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Replicative fitness of transmitted HIV-1 drives acute immune activation, proviral load in memory CD4+ T cells, and disease progression

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HIV-1 infection is characterized by varying degrees of chronic immune activation and disruption of T-cell homeostasis, which impact the rate of disease progression. A deeper understanding of the factors that influence HIV-1–induced immunopathology and subsequent CD4+ T-cell decline is critical to strategies aimed at controlling or eliminating the virus. In an analysis of 127 acutely infected Zambians, we demonstrate a dramatic and early impact of viral replicative capacity (vRC) on HIV-1 immunopathogenesis that is independent of viral load (VL). Individuals infected with high-RC viruses exhibit a distinct inflammatory cytokine profile as well as significantly elevated T-cell activation, proliferation, and CD8+ T-cell exhaustion, during the earliest months of infection. Moreover, the vRC of the transmitted virus is positively correlated with the magnitude of viral burden in naive and central memory CD4+ T-cell populations, raising the possibility that transmitted viral phenotypes may influence the size of the initial latent viral reservoir. Taken together, these findings support an unprecedented role for the replicative fitness of the founder virus, independent of host protective genes and VL, in influencing multiple facets of HIV-1–related immunopathology, and that a greater focus on this parameter could provide novel approaches to clinical interventions.

HIV-1 | Gag | replicative capacity | immune activation | pathogenesis

From the start of the AIDS epidemic, HIV-1 infection has been characterized by a steady decline in CD4+ T cells that results in a state of overt immunodeficiency marked by an increased susceptibility to opportunistic infections and malignancies (1). Although a majority of HIV-1–infected individuals eventually progress to AIDS during their lifetime, they do so at drastically different rates (2). Several immunological abnormalities during HIV-1 infection have been identified that correlate with disease progression, such as a rapid and robust expression of proinflammatory cytokines, chronic immune activation, cellular exhaustion, and infection of vulnerable memory CD4+ T-cell subsets important for maintaining T-cell homeostasis (3). The degree to which these pathogenic mechanisms are triggered early in infection may explain the varying rates of disease progression among individuals.

It has been shown that the magnitude of immune activation during HIV-1 infection is established early, is relatively stable over time, and predicts the rate of disease progression better than viral load (4, 5). Successful antiretroviral treatment (ART) of HIV-1–infected individuals reduces viremia to undetectable levels, restores CD4+ T-cell counts to some degree, and significantly prolongs life (6). Despite this, ART does not fully restore immune function, and levels of residual immune activation are associated with an increased risk of morbidity and mortality (7, 8). Moreover, even with successful ART, the virus is not fully eradicated and viral rebound occurs upon treatment interruption (9). Strategies aimed at mitigating persistent immune activation and eradicating the latent viral reservoir will contribute immensely toward improving the quality of life of HIV-1–infected individuals and will help to curb the epidemic. Thus, a better understanding of the mechanisms driving HIV-1–induced immunological abnormalities


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See Commentary on page 3591.

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and the processes by which they ultimately cause disease is crucial for unveiling novel avenues for pursuing these more advanced therapeutic interventions.

To date, research has primarily focused on identifying host factors that contribute to viral control and favorable disease outcomes, whereas viral characteristics have received less scrutiny (10, 11). HLA class I alleles such as HLA-B*57 and B*5801 have been shown to influence viral load and CD4+ T-cell decline through the induction of a strong CD8+ T-cell response that is able to target functionally vulnerable regions of the genome such as the structural protein Gag (10). The observation that not all individuals harboring such protective HLA class I alleles go on to become long-term nonprogressors suggests that other factors outside of host immunogenetics play a role in defining disease progression (12). Transmitted viral characteristics have been shown to impact viral load within heterosexual transmission pairs, suggesting that viral characteristics are heritable and can impact disease severity (11, 13, 14).

