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Dynamic Regulation of Host Restriction Factor Expression over the Course of HIV-1 Infection *In Vivo*

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In this study, we investigated the expression levels of host restriction factors in six untreated HIV-1-positive patients over the course of infection. We found that the host restriction factor gene expression profile consistently increased over time and was significantly associated with CD4$^+$ T cell activation and viral load. Our data are among the first to demonstrate the dynamic nature of host restriction factors *in vivo* over time.

Three decades of research have passed since the discovery of HIV-1, but to date there is no cure. Most infected individuals, if not given antiretroviral therapy (ART), will progress to AIDS within 10 years. With the introduction of ART, the morbidity and mortality associated with HIV-1 infection have decreased, but eradication remains difficult due to the persistence of viral reservoirs in the infected individual.

Many studies have associated the preferential *in vivo* control of HIV-1 with HLA or KIR alleles, such as HLA-B*57 (1–3), HLA-B*27 (4, 5), and KIR3DS1/KIR3DL1 (6, 7). A hierarchy of host alleles in relation to viral control has been established for the population groups studied (8). Among these, HLA-B*57 stands out as the major HLA allele associated with lower viral loads and elite viral control in Caucasians. However, these host genetic traits account for only about 25% of the variance in HIV-1 viral load, and it is not well understood why some HLA-B*57 individuals progress at a higher rate than do others who have the allele but are slower progressors. Some of this variation in disease progression among HLA-B*57 individuals may be due to viral escape mutations and avoidance of cytotoxic T-lymphocyte (CTL) control. However, the degree of variation suggests the existence of other mechanisms of viral control, including potentially novel mechanisms that have not been fully addressed.

Several cell-intrinsic host genes have been described as possessing potent antiretroviral activity *in vitro* and *in vivo* (9, 10). Restriction factors exert their function at different stages of the HIV-1 life cycle, and their expression varies significantly in different cells and activation states (11). Although most of the published literature describes their potency and mode of action *in vitro*, data describing the pathogenic consequences of intrinsic immunity against HIV-1 *in vivo* over time are largely lacking.

We recently reported that peripheral blood mononuclear cells (PBMC) from HIV-1-seronegative HLA-B*57 individuals possess significantly higher expression levels of anti-HIV-1 genes and that CD4$^+$ T cell activation levels strongly correlate with antiviral gene expression in HLA-B*57 individuals (12). More recently, we comprehensively quantified the overall restriction factor expression profile of primary cells in healthy individuals and demonstrated that cellular activation strongly induced the expression of anti-HIV-1 genes and that increased gene expression correlated with decreased *ex vivo* HIV-1 replication (11). We have also used this platform to analyze host restriction factor gene expression in a cross-sectional study of HIV-1 patients at different stages of disease (13).

In this study, we aimed to describe the kinetics of restriction factor expression over the natural course of HIV-1 infection. We selected six participants in the University of California San Francisco (UCSF) Options cohort who were enrolled within 6 months of their respective estimated dates of infection and were monitored while off antiretroviral therapy for a prolonged period of time.

We quantified the expression of 32 different anti-HIV-1 genes using a custom-made TaqMan array (11, 13) and measured the levels of CD4$^+$ T cell activation over 7 years. We found that overall, restriction factor gene expression consistently increased over time and was significantly associated with systemic CD4$^+$ T cell activation and viral load. Our data are among the first to demonstrate the dynamic nature of host restriction factors *in vivo* over time in HIV-1 infection.

All subjects were enrolled in the Options cohort at UCSF and were at least 18 years of age at the time of sample collection. The Options cohort consists of subjects in acute and early HIV-1 infection, with approximately 90% enrolled within 6 months of acquiring HIV-1 infection. All samples were obtained according to protocols approved by the Institutional Review Board (IRB) at UCSF. Written informed consent was obtained from all subjects, according to the Declaration of Helsinki. A full description of the cohort and each patient's characteristics are shown in Table 1.

