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Association between Latent Proviral Characteristics and Immune Activation in Antiretrovirus-Treated Human Immunodeficiency Virus Type 1-Infected Adults

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ABSTRACT

Generalized immune activation during HIV infection is associated with an increased risk of cardiovascular disease, neurocognitive disease, osteoporosis, metabolic disorders, and physical frailty. The mechanisms driving this immune activation are poorly understood, particularly for individuals effectively treated with antiretroviral medications. We hypothesized that viral characteristics such as sequence diversity may play a role in driving HIV-associated immune activation. We therefore sequenced proviral DNA isolated from peripheral blood mononuclear cells from HIV-infected individuals on fully suppressive antiretroviral therapy. We performed phylogenetic analyses, calculated viral diversity and divergence in the *env* and *pol* genes, and determined coreceptor tropism and the frequency of drug resistance mutations. Comprehensive immune profiling included quantification of immune cell subsets, plasma cytokine levels, and intracellular signaling responses in T cells, B cells, and monocytes. These antiretroviral therapy-treated HIV-infected individuals exhibited a wide range of diversity and divergence in both *env* and *pol* genes. However, proviral diversity and divergence in *env* and *pol*, coreceptor tropism, and the level of drug resistance did not significantly correlate with markers of immune activation. A clinical history of virologic failure was also not significantly associated with levels of immune activation, indicating that a history of virologic failure does not inexorably lead to increased immune activation as long as suppressive antiretroviral medications are provided. Overall, this study demonstrates that latent viral diversity is unlikely to be a major driver of persistent HIV-associated immune activation.

IMPORTANCE

Chronic immune activation, which is associated with cardiovascular disease, neurologic disease, and early aging, is likely to be a major driver of morbidity and mortality in HIV-infected individuals. Although treatment of HIV with antiretroviral medications decreases the level of immune activation, levels do not return to normal. The factors driving this persistent immune activation, particularly during effective treatment, are poorly understood. In this study, we investigated whether characteristics of the latent, integrated HIV provirus that persists during treatment are associated with immune activation. We found no relationship between latent viral characteristics and immune activation in treated individuals, indicating that qualities of the provirus are unlikely to be a major driver of persistent inflammation. We also found that individuals who had previously failed treatment but were currently effectively treated did not have significantly increased levels of immune activation, providing hope that past treatment failures do not have a lifelong "legacy" impact.

Generalized immune activation is a hallmark of HIV-1 infection. In this state, a variety of immune cells show an increase in expression of activation, proliferation, and apoptotic markers, cellular turnover with aberrant cell cycle regulation, production of proinflammatory cytokines, and increased lymphoid tissue fibrosis (1–6). Immune activation is strongly associated with HIV-1 disease progression; for instance, T cell activation, as measured by expression of CD38 and HLA-DR, is more predictive of $CD4^+$ T cell depletion and shorter survival than is the plasma viral load (7, 8). Furthermore, the level of immune activation early in HIV-1 infection as measured by $CD8^+$ T cell activation predicts $CD4^+$ T cell loss independently of plasma HIV-1 RNA levels (9). Suppression of viral replication with effective antiretroviral treatment (ART) reduces immune activation, but even effective ART regimens are unable to reduce the levels of immune activation in HIV-infected individuals to levels seen in healthy individuals (1). Since this inflammation is linked to poor health outcomes, including elevated risks of death, cardiovascular disease (CVD), neurocognitive impairment, osteoporosis, and frailty even in treated individuals (1, 2, 10–22), understanding the mechanisms behind immune activation may lead to new treatments to improve the quality and length of life for HIV-1-infected individuals.

Viral characteristics such as the presence of drug resistance mutations, coreceptor tropism, and the diversity of the proviral population are associated with HIV-1 disease progression (23–

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27). For example, individuals initially infected with a more diverse viral population undergo more rapid HIV-1 disease progression (23, 24). However, it is unclear whether these viral characteristics drive poor health outcomes by influencing levels of immune activation. Several studies suggest that in viremic individuals, the presence of drug resistance mutations is associated with lower levels of inflammation. For instance, the number of mutations conferring drug resistance was inversely correlated with levels of the proinflammatory cytokines interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and tumor necrosis factor receptor II (TNF-rII), independent of HIV-1 RNA levels (28). In addition, individuals with documented drug resistance have lower levels of $CD4^+$ and $CD8^+$ T cell activation, experience lower rates of $CD4^+$ depletion, and progress more slowly to AIDS than do untreated HIV-infected individuals, independently of plasma HIV RNA levels (28–32). The reduced ability of drug-resistant viruses to contribute to disease progression may result from particular drug resistance mutations that impede viral replicative capacity, thus decreasing the activation of bystander T cells (29, 33). Conversely, the presence of CXCR4-tropic viruses was associated with higher levels of inflammatory markers in one study (34) but with no difference in levels of inflammatory markers in another (35). However, few studies have evaluated associations between viral characteristics and inflammation.