Moreover, we recently showed that attenuated viral replicative capacity (vRC) of the transmitted virus, defined in vitro by the Gag sequence, was associated with a significant delay in CD4+ T-cell decline in individuals recently infected with HIV-1 subtype C (15). Because this clinical benefit appeared to be partially independent of set point viral load (SPVL), we hypothesized that high levels of transmitted/founder virus replication might initiate irreversible pathogenic events early in infection. Specifically, we hypothesized that high vRC might lead to exacerbated immune activation, elevated cellular dysfunction, and increased infection of memory CD4+ T-cell subsets, which in total might dictate the kinetics of subsequent disease progression (15).

To test this hypothesis, we have studied a unique cohort of 127 Zambian seroconverters acutely infected (median 46 d post-infection) with HIV-1 subtype C that have up to 6 y of longitudinal follow-up. We show here that transmission of high-vRC HIV-1 is associated with a distinct inflammatory profile marked by significantly higher levels of proinflammatory cytokines, increased cellular immune activation and exhaustion, and higher levels of proviral burden in naive and central memory CD4+ T-cell subsets at early time points after infection. Thus, the replicative capacity of transmitted HIV-1, defined by the structural protein Gag, is a critical factor in defining early immune activation, the preservation or loss of CD4+ T-cell homeostasis, and the subsequent trajectory of disease. To better understand interventional strategies such as antiretroviral therapy or vaccine-induced immunity that impact these early events and that attenuate early viral replication will have a significant effect on the development of clinical disease.

Results

Viral Characteristics Determine HIV-1 Pathogenesis. Previously, our work, and that of others, showed that transmitted viral characteristics significantly correlate with early SPVL (11, 13, 14) as well as CD4+ T-cell decline up to 3 y postinfection (15). Here, we sought to determine the underlying mechanisms by which vRC of transmitted HIV-1 impacts the trajectory of CD4 decline even in the context of viral control by previously identified host factors, such as protective HLA alleles, that also impact disease progression.

To assess the impact of the transmitted gag sequence on replicative capacity, we amplified the gag gene from plasma virus during acute infection time points (median 46 d after estimated date of infection), generated replication-competent virus by cloning the gag gene into a common proviral backbone (M4), and measured vRC in an in vitro cell culture assay as described previously (15, 16). In comparing six Gag-M4 chimeric viruses to replication-competent virus by cloning the gag gene into a common proviral backbone (M4), and measured vRC in an in vitro cell culture assay as described previously (15, 16).

In this cohort of 127 acutely infected individuals from Zambia, low vRC significantly delayed the time to CD4+ T-cell counts <300 for up to 5 y postinfection (Fig. L14, P = 0.002). The clearest benefit is observed with the lowest vRC tercile compared with the middle and highest tercile. A significant benefit remained even down to CD4+ T-cell counts of <200, the clinical definition of AIDS, when individuals infected with intermediate and highly replicating viruses were combined into one group (Fig. L1B, P = 0.03).

We have previously shown an association between early SPVL and vRC (15). Thus, we sought to more definitively determine if the replication capacity defined by the gag gene affected CD4 decline in a manner linked to, or independent of, the well-documented effect of early SPVL on subsequent disease progression. In this cohort, we found SPVLs >10^5 RNA copies/mL to be associated with poor outcomes for all volunteers (Fig. L1C, P <0.0001); however, vRC significantly dichotomized the trajectory of CD4 decline (P <0.0001) in individuals with SPVLs <10^5 (Fig. L1D), a majority (77%) of the cohort. This suggested independent, but additive effects, of both vRC and early SPVL on HIV disease progression. Moreover, whereas carriage of B*57/B*5801 alleles protects against CD4 decline in this cohort (Fig. L1E, P = 0.006), vRC significantly dichotomized disease trajectories of those with these protective HLA alleles (Fig. L1F, P = 0.04). This is confirmed in a multivariable Cox proportional hazards model assessing the relative risk of vRC in the context of other well-established predictors of HIV disease progression. We find that low vRC, early SPVL, and canonical protective HLA class I alleles (B*57, B*5801) were each highly significant independent predictors of CD4 decline (Table 1). Of note, the protective effect of being infected with low-RC viruses as opposed to high-RC viruses was similar to that of HLA-B*57 or B*5801 alleles, as evidenced by their similar hazard ratios (Table 1).