Six HIV-1-positive subjects were monitored longitudinally from early infection (median, 10 weeks [range, 9.4 to 10.8 weeks]

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TABLE 1 Patient characteristics

Patient ID	Gender	Risk factor	Time point (wpi)[a]	VL (copies/ml)	Baseline value				Therapy[c]
					CD4 (cells/mm^3)	CD8 (cells/mm^3)	Study period (wpi)[b]	CCR5 genotype	
P1	Male	MSM	10	7,728	555	660	10–347	CCR5/CCR5	Wks 13–72 and 321–333
P2	Male	MSM	10	4,973	570	665	15–160	CCR5/CCR5	Naive
P3	Male	MSM	10	<50	250	350	39–233	CCR5/CCR5	Naive
P4	Male	MSM	11	293	1,452	1,155	107–316	CCR5/CCR5	Naive
P5	Male	IDU	10	1,533	1,102	928	13–261	CCR5/CCR5	Wks 18–20
P6	Male	MSM	10	10,861	833	531	229–400	CCR5/CCR5Δ32	Naive

[a] Date of first available CD4 and VL measurements. wpi, weeks postinfection.
[b] Time interval for longitudinal PBMC samples used in analysis.
[c] Data for all time points analyzed were from therapy-naive patients or patients who were off therapy. Samples from the treatment period were excluded.

postinfection) up to 7 years. For each subject, the time since infection was estimated based on documented dates of negative and positive serologic testing (enzyme immunoassay, Western blotting) and HIV-1 RNA testing and on the results of detuned/less-sensitive immunoassay testing as previously described (14, 15). Five individuals were men who have sex with men (MSM) and one was an injecting drug user (IDU). Patients with identification codes (IDs) P2, P3, P4, and P6 were therapy naive, and P1 and P5 received ART from weeks 13 to 72 and 321 to 333 and weeks 18 to 20, respectively. Samples from the treatment period were excluded from this study. The sampling times were divided into three different intervals, corresponding to the infection stage: <1 year (10 to 39 weeks), 1 to 3 years (73 to 149 weeks), and >3 years (160 to 400 weeks). Patient 6 had the genotype CCR5/CCR5Δ32, and all other patients were CCR5/CCR5 homozygotes.

We quantified the expression of 32 different anti-HIV-1 genes in PBMC. Briefly, total RNA was extracted from whole PBMC using TRIzol, followed by DNase treatment. DNase-treated RNA was transcribed into cDNA using random primers. Quantitative real-time PCR utilizing a custom-made TaqMan low-density array (TLDA) and thermal cycling was performed using an Applied Biosystems ViiA7 real-time PCR system. A panel of 6 housekeeping genes was included in the TLDA plates (GAPDH, 18S, ACTB, PPIA, RPLP0, and UBC), and RPLP0 was identified as the most stably expressed gene using the GeNorm algorithm (16). All gene cards (Applied Biosystems) used for quantification have been previously described in reference 11.

As we consistently observed in previous studies (11, 13), members of the IFITM family, SAMHD1, and TRIM22 were the most highly expressed genes in our restriction factor panel. We found that the expression of restriction factors increased over the course of natural HIV-1 infection, with more a pronounced upregulation after 3 years postinfection (Fig. 1). We observed statistically significant (paired *t* test) upregulation in the expression of TRIM22, SAMHD1, MOV10, HERC5, and RNAseL, and the expression of several other restriction factors approached significance (APOBEC3C, APOBEC3H, TRIM5, TRIM14, TRIM21, EIF2AK2, SLFN11, and IFITM family members). Interestingly, the expression of several host restriction factors remained stable throughout the study period (TRIM26, TRIM28, PAF1, RTF1), and in contrast, we found that the expression of TRIM11 and CDKN1A/p21 decreased over time. Although the expression of the latter two restriction factors did not reach significance (*P* values of 0.19 and 0.15, respectively), the lack of statistical significance is likely to be due to limited sample size.

We then implemented an intuitive mathematical construct to represent the overall cumulative anti-HIV-1 restriction capacity associated with each sample. This metric, cumulative restriction or CuRe score, first described by our group in reference 13, depicts the cumulative fold difference in antiviral gene expression with respect to a reference individual. We plotted the individual CuRe scores for each patient together with the viral load (VL) and observed that the kinetics of cumulative restriction factor gene expression mirror the kinetics of viral replication (Fig. 2A). To evaluate the association between CuRe score and VL, we applied linear regression and then stratified permutation. Since we obtained repeated measures from different individuals, we did not assume the data were independent when deriving the inference. Therefore, we tested the conditional independence of the log viral load and CuRe score for each individual and found it to be statistically significant (estimate of 1.1726; *P* value, 0.033). The estimate is described as the ratio change in 1 standard deviation (SD) unit increase in log viral load.

