-suppressed and HIV-negative groups, although the difference with HIV-negative group did not reach statistical significance (Figure 1D). With respect to EC and VC, p21 and MCPIP1 mRNA expression levels were equally significant. Of note, the expression of other antiretroviral RF shows no statistical differences between the HIC and control groups, with exception of IFITM1 that was significantly elevated (1.15-fold increase; P = 0.03) in HIC in comparison to the HIV-negative group (Supplementary Figure S1). Moreover, we found a significant positive correlation between p21 and MCPIP1 mRNA expression ($r \geq 0.57$; $P \leq 0.014$) independently of the VL, treatment condition and HIV status (Figure 2). With respect to other RF, only a modest, but significant, negative correlation between p21/MCPIP1with APOBEC3G mRNA in HIC was observed (Supplementary Figure S2).

To explore the potential relationship of p21 or MCPIP1 expression with immune activation and inflammation, we measured the frequency of $CD4^+HLA\text{-}DR^+CD38^+$ T cells and plasma sCD14 levels (monocyte activation) in our cohort. Frequencies of activated $CD4^+$ T cell populations in VC and ART-suppressed subjects were similar; nevertheless, higher than in EC ($P < 0.0001$) and HIV-negative (P = 0.0002) individuals (Figure 3A). The median concentration of sCD14 in plasma was not significantly different across the groups (Figure 3B). The mRNA levels of p21 were positively associated with activated $CD4^+$ T cells levels in HIC (r = 0.53; P = 0.016) and EC (r = 0.68; P = 0.017) (Figure 3C). No significant correlations between mRNA levels of MCPIP1 and $CD4^+$ T cell activation were observed for HIC or EC subsets (Figure 3D). Levels of sCD14 were positively correlated with both p21 (r = 0.74; P = 0.005) and MCPIP1 (r = 0.58; P = 0.04) mRNA levels only in the EC subset (Figure 3E and F). No significant correlations between mRNA levels of p21/MCPIP1 and $CD4^+$ T cell activation or sCD14 levels were observed when ART-suppressed and HIV-negative individuals were included (Supplementary Figures S3). Multivariate analysis showed that the upregulation of MCPIP1 was positively associated with the increase of p21 expression in HIC (1.44-fold increase; P = 0.0035) (Supplementary Figure S4A). The frequency of activated $CD4^+$ T cells also was positively associated with the increase of p21 expression in both EC and VC (1.48-fold increase; P = 0.0116), although this increase of the p21 expression was down-regulated by the increase of activated $CD4^+$ T cells in VC when compared to EC (1.30-fold decrease by an increase of 1% $CD4^+HLA\text{-}DR^+CD38^+$ T cells; P = 0.0284) (Supplementary Figure S4B). Overall, the model was highly significant (P = 0.003) and could explain as much as 70% ($R^2 = 0.492$) of p21 expression.

4 Discussion

In this study, we observed that p21 and MCPIP1 mRNA expression were significantly increased in PBMC of HIC compared to cells of HIV-negative and -positive/ART-suppressed individuals. The elevated expression of p21 in PBMC of HIC had already been previously described (3,4,6,9,16). Originally, this is the first study to show overexpression of MCPIP1 alongside with p21 in these individuals. The mRNA levels of p21 and MCPIP1 were positively correlated in HIC as well as in HIV-positive and –negative individuals. This supports a coordinated expression of these cellular genes in different settings, consistent with what has been shown for a renal carcinoma cell line (19).

Other host RF, such as SAMHD1, SLFN11, and Mx1, previously found to be overexpressed in $CD4^+$ T cells from HIC (3,7,8), were not up-regulated in PBMC from our HIC cohort, which may reflect differences in the cellular populations analyzed (8). A recent study (10), however, described upregulation of tetherin and SLFN11 in PBMC from HIC, supporting that the contrasting findings across studies might also reflect inter-cohort diversity (1). Indeed, we detected in our HIC cohort a few individuals that displayed mRNA levels of SAMHD1 and/or SLFN11 well above the normal range (Supplementary Figure S1). These observations suggest that there might not be a unique host RF expression signature common to all HIC, but that different combinations of host RF could be associated with natural control of HIV-1 replication in distinct individuals. Thus, the particular set of increased host RF may vary across different HIC cohorts.