Taken together, these data firmly establish vRC as a distinct contributor to HIV disease progression. Moreover, they suggest that vRC may modulate innate immune events very early after infection, which could alter both the establishment of an inflammatory state and the development of an effective adaptive immune response capable of controlling viremia. To further test this hypothesis, we assessed early levels of circulating inflammatory cytokines, immune activation, and exhaustion in T-cell compartments, as well as viral burden in different CD4+ T-cell subsets.

Viral Replicative Capacity Alters Early Inflammatory Cytokine Profiles. Acute HIV infection is characterized by a rapid and robust expression of type I interferons (IFN-I), IFN-I–stimulated genes, and inflammatory cytokines (17). Disruption of the gut-associated lymphoid tissue (GALT) and subsequent microbial translocation have also been shown to contribute significantly to this inflammatory state, possibly through a positive feedback loop (18). This inflammatory response, particularly during chronic infection, contributes to disease progression (19, 20). Therefore, we analyzed the levels of 16 inflammatory cytokines, chemokines, and markers of gut damage and microbial translocation at or before seroconversion to assess the effect of vRC on the early inflammatory milieu [n = 33; previously dichotomized into low-and high-vRC phenotypes (15)]. We found that vRC was positively correlated with a number of inflammatory cytokines (Table 2), most notably IL-6 and IL-1p, two proinflammatory cytokines previously implicated in driving aberrant CD4+ T-cell turnover and impairing homeostatic proliferation (21). Of note, vRC was also strongly correlated with elevated levels of IL-10, an important antiinflammatory cytokine linked to T-cell dysfunction in HIV infection (22, 23).
Because acute HIV-1 infection is associated with a complex cytokine storm (17), we aimed to define distinct inflammatory “profiles” that could explain the impact of vRC, by using an unsupervised data reduction tool, principal component analysis (PCA), which groups linear variables into combinations, termed principal components (PCs) (Fig. 2A). Strikingly, principal component 1 (PC1), which describes the greatest variation in the dataset, significantly correlates with vRC, in that individuals with positive loadings for PC1 (elevated levels of inflammation) tend to have higher vRC, whereas those with negative loadings (low levels of inflammation) are significantly enriched for poorly replicating viruses (Fig. 2B, P = 0.0002). Moreover, principal component 2 (PC2), which by definition is uncorrelated with PC1, describes the second-greatest variation in the data and is significantly correlated with SPVL (Fig. 2C, P = 0.01) but not with vRC. The differences in analyte loadings between PC1 and PC2 are shown in Fig. 2D, and the inflammatory cytokines that substantially contribute to PC1 and -2 are depicted schematically in Fig. S2. This result further highlights the independence of factors associated with initial viral replication and subsequent adaptive immune control of SPVL. Moreover, it demonstrates that viruses with high vRC are correlated with a distinct inflammatory cytokine profile characterized by a heightened type I and II IFN response and elevated levels of key inflammatory cytokines such as IL-6 and IL-1β.

**Replicative Capacity Is Associated with Levels of CD8+ T-Cell Activation and Exhaustion.** Chronic immune activation is a hallmark of HIV-1 infection; it often persists following ART and is a more reliable predictor of disease progression than viral load (4, 5). Therefore, we assessed the impact of vRC on levels of cellular immune activation by measuring the coexpression of CD38 and HLA-DR on CD8+ T cells isolated within 3 mo postinfection for a subset of individuals under study (n = 33). We found that vRC is positively correlated with the expression of CD38 and HLA-DR on CD8+ T cells (Fig. 3A, P = 0.002). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03). Consistent with previous studies (4, 24), we observe that higher CD8+ T-cell activation in this cohort is associated with faster CD4+ T-cell decline (Fig. 3B, P = 0.03).
Table 1. Host and viral characteristics independently predict CD4+ T-cell decline