We then used flow cytometry to determine the relative levels of $CD4^+$ T cell activation in the six patients over the study period, as previously described in references 12 and 13. T cell activation levels are reported as a percentage of $CD4^+$ T cells coexpressing HLA-DR and CD38. The overall restriction factor gene expression profile followed a trajectory over time that was similar to that of $CD4^+$ T cell activation (Fig. 2B). The association between CuRe score and $CD4^+$ T cell activation was found to be statistically significant (estimate of 1.2498; *P* value, 0.009). These data are in alignment with our previous *in vivo* and *in vitro* observations suggesting that host restriction factor expression typically scales with levels of cellular activation (12, 13).

In order to better understand the kinetics of expression of each individual restriction factor gene in each patient over the course of disease, we created a heat map (Fig. 2C). Gene expression from the earliest time point acquired for each individual was arbitrarily normalized to 1, and subsequent fold expression was calculated. As reflected in the CuRe score, the expression of most genes increased over time. We detected strong upregulation of APOBEC3D, APOBEC3F, TRIM5, TRIM14, and SAMHD1. The expression of several genes remained relatively stable or underwent limited upregulation over the course of disease (e.g., PML/TRIM19, TRIM26, TRIM28, PAF1, RTF1, MOV10). Overall, the heat map analysis illustrates the dynamic