Improvements in ART regimens now allow for the majority of HIV-infected patients under care to maintain virologic suppression (36). Understanding the mechanisms underlying HIV-associated chronic inflammation in treated disease—including the role of the latent proviral reservoir that is maintained despite effective treatment—could inform the design of ART strategies and the development of novel therapeutic strategies to reverse chronic inflammation and improve health outcomes. Several studies suggest that low-level HIV-1 replication in the plasma may persist despite suppressive treatment (37–42). In fact, approximately 75% of individuals who have "suppressed" HIV-1 RNA levels using standard assays have detectable viremia with an ultrasensitive single-copy assay (43). While two studies suggest that this low-level viral replication may drive immune activation in treated individuals (44, 45), in another study no such association was noted (46). Overall, the existing data seem to indicate that low-level plasma viremia may explain some, but not all, of the persistent immune activation. The proviral population in HIV-infected individuals serves as an archive of circulating plasma viruses from throughout the course of infection (47–50) that does not undergo significant viral evolution once treatment has begun (51–55). Thus, examination of the proviral population provides the opportunity to examine whether certain viral characteristics, established early in infection, are associated with ongoing inflammation despite effective treatment. For instance, viral diversity is associated with disease progression in untreated individuals, but the extent to which a diverse proviral population is associated with ongoing inflammation remains unknown. We hypothesized that viral characteristics such as increased diversity, divergence (the average distance of an individual's sequences from their calculated most recent common ancestor [MRCA] as a measure of intrasubject evolution), and frequency of CXCR4-tropic viruses are associated with increased immune activation. To address this hypothesis, we undertook a uniquely comprehensive evaluation of viral characteristics and immune status in a cohort of individuals with currently undetectable viral loads on effective ART. We examined proviral characteristics, including diversity, divergence, ARV resistance, and coreceptor tropism. To assess immune status, we evaluated immune cell phenotype, serum cytokine levels, and immune cell function by phosphor-specific flow cytometry (56, 57). Since half of the subjects had a history of virologic failure (VF), defined as documented resistance as a result of therapy or as a viral load greater than or equal to 200 copies/ml after 6 or more months of therapy, we also examined whether a history of failed therapy was associated with a lasting impact on the immune status.

MATERIALS AND METHODS

Study population and sample collection. Subjects were drawn from the Stanford HIV Aging Cohort (SHAC), an ongoing prospective clinical cohort of virologically suppressed HIV-infected individuals. Sixteen subjects, all with undetectable viral loads for at least 6 months prior to the beginning of the study, were selected. Eight subjects had a history of virologic failure requiring alterations in their ART regimens to maintain suppression. Blood samples were collected between June and August 2009, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-Paque). Samples were cryopreserved in 90% heat-inactivated fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) and thawed for immune profiling and for characterization of the latent provirus.

Immune profiling. Comprehensive immune profiling was performed at the Human Immune Monitoring Center at Stanford University (http://iti.stanford.edu/himc/flow-cytometry.html) as described previously (57). Briefly, cryopreserved PBMC samples were thawed and evaluated by flow cytometry to determine the distribution of immune cell subsets, including enumeration of T, T regulatory (T_{reg}) cells, B cells, dendritic cells, monocytes, NK cells, differentiation into T_h1, T_h2, and T_h17 cell subsets, and T cell activation status. Intracellular signaling responses in T cells, B cells, and monocytes were assessed by phospho-flow cytometry to evaluate pSTAT-1, pSTAT-3, pSTAT-5, pERK1/2, pp38, and pPLCγ2 levels in response to IL-2, IL-6, IL-7, IL-10, IL-21, alpha interferon (IFN-α), and IFN-γ as described previously (57). Finally, a human 51-plex Luminex immunoassay was used to assess cytokine levels in plasma from these subjects.

Proviral sequence determination. For evaluation of HIV proviral DNA sequences, a single vial of PBMCs was thawed and DNA was extracted from 2 million to 6 million PBMCs using the QIAamp DNA mini-kit (Qiagen) according to the manufacturer's instructions. The open reading frame containing the region of *env* from V1 to V5 and the open reading frame containing the protease (PR) and reverse transcriptase (RT) regions of *pol* were amplified from subject proviral DNA with *Taq* DNA polymerase (Fisher Scientific) by nested PCR using the primers listed in Table S1 in the supplemental material, with ACH-2 proviral DNA (Qiagen) as a positive control and water and genomic DNA from HIV-uninfected individuals as negative controls. The *env* and *pol* PCR products were confirmed by agarose gel electrophoresis. The *env* and *pol* genes were amplified by limiting dilution PCR such that less that 50% of the reactions had a product to confirm single genome amplification. Unincorporated primers and deoxynucleoside triphosphates (dNTPs) were removed from amplified products in a single-step enzymatic reaction using ExoSAP-IT (Affymetrix) according to the manufacturer's instructions. The products were then sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Invitrogen) and sent to Molecular Cloning Laboratories (MCLAB; South San Francisco, CA) for fragment analysis. The sequencing primers are detailed in Table S1 in the supplemental material. The sequence data were assembled and edited in Sequencher version 5.1 (Gene Codes Corporation, Ann Arbor, MI). Sequences were entered into the NCBI Basic Local Alignment Search Tool (BLAST), as well as into the NCBI Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi), to confirm their identity and the absence of any contamination. Sequences with mixed peaks were excluded.

Sequence and phylogenetic analyses. The sequences of the V1 to V5 and PR-RT amplicons, along with the corresponding regions of the