<table>
<thead>
<tr>
<th>Factors tested</th>
<th>Cox proportional hazards model, *time to CD4 &lt;300</th>
<th>P value</th>
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<tr>
<td>Female</td>
<td>HR = 1.10; 95% CI = 0.67–1.70; P = 0.78</td>
<td></td>
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<tr>
<td>Low vRC, lowest tercile</td>
<td>0.48; 95% CI = 0.28–0.80; P = 0.004</td>
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<tr>
<td>B+575/S081</td>
<td>0.45; 95% CI = 0.23–0.81; P = 0.006</td>
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<tr>
<td>Set point VL†</td>
<td>10.00; 95% CI = 2.86–44.1; P = 0.0004</td>
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*Multivariable Cox proportional hazards model with an endpoint defined as a single CD4+ T-cell count reading below 300 (n = 127).
†Set point VL was defined as the earliest nadir viral load, between 3 mo and 9 mo postinfection, which remained stable for subsequent viral load readings.

reported previously (Fig. 4F, P < 0.0001) (30, 32). Furthermore, individuals infected with poorly replicating viruses displayed expression levels of CD38/HLA-DR and Ki67 on their CD4+ T cells, which, although somewhat elevated, for the most part did not differ significantly compared with those of HIV-uninfected Zambians (Fig. 4, B, D, and E). Thus, these results suggest that, shortly after transmission, individuals infected with low-vRC viruses preserve an immune system more similar to that of a healthy individual.

Despite the stratification of these individuals into those infected with low- and high-RC viruses, an analysis of VL at the time these PBMC samples were collected did not reveal any significant difference in mean VL between the two groups (mean VL for low vRC = 4.5, mean VL for high vRC = 4.8; P = 0.26; Student’s t test).

Early Inflammatory Cytokine Profiles Associated with vRC Are Linked to Activated T-Cell Phenotypes. To determine whether changes in T-cell activation are associated with the distinct inflammatory cytokine profiles associated with vRC, we compared individuals with positive (increased inflammatory cytokine levels) and negative loadings for PC1 (Fig. 2). Individuals with positive loadings for PC1 presented with significantly increased CD8+ T-cell activation (Fig. 5A, C, P < 0.0001) and exhaustion (Fig. 5, B and D). However, no significant difference in mean VL between the two groups (mean VL for high vRC = 4.8; P = 0.26; Student’s t test).

High vRC Is Associated with Increased Proviral Burden in CD4+ T-Cell Subsets. Establishment of the latent HIV reservoir occurs early during acute infection and sets the stage for viral persistence. Thus, even in the context of suppressive antiretroviral therapy, HIV-1 cannot be fully eradicated (9, 33, 34). To determine whether the replicative capacity of the transmitted/founder virus influences the proviral DNA burden during early infection, levels of cell-associated viral DNA were measured in naive, central memory, and effector memory CD4+ T-cell subsets sorted from PBMCs isolated 3 mo postinfection (n = 21). Despite no significant difference in mean VL between the two groups at this time point (mean VL for low vRC = 4.4, mean VL for high vRC = 4.8;
Discussion

Recent studies have implicated transmitted viral characteristics in explaining the heritability of HIV-1 pathogenesis (11, 15, 35), and the results delineated above argue that a major component of this heritability is the replicative capacity of the transmitted/founder virus. Moreover, in this cohort of Zambian seroconvertors, acutely infected with HIV-1 subtype C, we demonstrate that vRC is a key contributor to CD4+ T-cell decline that can only be modulated but not reversed by the protective effects of HLA class I alleles such as B*57 and B*5801. This role in disease pathogenesis is consistent with studies of the lymphocytic choriomeningitis virus (LCMV) infection model, where the higher replicative capacity LCMV clone 13 strain goes on to induce chronic infection whereas the more attenuated Armstrong strain is quickly contained by the immune system (36). Although we were unable to accurately measure peak viral load in this cohort of HIV-1 acutely infected individuals, it seems likely that viruses with high replicative capacity have the potential to induce much higher levels of peak viremia, which then induces exacerbated immunopathology that cannot be completely reversed by the immune response. Thus, it is clear that early interactions between the virus and the host have the potential to set in motion a series of events that ultimately define the trajectory of disease progression.