In contrast to p21 and MCPIP1, the other antiretroviral RF analyzed here are interferon (IFN)-stimulated genes (ISGs) (25). In the chronic phase of HIV−1 infection in viremic untreated patients, most ISGs (mainly those responding to IFN type I) are upregulated in $CD4^+$ T cells (26–28) and their expression is positively correlated with the percentage of activated T cells and negatively correlated with $CD4^+$ T cell counts (26–30). We found no significant upregulation of ISGs in our HIC cohort, likewise other studies (3,27,28,31), except for IFITM1 that is also induced by type II IFN (32), indicating that another pathway may have stimulated its expression. These results suggest that residual or low-level viremia might not be enough to induce a generalized upregulation of ISGs during chronic infection (28). In addition, MCPIP1 negatively regulates the NF-κB cascade (33,34) and its overexpression in HIC may also contribute to limit the chronic overexpression of ISGs in HIC.

Our results confirm previous observations that levels of p21 transcripts are positively correlated with $CD4^+$ T cell activation in EC and HIC groups (3) and further suggest a positive correlation of p21 and MCPIP1 mRNA with sCD14, a marker of monocyte activation, in EC. Increased expression of p21/MCPIP1 associated with T cell and/or monocyte activation seems to be a unique characteristic of HIC/EC, because similar correlations were not observed for HIV-infected/ART-suppressed or HIV-negative subjects. Furthermore, previous studies have shown that viremic progressors displayed reduced levels of p21 even though cellular activation was much higher than in HIC or HIV-uninfected individuals (6). These results suggest that p21/MCPIP1 overexpression may be a distinctive homoeostatic innate response of HIC to limit the deleterious effects of chronic immune activation driven by HIV-1 infection.

Selective upregulation of p21 and MCPIP1 in $CD4^+$ T, macrophages and/or dendritic cells may directly limit HIV-1 replication by 1) reducing the reverse transcription and chromosomal integration of HIV-1 in quiescent cells and thus limiting the size of the latent proviral reservoir (11–13,35–37); 2) restricting HIV-1 LTR transcription (33,34,38,39); and, 3) degrading viral mRNA and miRNA (17,40–42). Upregulation of p21 and MCPIP1 may also indirectly limit HIV-1 replication and further prevent $CD4^+$ T cells loss by reducing chronic IFN-I signaling, generalized inflammation and over-activation of the immune system (43–52), without affecting the activation of antiviral cellular responses. Although the enhanced antiviral and anti-inflammatory state may not be enough to fully restrict HIV-1 replication (53), it could act in concert with other innate and adaptive immune mechanisms to control HIV replication in HIC.

The enhanced expression of a few select host RF, including p21, was strongly associated with reduced $CD4^+$ T cell-associated HIV RNA during ART, indicating that this host RF may contribute to the control of viral expression and ongoing replication during ART (54). Another study demonstrates that atorvastatin, a lipid-lowering medication, exert a broad spectrum of anti-

inflammatory functions and further reduced HIV infection in both rested and activated $CD4^+$ T cells *in vitro* via p21 upregulation (14). Interestingly, atorvastatin was found to up-regulates p21 through a p53 independent pathway, which is consistent with a potential role of MCPIP1 in that antiviral mechanism. These observations suggest that pharmacological manipulation of p21 and MCPIP1 may open novel therapeutic perspectives to prevent HIV-1 replication and to attenuate HIV-associated inflammation and immune activation during ART.

An important limitation of our study is the impossibility of assigning which cell(s) population(s) has increased expression of p21 and MCPIP1 in HIC. The expression profile of many RF and ISGs may be different between $CD4^+$ T cells and monocytes (8), suggesting that the individualization of these cell types might better decipher the mechanisms of RF regulation in the setting of natural control of HIV-1 infection. Another potential limitation is that only RF mRNA levels were analyzed. Previous studies showed that p21 mRNA levels mirror p21 protein levels in $CD4^+$ T cells from HIC (6) and that MCPIP1 mRNA levels reflect MCPIP1 protein levels in HCV-infected hepatoma cells (55). Although these evidences indicate a close match between transcripts and protein expression levels, measuring the levels/activity of p21 and MCPIP1 proteins in cells from HIC should also help to elucidate the relevance of these RF for HIV control.