subtype A reference sequences 92ug037 (U51190.1) and Q23-17 (AF004885.1), the subtype B reference sequences HXB2 (K03455.1) and BK132 (AY173951.1), the subtype C reference sequences 92BR025 (U52953.1) and ETH2220 (U46016.1), the subtype D reference sequences 94UG114 (U88824.1) and ELI (K03454.1), and the subtype H reference sequence 90CF056.1 (AF005496) (all accession numbers are from GenBank), were manually aligned in Geneious version 6.0.6 (Biomatters Limited, Auckland, New Zealand) using a ClustalW multiple alignment with a gap open penalty of 15 and a gap extension penalty of 6.66. The Hypermut tool of the HIV database maintained by the Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html) was used to detect APOBEC-induced hypermutated sequences with a *P* value of less than 0.05, which were excluded from phylogenetic analyses. The PR-RT and the V1 to V5 sequences of HXB2 were used as the reference sequences for this analysis. In total, nine *env* sequences were removed for hypermutation, one each from subjects 81, 82, 83, 84, 86, 91, 93, 96, and 98. Six *pol* sequences were removed, from subjects 84 (2 sequences), 86 (1 sequence), 89 (2 sequences), and 91 (1 sequence). Neighbor-joining, bootstrapped (over 100 iterations) phylogenetic trees for *pol* and *env* were built in Geneious using the HKY genetic distance model, with the PR-RT and V1 to V5 sequences of 90CF056.1 used as the outgroup. The DIVEIN program (http://indra.mullins.microbiol.washington.edu/DIVEIN/diver.html) was used to determine pairwise diversity and divergence from the MRCA for both *env* and *pol*. Calculations were made with the HKY85 substitution model with a fixed transition-to-transversion ratio of 4.

Determination of coreceptor tropism. Geno2pheno [coreceptor] version 2.5 (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php; Max-Planck-Institut Informatik, Germany) was used to determine coreceptor tropism of the V3 region of *env*, with a false-positive rate of 5%. The proviral population of each subject was assigned an aggregate label based on the tropism of the individual sequences: a population with only R5-tropic viruses was labeled R5, a population with only X4-tropic viruses was labeled X4, and a population with at least one X4-tropic virus was labeled dual.

Determination of drug resistance score. The average drug resistance score for the *pol* sequences obtained from each subject was determined by the Stanford HIV Drug Resistance Database version 6.3.0 (http://hivdb.stanford.edu/index.html, Stanford University, Stanford, CA). Resistances to lopinavir/ritonavir (LPV/r), lamivudine (3TC) and abacavir (ABC), and efavirenz (EFV) were chosen to represent resistance to protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), and nonnucleoside reverse transcriptase inhibitors (NNRTIs), respectively, for subsequent analyses.

Statistical analysis. Cytokine data were log transformed prior to analysis, and phospho-flow data were normalized and transformed as previously described (57). Spearman's rank correlation was applied to characterize the relationship between immune parameters and viral features. The Wilcoxon rank sum test was used to compare immune features between subjects with and without a history of virologic failure. As numerous features were involved in addressing each of these research questions, numerous corresponding hypotheses were tested. To account for the issue of multiple testing, we controlled the false-discovery rate to be no more than 10% for each of the two main research questions being addressed. Statistical analyses were performed by the statistical package R (http://www.r-project.org/) and Excel (Microsoft Corporation).

Nucleotide sequence accession numbers. The *pol* sequences were deposited into GenBank with accession numbers KJ528594 through KJ528716. The *env* sequences were deposited into GenBank with accession numbers KJ528717 through KJ528870.

RESULTS

Characteristics of subjects. The study cohort consisted of 16 HIV-infected individuals from the Stanford HIV Aging Cohort (SHAC), an ongoing prospective clinical cohort of virologically suppressed HIV-infected individuals (Table 1). Enrollment criteria included ART adherence and an undetectable HIV-1 viral load for at least 6 months. The subjects ranged in age from 30 to 78 years of age, with nadir CD4 counts from 0 to 584 $CD4^+$ T cells/μl. The mean contemporaneous CD4 count was 653 cells/μl (range, 206 to 1,221 cells/μl), and the mean length of suppression was 38.5 months (range, ≥6 to 137 months). Although all subjects had viral loads below the limit of detection for at least 6 months at the time of the study, eight of the subjects had a clinical history of virologic failure (VF). The subjects with a history of VF tended to be older (mean age, 57 versus 45 years) and have a lower nadir CD4 count (208 versus 248 cells/μl) and current CD4 count (519 versus 786 cells/μl) (Table 1).

TABLE 1 Subject demographics

Group	ID[a]	Age (yr)	Nadir CD4 count (cells/μl)	Current CD4 count (cells/μl)	Length of suppression (mo)
No history of VF	84	57	400	594	≥6
	87	56	288	520	41
	88	40	3	1,221	137
	93	35	279	438	13
	95	59	189	618	≥6
	96	40	362	1,015	19
	99	37	220	805	≥6
	100	40	246	1,080	17
	Mean	46	248	786	31
History of VF	81	54	413	469	13
	82	56	584	898	87
	83	78	0	545	30
	86	70	140	452	36
	89	30	155	573	46
	91	38	138	474	70
	92	64	82	205	23
	98	67	150	539	66
	Mean	57	208	519	46

[a] ID, identification.

Diversity and divergence in *env* sequences from virologically suppressed adults. Neighbor-joining, bootstrapped phylogenetic trees of HIV-1 env V1 to V5 regions were used to quantify diversity and divergence (Fig. 1). Most subjects' sequences formed a monophyletic cluster, with the notable exception of subject 96, in whom two amplicons formed a unique cluster (Fig. 1). These two sequences did not cluster with any other subject, nor were they identified as known sequences by BLAST searching, indicating that subject 96 was most likely dually infected or superinfected with two unique strains from different partners. We were unable to obtain additional samples from this subject to confirm the dual infection. The subjects exhibited a wide range of diversity (0.35% to 5.0%) and divergence (6.8% to 13.0%) in their proviral populations (Table 2). These data indicate that a wide range of diversity in *env* sequences is observed even in individuals on effective therapy and with suppressed plasma viral loads.