The first interactions between HIV-1 and the host immune response occur at the interface of the virus and the innate immune system. Viral replication results in an initial cytokine storm that is thought to be largely deleterious (17). We show here that the replicative capacity of HIV-1 modulates the magnitude and composition of the early inflammatory milieu, perhaps through differential activation of innate sensing mechanisms that lead to multiple inflammatory consequences. Notably, the inflammatory state associated with high vRC is characterized by elevated levels of key mediators, such as IFN-α and IL-1β, as well as increased production of type I (IFN-α2) and type II (IFN-γ) interferons. Positive loadings for PC2 are characterized by increased levels of IFN-stimulated genes (ISGs) by the time of seroconversion, consistent with what is observed in pathogenic SIV infection. Additionally, vRC was positively correlated with levels of IL-1β, a highly inflammatory cytokine associated with pyroptosis, an inflammatory form of programmed cell death recently linked to abortive HIV infection of quiescent CD4+ T cells (39). It is likely that increased viral production during the acute phase of HIV-1 infection increases the occurrence of abortive infection. This would escalate pyroptotic cell death, drive the production and release of IL-1β, and serve to foment immune activation and exacerbated immunopathology.
Enhanced production of IL-10 was also observed in individuals infected with high-RC viruses. Although IL-10 is traditionally seen as an antiinflammatory cytokine capable of reducing inflammation, in this setting it may be a surrogate of hyperactivation of the innate immune response. Previous studies demonstrated that increased PD-1 expression by monocytes, due to the presence of microbial products and inflammatory cytokines in HIV-1 infection, can lead to the production IL-10, which further disrupts T-cell function (23). Moreover, IL-10 blockade restored the antigen-induced proliferation of HIV-specific CD4+ and CD8+ T cells in vitro, as well as the production of effector cytokines by HIV-specific CD4+ T cells (22). Thus, in the present study, enhanced IL-10 production may reflect a dysregulated innate and adaptive immune response brought on by intensified viral replication during acute infection.

Previous studies showed that the level of immune activation is established very early after HIV-1 infection and predicts disease progression even when viral loads are suppressed either immunologically or by ART (4, 5, 8). For the first time to our knowledge, we demonstrate that levels of aberrant immune activation in CD8+ and CD4+ T cells can be significantly attributed to the replicative capacity of the transmitted virus. We further link the increased activation, exhaustion, and proliferation of T cells to inflammatory cytokine profiles, suggesting a mechanism by which vRC, through induction of an early innate immune response, influences levels of chronic cellular immune activation. This is supported by previously reported associations between CD8+ T-cell activation and the presence of inflammatory cytokines (40, 41).

Whether the elevated inflammatory response and exacerbated immunopathology associated with highly replicating viruses are the consequence of molecular signatures specific to Gag and TRIM5 sensing (42) or higher overall antigen load remains to be determined. Increased vRC likely results in elevated levels of...
HIV-1 epitope presentation and recognition by CTLs, which in turn could lead to aberrant T-cell activation via T-cell receptor (TCR) overstimulation (43). In support of this hypothesis, we found vRC to be positively correlated with PD-1 expression on CD8+ T cells. Because PD-1 expression in chronic HIV infection has previously been shown to be most pronounced on HIV-specific CD8+ T-cell populations (25, 26), this is consistent with increased circulating antigen being a factor linking vRC to increased markers of immune activation and exhaustion in the CD8+ T-cell compartment. It is important to note, however, that loss of protective CD8+ PD-1+/CD57+ cells at these early time points is strongly associated with increased vRC and to a lesser degree with contemporaneous VL, suggesting that it is not simply antigen load that defines this pathogenic effect. A key factor governing this observation may be that vRC is a heritable characteristic of the virus that remains relatively stable at these early times after infection, whereas, in contrast, VL is in significant flux due to the onset of the adaptive immune response. Thus, vRC, as a measurable phenotypic trait, may provide a correlate of viral damage to the immune system in tissues or at even earlier times where and when VL is difficult to measure.