In summary, our data confirm the high levels of p21 mRNA expression and shows for the first-time the concurrent overexpression of MCPIP1 mRNA in HIC. Moreover, we found a positive correlation between p21 and MCPIP1 transcripts in HIC, indicating a possible synergistic effect of both innate host RF on natural suppression of HIV-1 replication *in vivo*. Further studies are needed to better understand the role of p21 and MCPIP1 in the natural control of HIV-1 replication and disease progression in HIC. These findings may also have important implications for the development of new immune-based therapeutic strategies for a functional cure of HIV-1 infection.

5 Figure legends

Figure 1. p21 and MCPIP1 mRNA levels are up-regulated in PBMC from HIC. Boxplots represent the interquartile and sample median (central solid black line) of the relative changes (fold-change values relative to the mean of HIV-1-uninfected (NEG) subjects) of p21 and MCPIP1 expression comparing NEG and ART-suppressed subjects (ART) with HIV controllers (HIC) (A and B) or elite (EC) and viremic (VC) controllers (C and D). P-values < 0.05 were considered statistically significant.

Figure 2: **p21 and MCPIP1 mRNA levels in PBMC from HIC are positively correlated.** The p21 and MCPIP1 normalized expression correlations were calculated considering all groups (A), HIV-infected (B), HIC (C), and EC (D). The points' colors indicate the patient group, accordingly to the legend. Correlation coefficients (Spearman's ρ) are shown in the upper right corner of each graph. P-values < 0.05 were considered statistically significant.

Figure 3. p21 transcripts are positively correlated with $CD4^+$ T cell and monocyte activation while MCPIP1 transcripts are positively correlated only with monocyte activation in EC. The mean of activated $CD4^+$ T cells ($HLA\text{-}DR^+CD38^+$) counts (A) and soluble CD14 (sCD14) in plasma (B) were compared for each group. The color of each dot represents the group as indicated in the legend at right. P-values < 0.05 were considered statistically significant. The correlations were made evaluating the relationship between activated $CD4^+$ T cells (C and D) or sCD14 levels (E and F) with the normalized expression of p21 and MCPIP1 for EC and HIC groups. The points' colors present in each graph indicate the groups present according to the legend. Correlations coefficient (Spearman's ρ) are shown in the upper left corner of each graph.

6 Ethics Statement

This study was carried out in accordance with the recommendations of the ethical committee of Instituto Nacional de Infectologia Evandro Chagas (INI-Fiocruz) that approved the study protocol (CAAE 1717.0.000.009-07). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Author Contributions

GB and TMLS conceived and designed the study and supervised the experiments. SSDA conducted experiments and analyzed the data together with MR-A and GB. FH performed the $CD4^+$ T cell and monocyte activation assays. ED collaborated with mRNA gene-expression analysis. BH, BG, and VGV conducted patient recruitment and follow-up. FH, ED and MGM provided intellectual input for results interpretations. SSDA, GB and MR-A wrote the first draft and all authors assisted with the writing and approved the final manuscript.

9 Acknowledgments

The authors thank the patients, who participated in the study, as well as all the technical staff involved in the clinical follow-up of these patients. We also thank Ms Marilia Alves Figueira de

Melo for the excellent technical support in RNA quantification and integrity analyses and the Plataforma de PCR em Tempo Real – RJ (RPT09A) – FIOCRUZ and Plataforma de Sequenciamento de Ácidos Nucléicos de Nova Geração – RJ (RPT01J) – FIOCRUZ.