Diversity, divergence, and drug resistance mutations in *pol* sequences from virologically suppressed adults. As with env, phylogenetic trees were constructed based on *pol* sequences in proviral DNA (Fig. 2). Amplification was unsuccessful for subject 88. The *pol* sequences exhibited less diversity and divergence than the *env* sequences, though a range in diversity (0% to 2.66%) and

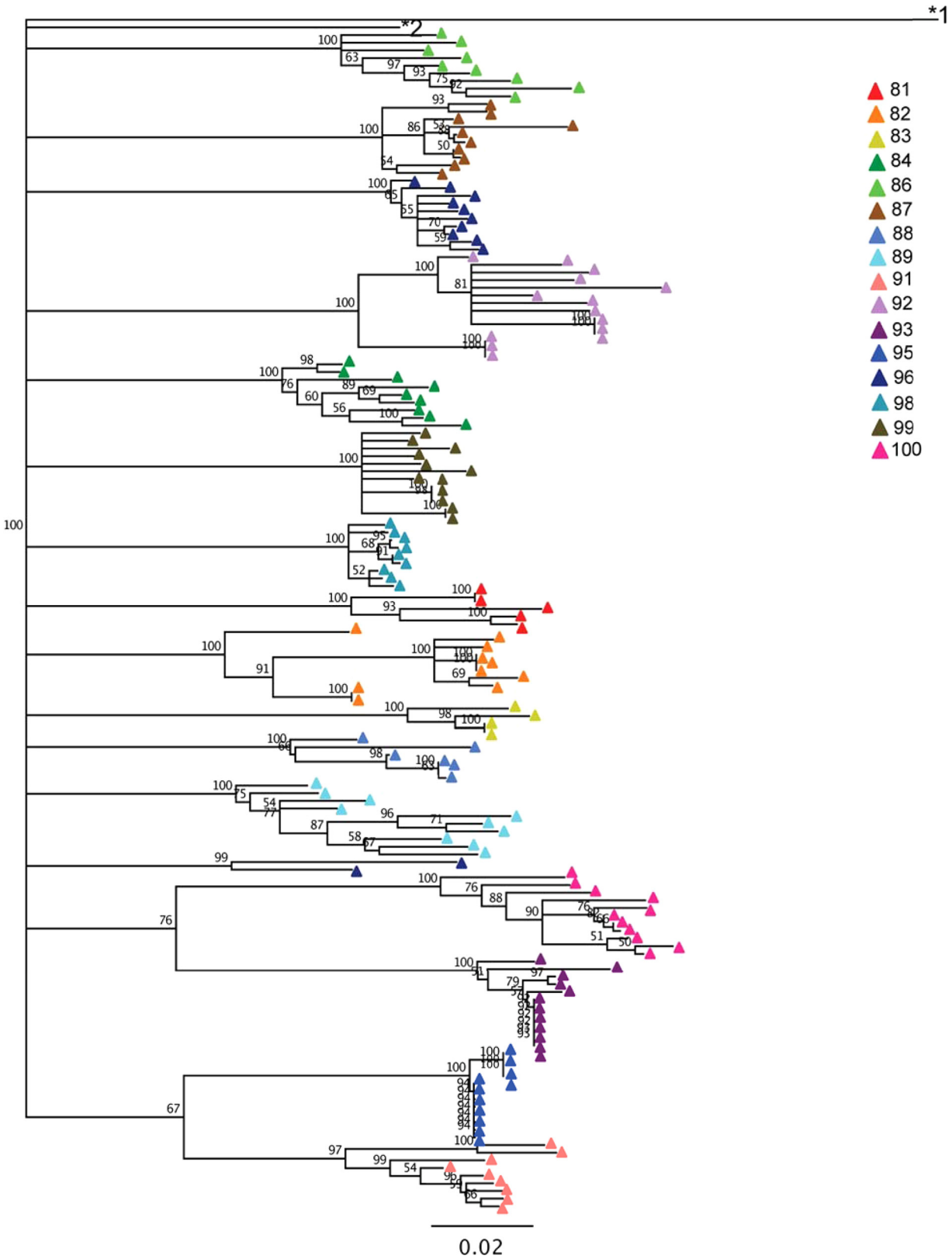

FIG 1 Neighbor-joining, bootstrapped phylogenetic tree of *env* sequences. Sequences are colored by subject. The numbers at the nodes indicate the percentage of bootstrap replicates (100 iterations total). The subtype H reference sequence 90CF056.1 (GenBank accession number AF005496) and subtype B reference sequence HXB2 (GenBank accession number K03455.1) are labeled *1 and *2, respectively.

TABLE 2 *env* diversity and divergence and coreceptor tropism[a]

ID	History of VF	*env* diversity (%)		*env* divergence (%)		Coreceptor tropism
		Mean	SD	Mean	SD	
84	0	3.52	1.07	8.18	0.89	Dual
87	0	2.53	1.19	9.78	0.56	R5
88	0	2.98	2.07	8.55	0.83	Dual
93	0	1.28	1.24	11.11	0.37	X4
95	0	0.35	0.36	10.96	0.32	R5
96	0	4.54	4.99	9.89	0.56	R5
99	0	1.71	0.69	9.13	0.51	R5
100	0	3.66	1.36	12.98	0.71	Dual
81	1	4.52	2.15	9.72	0.55	R5
82	1	3.70	2.50	9.19	1.44	R5
83	1	2.61	1.58	9.85	0.45	Dual
86	1	3.98	1.20	6.78	1.02	R5
89	1	5.00	1.37	8.74	1.88	Dual
91	1	3.91	2.60	10.80	0.95	R5
92	1	4.45	2.05	12.03	1.23	R5
98	1	1.08	0.32	7.47	0.20	R5
Mean	NA	3.11	1.67	9.70	0.78	NA