Finally, we demonstrated that the amount of HIV-1 DNA harbored by both TCM and naive (CD27+/CD45RO−) CD4+ T cells is highly associated with viral replicative capacity. It is possible that the increased levels of HIV-1 DNA found in the “naïve” CD4+ T cells could be due in part to the presence of T memory stem cells (TSCM) that would be found within our sorted naïve T-cell populations (44, 45). These long-lived memory T cells with stem cell-like properties are susceptible to HIV-1 or SIV in pathogenic infections but are spared in non-pathogenic SIV disease (46). Similarly, the long-lived TCM CD4+ cells are spared in nonpathogenic SIV infection, and infection of this subset has been linked to the immunopathology of HIV-1 infection (30, 47). Moreover, recent evidence demonstrates that reduced infection of TSCM and TCM CD4+ cells is associated with a nonprogression phenotype in viremic individuals (48). The results of the present study provide further evidence that HIV-1 infection of these cell types occurs early, is linked to increased CD4+ T-cell activation, and in TCM significantly predicts disease progression, perhaps by disrupting the capacity to renew the CD4+ TTEM population (30). TCM CD4+ cells have also been highlighted as an integral population for the maintenance of latency and viral persistence (33). Our findings suggest that the extent to which HIV infects this population and establishes a latent reservoir might be influenced by characteristics of the transmitted/founder virus.

Fig. 4. Viral RC is associated with increased cellular activation and proliferation in CD4+ T-cell memory subsets. Cryopreserved PBMCs isolated at 3 mo after the estimated date of infection from individuals with high vRC (n = 13) and low vRC (n = 6) as well as cryopreserved PBMCs from HIV-negative Zambians (n = 14) were stained for markers of activation and cellular turnover and for delineation of memory T-cell subsets. Statistical comparisons were made using the Student’s t test. (A and B) Comparison of the percentage of CD4+ TEm (A) and TCM (B) coexpressing the activation markers CD38 and HLA-DR in HIV-negative Zambians and individuals infected with either low-RC or high-RC viruses. (D and E) Comparison of the percentage of CD4+ TEm (D) and TCM (E) positive for Ki67 in HIV-negative Zambians and individuals infected with either low-RC or high-RC viruses. (C and F) Kaplan–Meier survival analyses (with an endpoint defined as CD4+ T-cell counts <300) depict associations between the percentage of memory CD4+ T cells expressing markers of activation (C) and proliferation (F) and the decline in total CD4+ T cells. Kaplan–Meier survival statistics were generated from the log-rank test.

E1486 | www.pnas.org/cgi/doi/10.1073/pnas.1421607112

Claiborne et al.
Taken together, these results support an unprecedented role for the viral replicative capacity of the transmitted/founder virus, in determining the early inflammatory state and general immune dysfunction of HIV-1–infected individuals as well as their subsequent disease trajectory. The data presented here suggest that viral characteristics can provide independent information about an individual’s risk for disease progression and point to a previously unidentified target for interventions to reduce immune activation and viral burden. Vaccine-induced immune responses or interventions that effectively attenuate vRC in the earliest stages of infection could not only have a dramatic impact on viral control and disease progression, but, based on our recent findings (35) also impact the efficiency of subsequent transmission to other partners. Moreover, reducing the size of the viral burden in key T-cell populations before antiretroviral treatment could augment cure strategies aimed at eliminating the latent reservoir.

Fig. 5. Inflammatory cytokine profiles associated with vRC correlate with T-cell activation. Inflammatory cytokines measured at seroconversion in 33 acutely infected individuals were used to define distinct cytokine profiles via principal Component Analysis (Fig. 2). In this group of 33 individuals, a subset of 18 and 19 individuals had also been immunophenotyped for CD8+ and CD4+ T-cell activation, respectively. Positive loadings (elevated inflammatory cytokines) were defined as a PC1 score >0 and negative loadings were defined as a PC1 score <0. (A–C) The differences in CD8+ T-cell activation in individuals with positive (elevated inflammatory cytokines) or negative loadings for PC1. (D and E) The differences in CD4+ T-cell activation in individuals with positive (elevated inflammatory cytokines) or negative loadings for PC1. The left-hand y-axis displays the percentage of positive cells for A–D; the right-hand y-axis displays the percentage of Ki67+ cells for E. (*P < 0.05, **P < 0.01, ***P < 0.001; statistical comparisons were made using Student’s t test, P values are one tailed.)