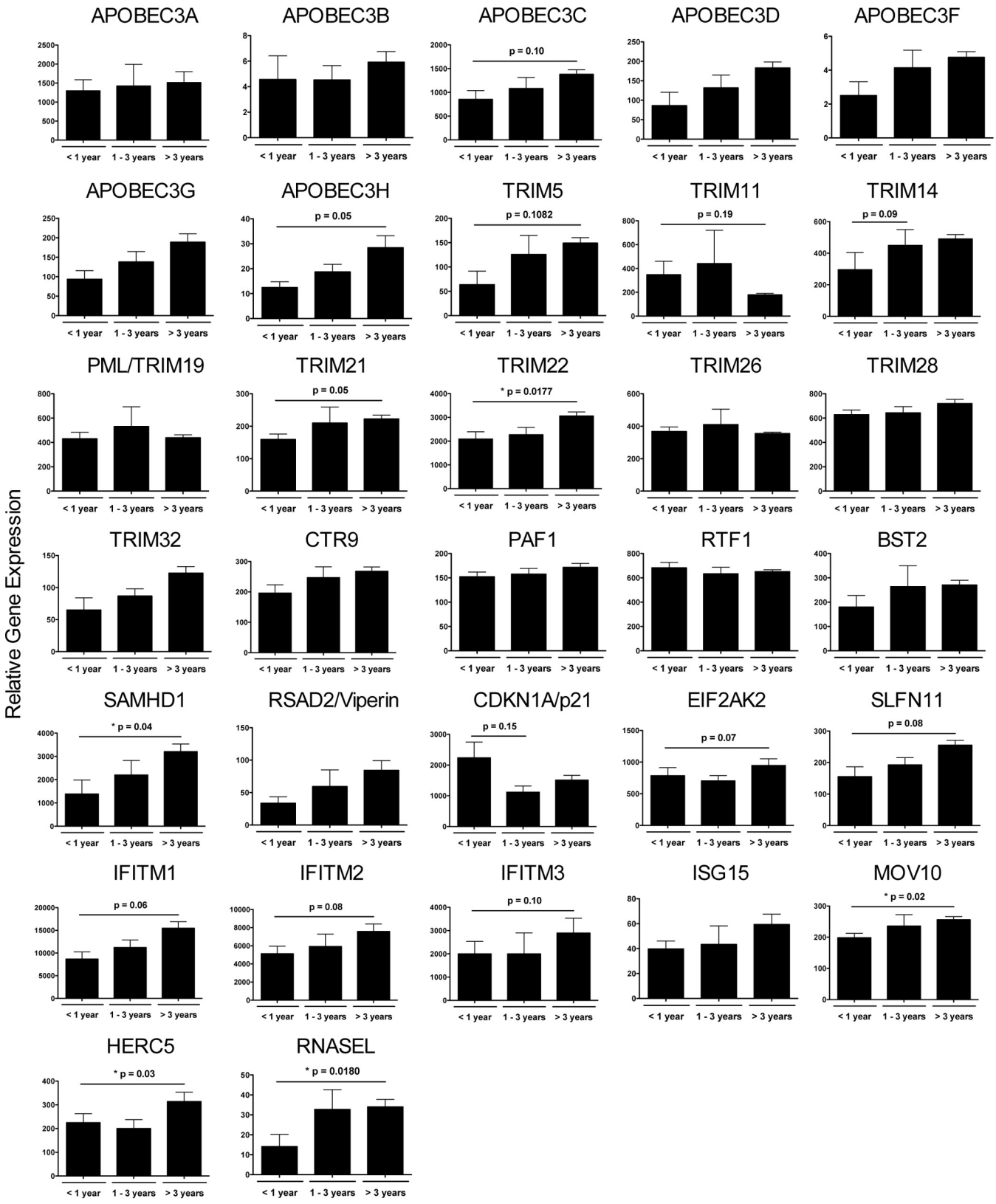

FIG 1 Relative gene expression of individual restriction factors in PBMC from six HIV-1-infected subjects from the Options cohort. The sampling times were divided into three different intervals, corresponding to the infection stage: <1 year, 1 to 3 years, and >3 years. RNA extraction using TRIzol was followed by cDNA synthesis. Quantitative real-time PCR was performed using custom-made TaqMan low-density arrays (TLDA). Thermal cycling was performed using an ABI ViiA7 real-time PCR system. A panel of 6 housekeeping genes was included in the TLDA plates, and RPLP0 was identified as the most stably expressed gene and was used for normalization of results by the comparative threshold cycle (C_T) method. Values represent means plus standard errors of the means (SEM). Statistical analysis was performed using a paired *t* test. A *P* value of <0.05 was considered significant.