10 Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ (grant number E-26/110.123/2014) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grant Number 401220/2016-8). SSDA was supported by funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ. ED was financed by a Postdoctoral fellowship from the "Programa Nacional de Pós-Doutorado (PNPD)" by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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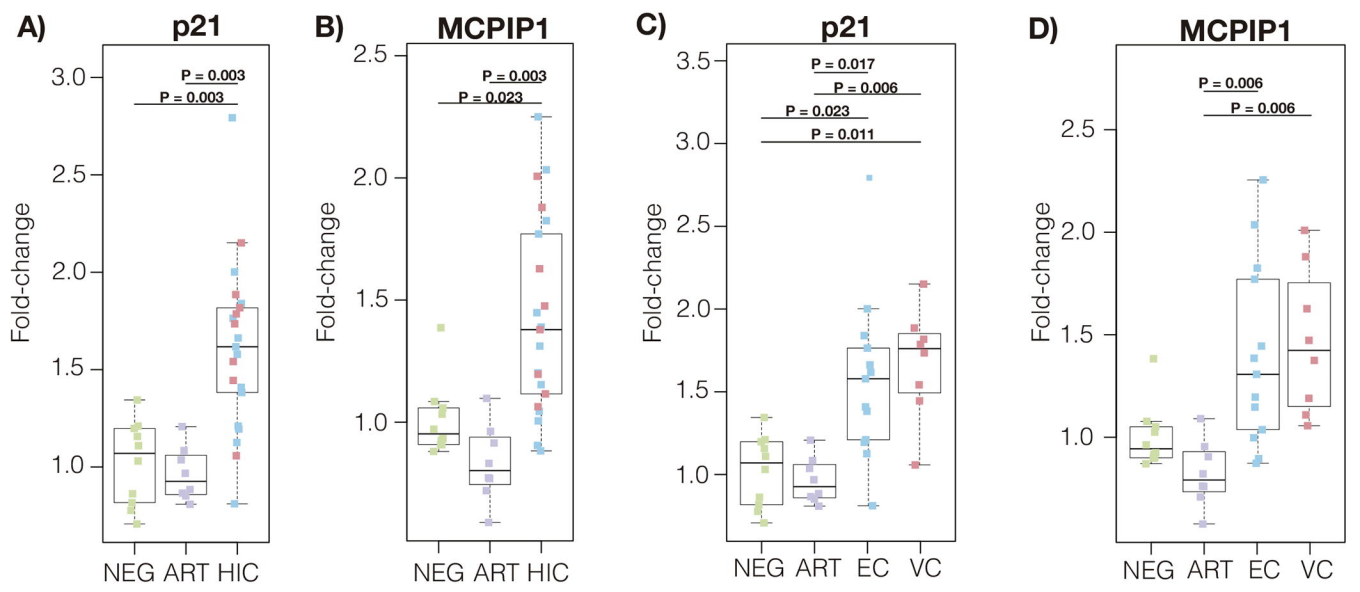
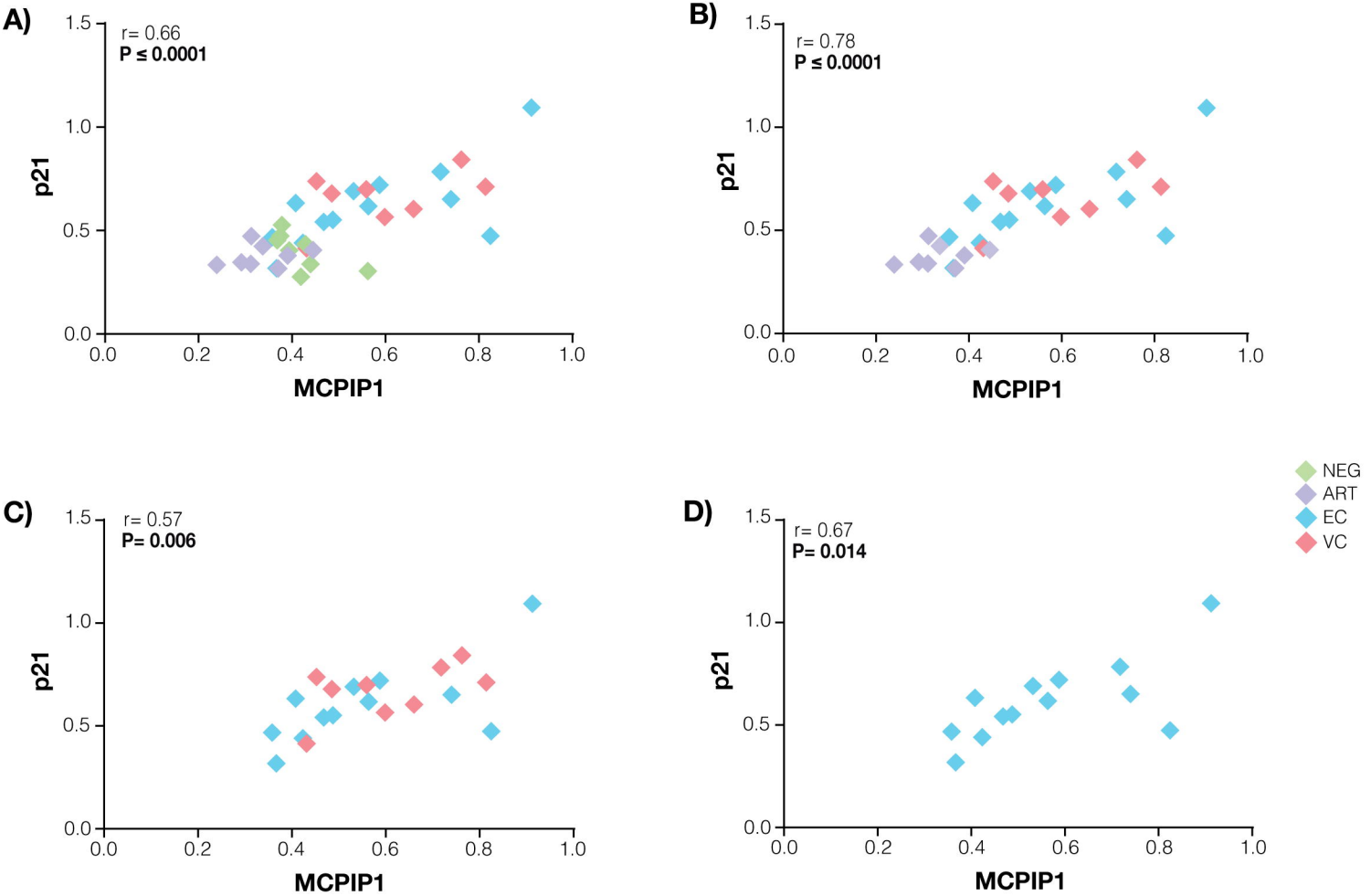
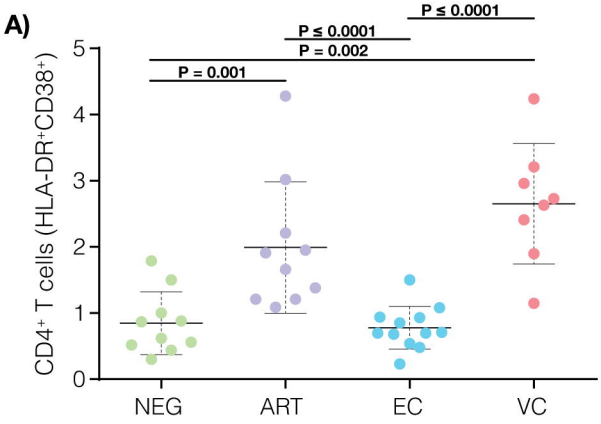
Table 1. Main clinical and epidemiologic characteristics of individuals of this study.