[a] ID, identification; VF, virologic failure (1 = true); NA, not available (we were unable to successfully obtain any amplicons).

divergence (2.83% to 5.97%) was still observed (Table 3). Notably, subject 83 demonstrated no *pol* diversity (mean = 0; standard deviation [SD] = 0) or variability in divergence (mean = 4.2%; SD = 0%), indicating that all of the amplicon sequences were identical. Subject 83 had a nadir $CD4^+$ T cell count of 0/μl, perhaps indicating that a population bottleneck substantially reduced the diversity of the proviral population. However, this phenomenon was not reflected for *env*, for which both diversity (mean = 2.61%; SD = 1.58%) and divergence (mean = 9.85%; SD = 0.45%) were observed (Table 2).

The Stanford HIV Drug Resistance Database, which produces a numeric score in addition to the discrete resistance categories for each drug, was used to evaluate the presence of drug resistance mutations in the proviral population for each subject. Scores for 3TC, ABC, EFV, and LPV/r were used to capture low- and high-level resistance to nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors. ART resistance scores varied among the subjects, with the most resistance to 3TC (mean = 22.1), followed by ABC (mean = 21.7), EFV (mean = 10.4), and LPV/r (mean = 4.00). Several subjects, particularly subject 92, had markedly high levels of archived drug resistance (Table 3).

Proviral diversity and divergence do not correlate with demographic immune characteristics. Demographic data, immune cell phenotyping, serum cytokine levels, and phospho-flow analysis of intracellular signaling responses were used to determine whether these characteristics associate with proviral diversity and divergence in *env* and *pol* (Fig. 3; see also Fig. S1 to S5 in the supplemental material). The diversity and divergence in *env* and *pol* were positively correlated (Fig. 3). In addition, there were positive correlations between the percentage of total lymphocytes and the percentage of $CD4^+$ effector memory cells with proviral diversity and divergence. The percentage of monocytes was inversely correlated with proviral diversity and divergence. Proviral diversity and divergence were also weakly associated with decreased levels of $CD8^+$ T cell STAT-3 responses to IL-6 and IL-7. After control of the false-discovery rate to be no more than 10%, none of the associations reached statistical significance. In addition, none of the demographic characteristics such as age, nadir $CD4^+$ T cell count, or duration of infection were significantly associated with the proviral diversity or divergence in *env* or *pol*. Thus, overall there were no statistically significant correlations between proviral diversity and divergence and any demographic or immune characteristic (Fig. 3). In addition, neither ART resistance levels nor coreceptor tropism correlated with any of the immunological or demographic parameters (data not shown).

Virologic failure does not predict level of immune activation. As the characteristics of the virus were not significantly correlated with the level of ongoing immune activation in this cohort of well-controlled, HIV-infected individuals, we examined whether a clinical history of virologic failure was associated with immune activation. We performed Wilcoxon rank sum tests comparing the proviral and immune characteristics between subjects without and with VF while controlling the false-discovery rate to be no more than 10% (Table 4). A history of virologic failure was associated with a trend for increased *pol* diversity, increased drug resistance, and duration of infection, indicating that our data set was adequately robust to detect some viral characteristics associated with clinical history. However, for the remaining proviral and immune characteristics, individuals with a history of virologic failure were not significantly different from individuals without a history of virologic failure (Table 4 and data not shown). This result held even for immune characteristics that have previously been shown to be markers of HIV-associated immune activation, such as the percentage of CD38- and HLA-DR-expressing $CD4^+$ and $CD8^+$ T cells. Thus, a clinical history of virologic failure does not predict the level of immune activation in our cohort.

DISCUSSION

Recently, much HIV-1 research has focused on defining the mechanisms driving HIV-associated immune activation, which is associated with early manifestations of aging, such as increased risk of developing CVD. We report here an evaluation of the relationship between proviral characteristics and markers of immune activation in a cohort of virologically suppressed HIV-infected individuals on ART. Our study is the first to investigate HIV-associated immune activation in the context of proviral populations in effectively treated HIV-infected individuals and is the most comprehensive evaluation of immune function during chronic HIV-1 infection to date. As some studies have suggested that cryptic viral replication in virologically suppressed individuals may occur and contributes to chronic immune activation (44), and even fully virologically suppressed individuals have increased levels of soluble activation markers (58), we hypothesized that by examining the relationship between the proviral population and immune activation we could reveal previously undiscovered contributors to HIV-associated immune activation. We found that subjects, whether or not they had a history of virologic failure, exhibited a wide range of diversity and divergence in both *env* and *pol*. We also found that proviral diversity and divergence in *env* and *pol*, coreceptor tropism, level of drug resistance, and a history of virologic failure do not correlate with markers of immune activation. These results indicate that the nature of the latent virus does not appear to be a major driver of HIV-associated immune activation and its effect on the health of HIV-infected individuals, suggesting that