Fig. 6. Viral RC correlates with the burden of HIV-1 viral DNA in CD4+ TCM and TN. (A) Cryopreserved PBMCs isolated 3 mo postinfection (n = 21) were stained for markers to delineate memory phenotypes, and CD4+ T cells were sorted into three distinct populations, TN (CD27+, CD45RO−), TEM (CD27−/+, CD45RO+, CCR7−), and TCM (CD27−/+, CD45RO+, CCR7+). Cell-associated HIV-1 DNA was quantified by real-time PCR amplification of the HIV-1 subtype C integrase gene. Cell copy number was normalized based on real-time PCR amplification of the human albumin gene. (A) Bar graphs depicting the magnitude of viral burden in TN, TEM, and TCM between individuals infected with either low-RC or high-RC viruses. (B and C) In a majority of the same individuals (n = 18), a fraction of unsorted PBMCs were stained for markers of cellular activation and proliferation. (B) Correlation between the magnitude of viral burden in CD4+ TCM and the percentage of activated (CD38+ HLA-DR+) CD4+ TEM. (C) Correlation between the magnitude of viral burden in CD4+ TCM and the percentage of proliferating (Ki67+) CD4+ TEM. Correlation statistics were generated using linear regression. Solid lines indicate trend lines, and dashed lines represent 95% confidence bands. (D) A Kaplan–Meier survival analysis (with an endpoint defined as CD4+ T-cell counts <300) depicts the effect of viral burden in CD4+ TCM on total CD4+ T-cell decline. Kaplan–Meier survival statistics were generated from the log-rank test.
Materials and Methods

Study Subjects. All participants in the Zambia Emory HIV Research Project (ZEHRP) discordant couples cohort in Luaka, Zambia, were enrolled in human subjects protocols approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board. Before enrollment, individuals received counseling and signed a written informed consent form agreeing to participate.

The subjects included in this study were selected from the ZEHRP cohort based on being recently infected with HIV-1. All subjects were initially seronegative partners within serodiscordant cohabitating heterosexual couples that subsequently seroconverted. All subjects were antiretroviral therapy naïve and were identified a median of 46 (interquartile range = 33–49) d after the estimated date of infection (EDI). The algorithm used to determine the EDI has been previously described (49). All subjects were infected by HIV-1 subtype C viruses.

Generation of Gag-M4 Chimeras and Transmitted/Founder Full-Length Infectious Molecular Clones. Gag-M4 chimeras were generated from frozen plasma isolated at the seroconversion time point for 127 subjects as previously described (15, 16). Briefly, nested PCR primer sets were used to amplify the gag gene from patient plasma and cloned into the M4 provirus, using a restriction enzyme-based approach. Replication-competent virus was generated by transfection of 293T cells with proviral plasmids, and infectious units were assessed on the TZM-bl indicator cell line. GXR25 cells were infected at a constant multiplicity of infection of 0.05, and viral production in the supernatant was evaluated using a radiolabeled reverse transcriptase assay at days 2, 4, 6, and 8 postinfection. Replication capacity scores were generated by dividing the log10-transformed slope of replication from days 2-6 for each Gag-M4 chimera by the log10-transformed slope of replication of wild-type M4. The sequences of the cloned gag genes are available at GenBank under accession nos. KP715723–KP715849.