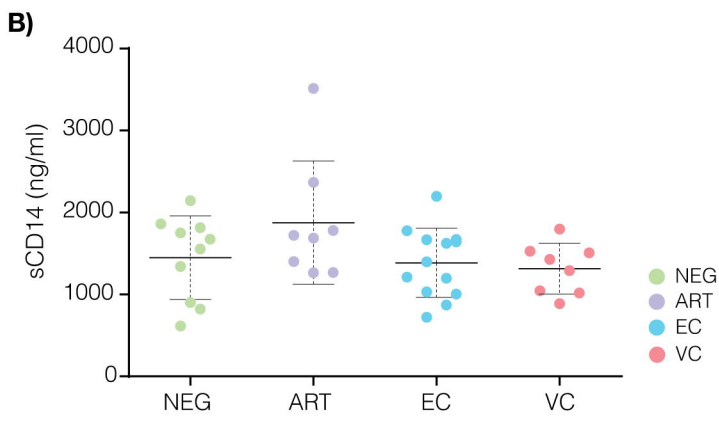
Characteristics	**HIC (n = 21)**		**ART-suppressed (n = 8)**	**HIV-1 negative (n = 10)**
	EC (n = 13)	**VC (n = 8)**		
Sex, no. (%)				
Female	10 (77)	3 (38)	4 (50)	6 (60)
Male	3 (23)	5 (62)	4 (50)	4 (40)
Age (years)*	45 (39-60)	43.5 (39-47)	47 (38-53)	47 (36-51)
Study point				
Time since HIV-1 diagnosis (years)	9 (5.5-15)	12.5 (7-16)	NA	-
$CD4^+$ T cell (cells/µl)	1027 (834-1255)	664 (563-1228)	889 (678-1097)	1043 (784-1581)
Plasma HIV RNA (copies/ml)	<50	641 (327-915)	<40	-
CD4/CD8 ratio	1.33 (1.24-1.61)	0.91 (0.67-1.23)	1.06 (0.73-1.5)	1.69 (1.62-2.07)