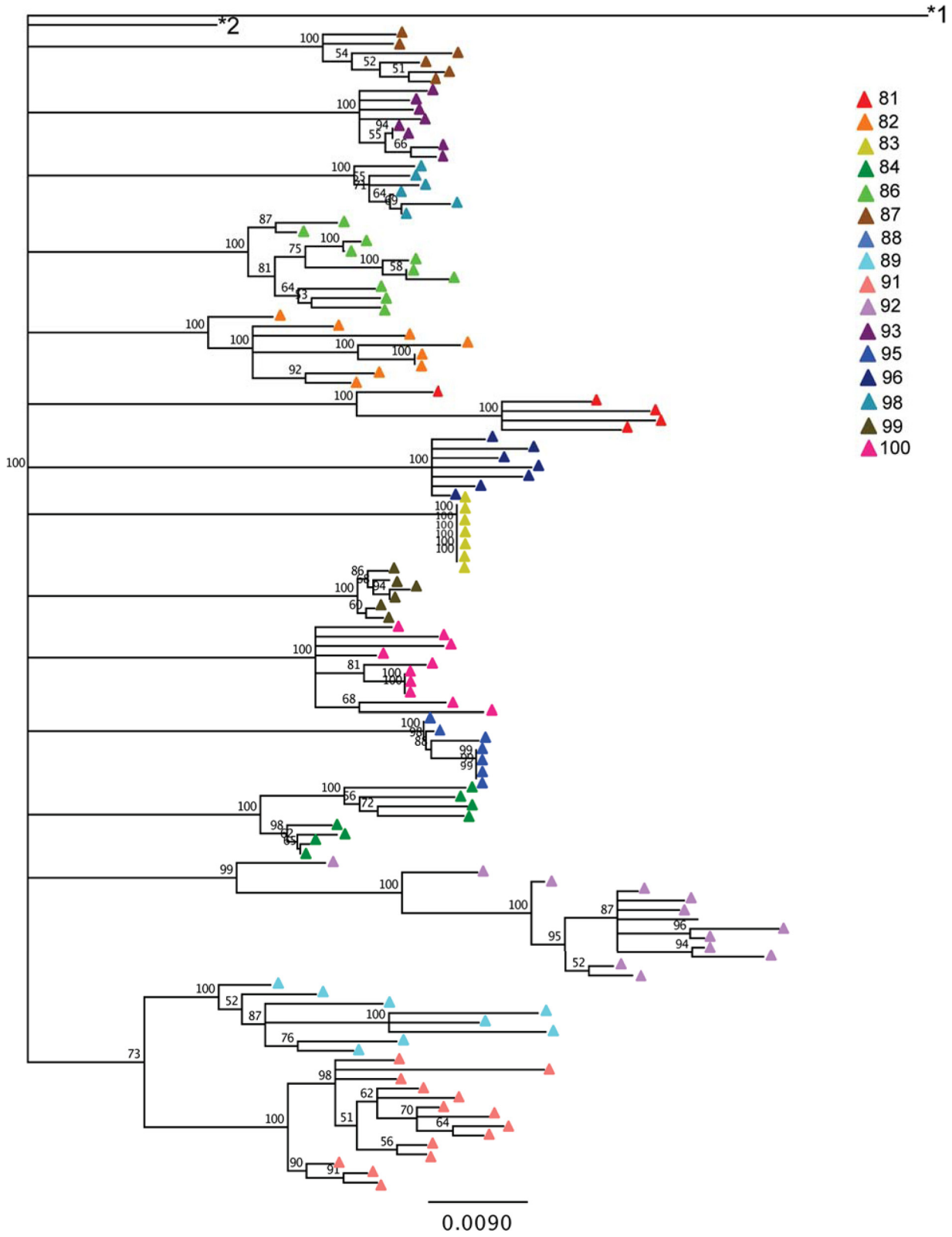

FIG 2 Neighbor-joining, bootstrapped phylogenetic tree of *pol* sequences. Sequences are colored by subject. The subtype H reference sequence 90CF056.1 (GenBank accession number AF005496) and subtype B reference sequence HXB2 (GenBank accession number K03455.1) are labeled *1 and *2, respectively.

TABLE 3 *pol* diversity and divergence and ART resistance scores[a]

ID	History of VF	*pol* diversity (%)		*pol* divergence (%)		Mean resistance score for:			
		Mean	SD	Mean	SD	LPV/r	3TC	ABC	EFV
84	0	1.82	0.72	3.39	0.72	0	3.13	16.25	0
87	0	1.33	0.42	4.04	0.23	0	0	0	0
88	0	NA	NA	NA	NA	NA	NA	NA	NA
93	0	0.78	0.26	3.59	0.19	0	1.88	1.88	0
95	0	0.43	0.35	4.58	0.23	0	2.86	2.86	0
96	0	0.91	0.24	4.89	0.26	0	0	10	0
99	0	0.41	0.12	3.62	0.08	0	0	0	3.33
100	0	1.50	0.63	3.69	0.31	1	0	0	1.5
81	1	2.46	0.61	5.78	0.91	0	60	35	70
82	1	2.07	0.66	3.71	0.56	1.25	33.13	18.75	0
83	1	0	0	4.20	0	0	75	75	0
86	1	1.71	0.61	3.16	0.40	0	29.5	26	10.5
89	1	2.66	0.86	3.77	0.96	0	13.57	22.86	12.86
91	1	1.67	0.60	4.37	0.58	0	33.93	40	6.43
92	1	2.16	1.34	5.97	1.23	57.69	78.85	76.13	51.92
98	1	0.82	0.35	2.83	0.24	0	0	0	0
Mean	NA	1.38	0.52	4.11	0.46	4.00	22.12	21.65	10.44

[a] ID, identification; VF, virologic failure (1 = true); NA, not available (we were unable to successfully obtain any amplicons).

other factors drive the immune activation that persists despite effective treatment.