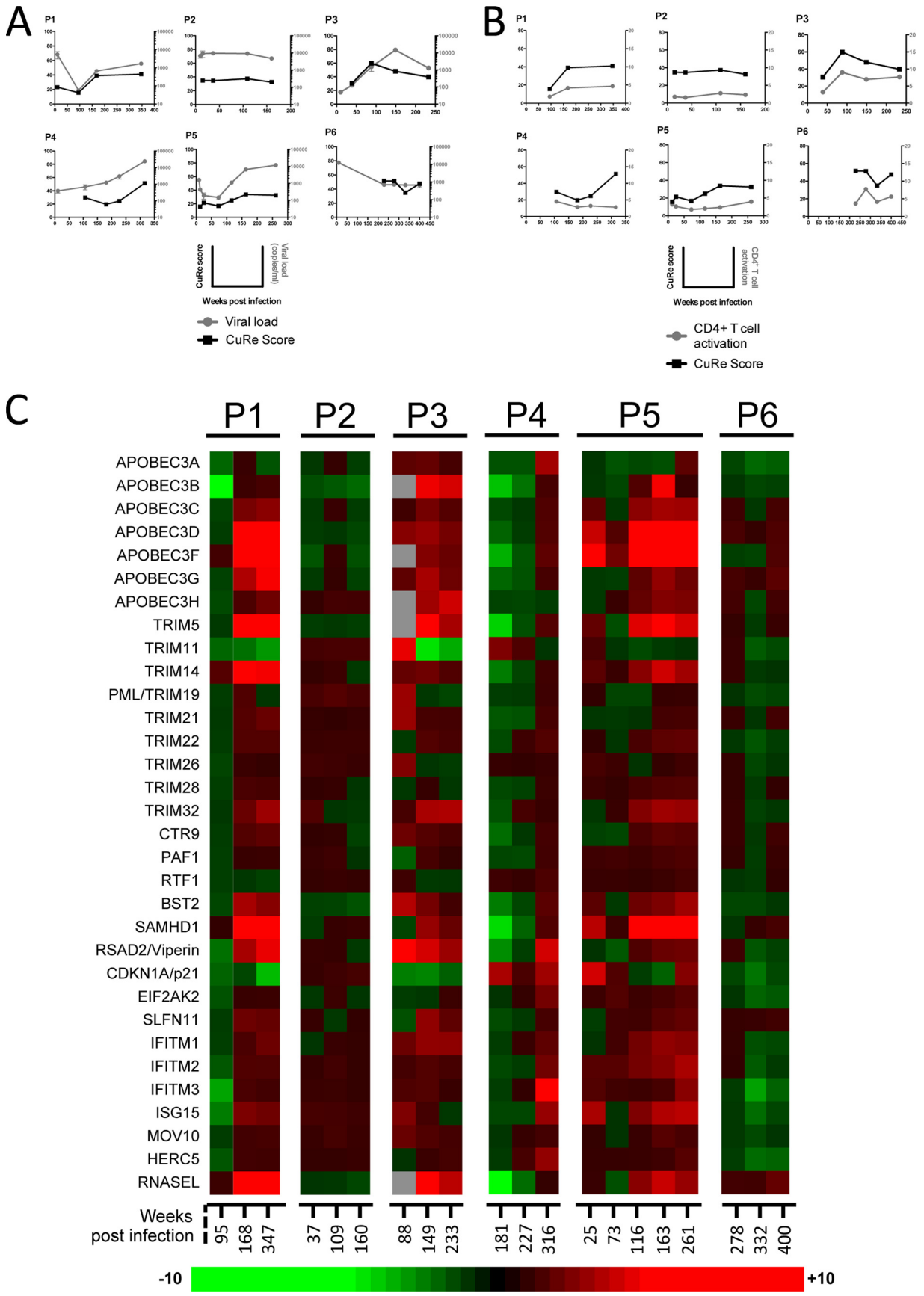

FIG 2 Kinetics of VL (A) and CD4$^+$ T cell activation (B) versus cumulative restriction (CuRe) score in PBMC from six HIV-1-infected subjects from the Options cohort over the course of the study. (C) Heat map illustrating the fold changes in individual restriction factor gene expression. Gene expression from the earliest time point for each individual was arbitrarily normalized to 1 and subsequent fold expression was calculated (on a scale of −10 to +10). Weeks postinfection are indicated at the bottom of the heat map. Green, downregulation; red, overexpression; and gray, undetermined.

regulation of host antiviral genes over the course of HIV-1 infection.

Our overall objective in this study was to investigate the dynamic regulation of host restriction factors over the course of HIV-1 infection *in vivo*. We observed that the expression of most restriction factors increased over time as the disease progressed, and the pattern of expression followed kinetics similar to those of $CD4^+$ T cell activation and viral replication. The observed kinetics of restriction factor expression levels over time suggests that cross-sectional studies of intrinsic immunity in HIV-1-infected individuals may need to account for duration of infection and cellular activation levels.

Our data complement earlier observations from our group suggesting that naturally occurring, activation-driven variation in restriction factor expression may not exert substantial effects on viral replication *in vivo*, while induction of these factors to supraphysiological levels in the absence of enhanced $CD4^+$ T cell activation results in profound viral suppression (17).

Interestingly, several host restriction factors seem to be constitutively expressed and unchanged over long periods of time. In addition, two of the genes in our panel, TRIM11 and CDKN1A/p21, exhibited inverse patterns in which their expression decreased as the disease progressed.

The overall increase in restriction factor mRNA may be result of the selection of long-lived cells with higher restriction factor gene expression. It is possible that within $CD4^+$ T cells there are long-lived subsets with high levels of expression of host restriction factors. Because of the intrinsic resistance against the virus in the latter subsets, cells could remain protected over time, in contrast to other subsets with lower levels of expression, which would be quickly killed by the virus. With the availability of specimens and a large number of cells, it will be possible to isolate different cellular subsets and understand the contribution of long-lived cells with higher restriction factor gene expression levels to HIV-1 pathogenesis.

To the best of our knowledge, our study is the first to comprehensively analyze the kinetics of restriction factor gene expression *in vivo* during the course of HIV-1 infection. It is critical to note that our data do not address the question of whether higher restriction factor expression levels during the earliest phases of acute HIV-1 infection have beneficial effects on HIV-1 disease outcomes, since we did not have access to samples during the first weeks of infection. As this study involved a small group of patients, validation of our findings with larger sample sizes should be conducted. In addition, analyzing the dynamics of host restriction factors in different cell types will undoubtedly lead to a better understanding of viral pathogenesis *in vivo*.

Further investigation of the kinetics of host restriction factors at the very early stages of HIV-1 infection may shed light into the role of these host genes in the establishment of the latent reservoir and overall rate of disease progression. Intervention at the early stages of HIV-1 infection is crucial and likely determines long-term disease outcomes. We hypothesize that induction of host restriction factor gene expression during acute infection, when levels of T cell activation are still relatively low, may curb the establishment of latent reservoirs and delay disease progression. Reduced colonization of viral reservoirs and constraint of HIV-1 replication by host-encoded antiviral genes may create a better immunological scenario for eradication strategies.

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The authors declare that they have no competing interests.

R.A.S.R. and M.A.-M. performed experiments; R.A.S.R., M.A.-M., S.K.P., and D.F.N. analyzed results. X.D. calculated CuRe scores. F.M.H. and C.D.P. coordinated patient recruitment. R.A.S.R., S.K.P., and D.F.N. designed the research and wrote the paper.

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