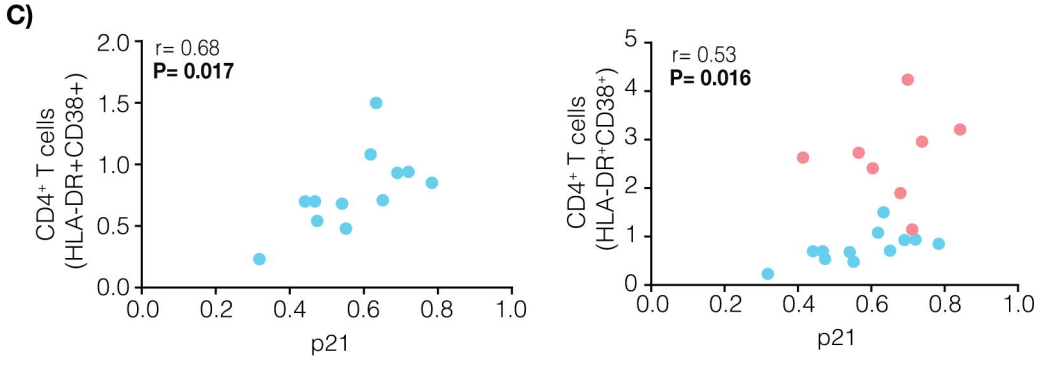
* Age at study point; Interquartile ranges are shown in parenthesis. HIC, HIV controllers; ART, antiretroviral therapy; EC, elite controllers; VC, viremic controllers. NA, not available.

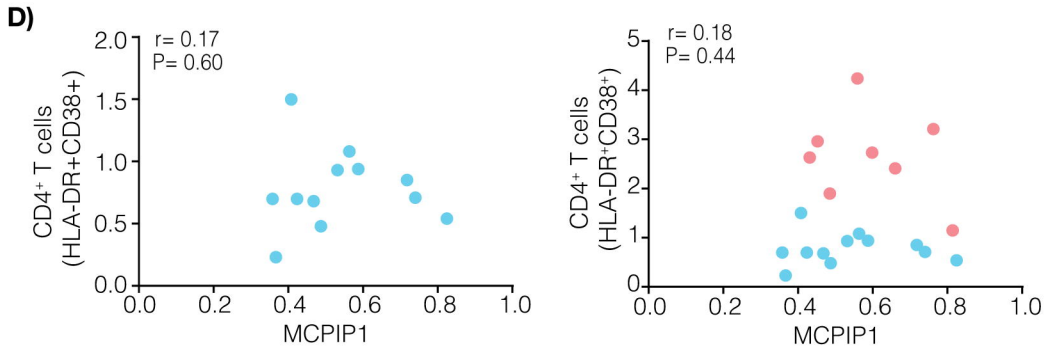

A)
p21
P = 0.003
P = 0.003
Fold-change
3.0
2.5
2.0
1.5
1.0
NEG ART HIC
B)
MCPIP1
P = 0.003
P = 0.023
Fold-change
2.5
2.0
1.5
1.0
NEG ART HIC
C)
p21
P = 0.017
P = 0.006
P = 0.023
P = 0.011
Fold-change
3.5
3.0
2.5
2.0
1.5
1.0
NEG ART EC VC
D)
MCPIP1
P = 0.006
P = 0.006
Fold-change
2.5
2.0
1.5
1.0
NEG ART EC VC