This evaluation of proviral diversity and divergence in the setting of effective ART provides new insight into the nature of the archived virus during long-term, fully suppressive treatment. Relatively few studies have evaluated such characteristics during treatment, particularly with modern regimens. Studies performed by Finzi et al. in 1997 and 1999 indicated that latent provirus represents a relatively stable population containing archived viruses from prior to initiation of therapy (47, 48). Subsequent studies have revealed that the proviral HIV-1 DNA sequences can remain dynamic even during antiretroviral therapy, acting as an archive of circulating plasma viruses found throughout the course of HIV infection (49, 50). However, given that HIV-1 diversity accumulates more quickly in acute infection (59), it is likely that much of the *env* and *pol* diversity and divergence observed in our cohort accumulated prior to ART initiation. We were unable to directly assess this, as we did not have a reliable history documenting the duration of HIV-1 infection prior to ART initiation.

Within this framework, the subjects in our study exhibited a wide range of both diversity and divergence in their proviral populations during effective treatment. The diversity we observed in both the *env* and *pol* genes was similar to the levels of proviral diversity previously reported during antiretroviral therapy (49, 60) but higher than the levels observed when treatment is started during acute infection (61). Thus, our data support the possibility that the proviral population may be very diverse and divergent even if the individual has been effectively treated with modern ART regimens. The lack of association between these viral characteristics and immune activation in our study suggests that even effective viral suppression may not affect already established immunological changes originating from HIV-1 infection.

Several prior studies suggest that factors other than proviral diversity and divergence, including coreceptor tropism and the presence of drug resistance mutations, are associated with immune activation. For instance, two studies suggested that coreceptor tropism in untreated, HIV-infected adults was associated with immune activation (34, 62). However, in both our study and a recent study by Saracino et al. (35), the presence of CXCR4-tropic viruses in proviral DNA was not associated with the level of immune activation in individuals with fully suppressed or very low viral loads. Thus, the association between CXCR4 tropism and inflammation appears to require active viral replication. Additionally, in viremic individuals, the presence of drug resistance mutations has been associated with lower levels of immune activation, rates of $CD4^+$ T cell depletion, and progression to AIDS, independently of HIV RNA levels (28–31). Our study was the first to examine archived drug resistance mutations in individuals on fully suppressive treatment, and we did not identify a significant association between drug resistance mutations and inflammation.

Interestingly, we also found that a clinical history of virologic failure was not significantly associated with increased levels of immune activation, suggesting that immunological changes established early during infection or in the pretreatment phase may set the stage for ongoing inflammation. Such changes appear to be unrelated to the viral characteristics and inadequate to control the infection regardless of magnitude. This finding is consistent with studies of blood and gut-associated lymphoid tissue sequences, in which no significant changes in diversity were observed after treatment initiation, and immune activation persisted despite ART (63).

The limitations of our study include our small sample size and our lack of longitudinal data. Assuming two normally distributed random variables, with our sample size of 16, we would have 88.2% power to detect a strong correlation, 0.7 or larger, but only 50.5% power to detect the more modest correlation of 0.5. We did find trends and/or statistically significant differences between subjects with and without a history of VF in *pol* diversity, ART resistance scores, and duration of infection. An increased sample size or longitudinal data may have nonetheless increased our ability to find significant associations between the nature of the provirus and immune activation and/or associations between the nature of the provirus and changes in immune activation over time. In addition, due to limitations in sample volume, we had a relatively

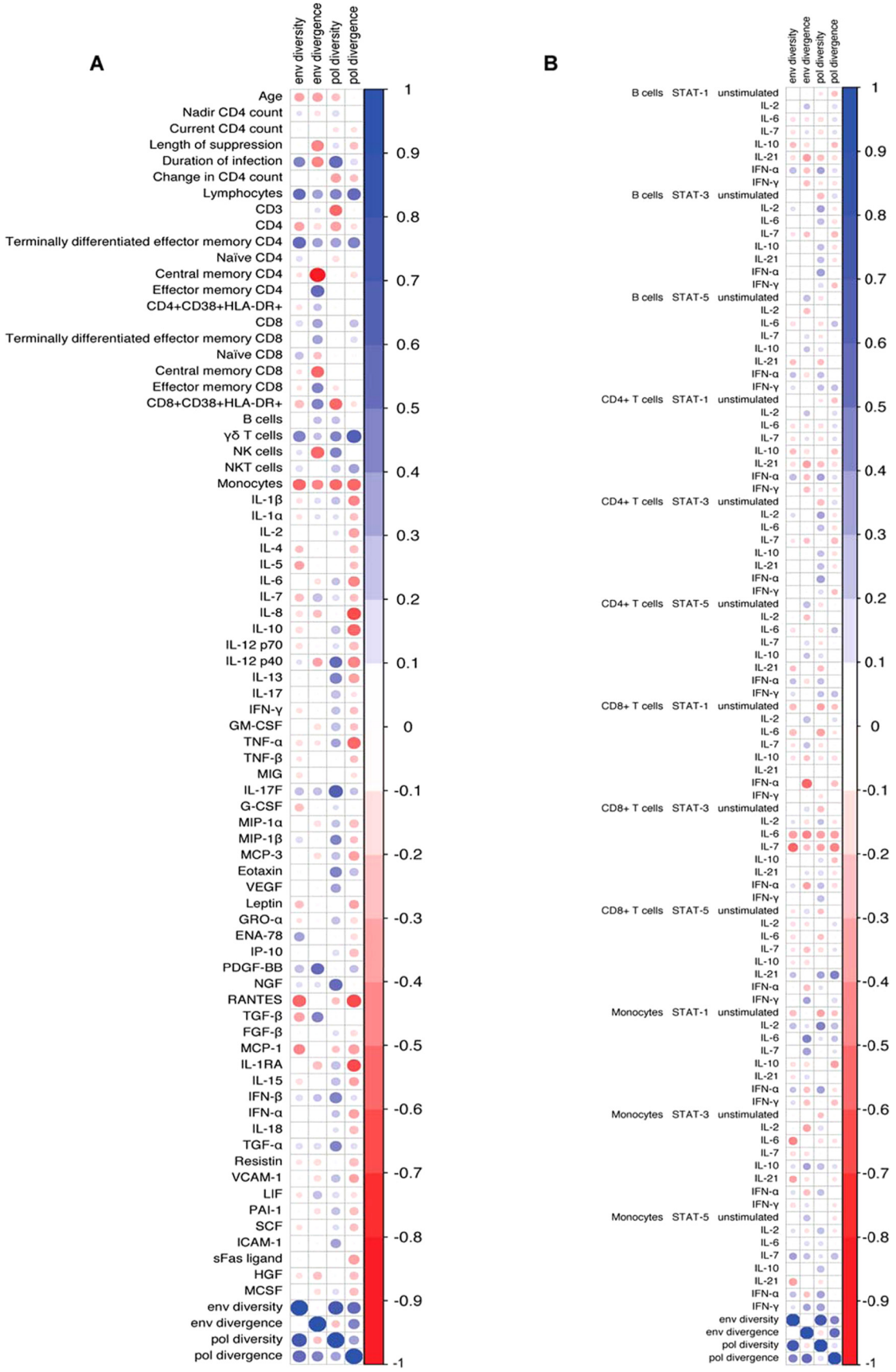

FIG 3 Correlation matrices of proviral characteristics and immune characteristics. The colored circles represent the Spearman's correlation coefficient, sized according the magnitude of the coefficient and colored according to the scale bar shown on the right. Positive correlations are blue and negative are red, and the size denotes the magnitude of the correlation. (A) Correlation matrix of proviral characteristics and cellular markers and plasma cytokine levels; (B) correlation matrix of proviral characteristics and signaling markers.

TABLE 4 Comparison of viral and immune characteristics between subjects without and with a history of virologic failure

Characteristic	No VF		VF		Wilcoxon *P* value	Adjusted *P* value[a]
	Mean	SD	Mean	SD		
% $CD3^+$	67.0	12.3	72.7	6.44	0.64	0.78
% $CD4^+$	30.2	9.36	26.8	12.51	0.54	0.78
% $CD8^+$	35.7	10.6	42.6	12.84	0.46	0.78
% HLA-DR^+ $CD38^+$ $CD4^+$	19.5	10.6	16.2	6.14	0.78	0.78
% HLA-DR^+ $CD38^+$ $CD8^+$	23.5	11.0	17.5	6.31	0.15	0.34
Plasma IL-6 level (pg/ml)	0.5	0.22	0.49	0.25	0.68	0.78
Baseline $CD4^+$ STAT-3 activity	0.750	0.53	0.76	0.5	0.78	0.78
$CD4^+$ STAT-3 activity in response to IL-6	−0.810	0.43	−0.82	0.44	0.78	0.78
env diversity (%)	2.57	1.62	3.66	1.72	0.12	0.31
env divergence (%)	10.1	0.60	9.32	0.96	0.28	0.56
pol diversity (%)	1.03	0.39	1.69	0.63	0.09	0.28
pol divergence (%)	3.97	0.29	4.22	0.61	0.69	0.78
LPV/r resistance score	0.140	0.38	7.37	20.3	0.56	0.78
3TC resistance score	1.12	1.45	40.5	28.4	0.008	0.09
ABC resistance score	4.43	6.31	36.7	26.8	0.01	0.09
EFV resistance score	0.690	1.29	19.0	26.8	0.08	0.28
Length of suppression (mo)	31	44.5	46	25.7	0.01	0.09
Duration of infection (yr)	11.6	5.01	18	4.6	0.04	0.17

[a] Adjusted *P* values account for a false-discovery rate of 10%.

modest number of HIV-1 sequences with which to calculate diversity and divergence for some individuals. However, exclusion of subjects with low numbers of sequences did not alter results (data not shown). Finally, viral characteristics that we did not measure, such as escape mutations from the immune response within each individual, could be the primary driving force behind immune activation. It is also important to note that researchers have yet to establish a common, widely accepted definition of immune activation that effectively encompasses molecular and cellular characteristics. Studies of HIV-associated immune activation have focused on different aspects of the immune system such as expression of activation markers on immune cells, cytokine levels, and functional responses. While numerous biomarkers, such as IL-6, soluble CD14, and HLA-DR^+ $CD38^+$ $CD4^+$ and $CD8^+$ T cells, predict disease progression, we have yet to discover how they interact with each other (1). A key strength of our study is that it is the most comprehensive evaluation of immune status and function performed to date with treated HIV-infected individuals, as it accounts for cellular subsets, signaling pathways, and cytokine profiles, while previous studies have focused on only one or two of these features at a time.

By showing that characteristics of the proviral population, including viral diversity, divergence, coreceptor tropism, and the frequency of drug resistance mutations, do not significantly correlate with levels of immune activation in this modestly sized cohort of ART-treated HIV-1-infected individuals, our study reveals that these viral characteristics may not be the most significant drivers of chronic immune activation. Furthermore, a clinical history of virologic failure does not permanently imprint the immune system through increased levels of immune activation if the virus is currently suppressed. Our study therefore has important implications for treatment decisions and outcomes, indicating that a history of virologic failure or high viral diversity is not inexorably linked to increased immune activation as long as appropriate suppressive ART regimens are provided. Future studies should extend these findings to larger longitudinal cohorts followed prior to and after successful viral suppression with ART.

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