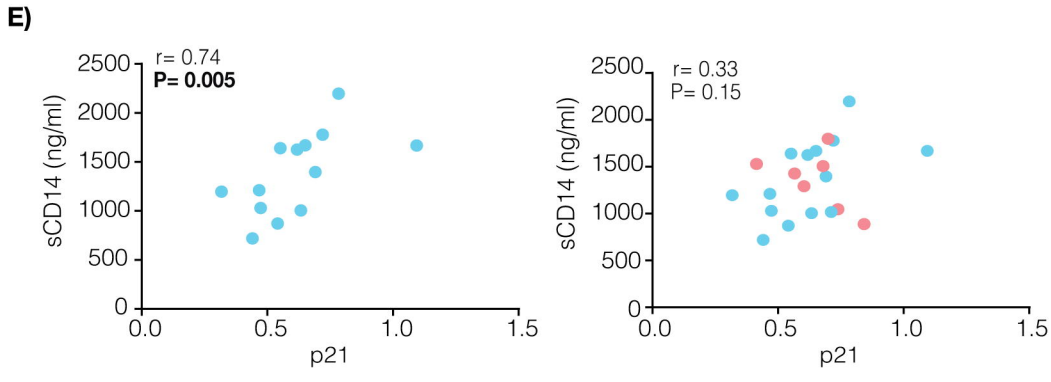

A)
r= 0.66
P ≤ 0.0001
p21
MCPIP1
B)
r= 0.78
P ≤ 0.0001
p21
MCPIP1
C)
r= 0.57
P= 0.006
p21
MCPIP1
D)
r= 0.67
P= 0.014
p21
MCPIP1
NEG
ART
EC
VC

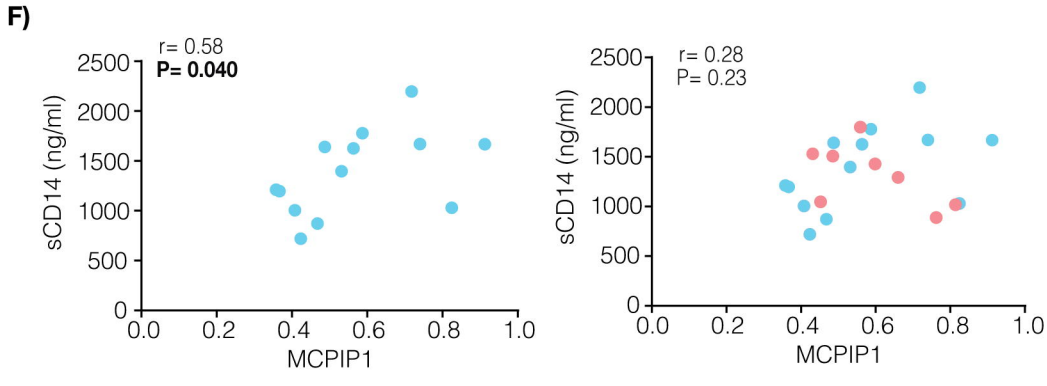

A)
P ≤ 0.0001
P ≤ 0.0001
P = 0.002
P = 0.001
CD4+ T cells (HLA-DR+CD38+)
NEG
ART
EC
VC


B)
sCD14 (ng/ml)
NEG
ART
EC
VC


C)
r= 0.68
P= 0.017
CD4+ T cells (HLA-DR+CD38+)
p21
r= 0.53
P= 0.016
CD4+ T cells (HLA-DR+CD38+)
p21


D)
r= 0.17
P= 0.60
CD4+ T cells (HLA-DR+CD38+)
MCPIP1
r= 0.18
P= 0.44
CD4+ T cells (HLA-DR+CD38+)
MCPIP1


E)
r= 0.74
P= 0.005
sCD14 (ng/ml)
p21
r= 0.33
P= 0.15
sCD14 (ng/ml)
p21


F)
r= 0.58
P= 0.040
sCD14 (ng/ml)
MCPIP1
r= 0.28
P= 0.23
sCD14 (ng/ml)
MCPIP1