Full-length genome cDNA synthesis and near full-length single genome amplification were performed as described previously (50) on seroconversion plasma samples for six individuals where Gag-chimera vRC had been measured. Sequencing of multiple amplicons allowed for confirmation of single-variant transmission by star-like phylogeny and to infer the near full-length sequence of the transmitted/ founder (TF) virus. Full-length genome infectious molecular clones of the entire TF sequence were constructed as described previously (50). Viral stocks were generated and infectious units assessed in the same way as Gag-M4 chimera viruses. Replication curves were generated by infecting PHA-stimulated PBMCs at a constant multiplicity of infection of 0.01. viral output was assayed and was assessed at days 0, 2, 4, 6, and 8 d postinfection via a radiolabeled reverse transcriptase assay, and replication scores were generated in the same way as for Gag-M4 chimeras.

Evaluation of Plasma Cytokines. Plasma levels of cytokines and chemokines were measured using MILLIPLEX Human Cytokine/Chemokine detection kits (Millipore). High sensitivity kits were used for measurement of IFNγ, IL-10, IL-6, and IL-7 and regular sensitivity kits were used for IFNα2, IL-10, IP-10, MCP-1, MMP-1, and TNFα and were used according to the manufacturer’s instructions. Samples were run in duplicate with all individuals on the same plate and wells with low bead count or coefficient of variation >30% were excluded from subsequent analysis. Plates were read on the Bio-Plex 3D Suspension Array System (Bio-Rad). Levels of sCD14 (R&D Systems), CRP (Millipore), sCD163 (Truillium Diagnostics), and D-dimer (American Diagnostica) were all measured using standard ELISA-based assays according to the manufacturer’s instructions. Measurement of LPS levels was performed using the LAL Chromogenic Endotoxin Quantification kit (American Diagnostica). Intestinal fatty acid binding protein (F-FABP) was measured using a commercially available ELISA DuoSet assay (R&D Systems) according to the manufacturer’s instructions with minor adjustments. Plasma samples were diluted to 10% (vol/vol) in diluent from the R&D Systems soluble CD14 ELISA kit (DC140) and plates were blocked with Sigma Blocking Buffer.

Flow Cytometry Analysis. Cryopreserved PBMCs isolated from 33 HIV-1–infected Zambians at a median of 49 d after the estimated date of infection were analyzed by flow cytometry to immunophenotype CD8+ T cells. The 12-parameter cytometric staining panel is depicted in Table S1. Frozen PBMCs were thawed, washed once with RPMI 1640 (Sigma) containing 20% FCS (Sigma), 1% M Hepes buffer (Sigma), 1% L-glutamine (Sigma), 1% penicillin–streptomycin (Sigma), and 1% sodium pyruvate (Sigma), and then rested overnight in 4 mL of R20 at 37 °C, 5% CO2, 95% humidity.

For each individual, 106 PBMCs per well were washed once with PBS (Sigma) before staining. When evaluating markers of T-cell cytotoxicity, an 11-parameter cytometric panel, shown in Table S2, was used. For the intracellular markers granzyme B and perforin, Cytofix/Cytoperm (BD) was used for permeabilization and staining per the manufacturer’s protocol.

Detection of Cell-Associated HIV-1 Viral DNA in CD4+ T-Cell Compartments. Cryopreserved PBMCs isolated from a subset of 21 HIV-1–infected individuals at 3 mo postseroconversion were stained to delineate memory CD4+ T-cell subsets, using the seven-parameter cytometric panel displayed in Table S4. Samples were sorted into naive CD4 T cells (CD3+, CD4+, CD8−, CD27+, CD45RO−), central memory CD4 T cells (CD3+, CD4+, CD8−, CD27−, CD45RO+, CCR7+), and effector memory CD4 T cells (CD3+, CD4+, CD8−, CD27+, CD45RO−, CCR7+), using a FACSAria II flow cytometer (Becton Dickinson). Sorted populations were checked for purity and were found to be between 94.5% and 99.4% specific for the population desired.

Live, sorted CD4+t-cell subsets were immediately lysed and genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Samples were eluted in nuclease-free water and analyzed for DNA concentration. DNA samples were analyzed by quantitative real-time PCR (qPCR) for total cell-associated HIV DNA. Approximately 10,000 cell equivalents of genomic DNA were loaded in a 50-μL qPCR reaction, using a custom-designed HIV clade C primer and probe set designed to detect HIV int sequences derived from the patient population. Albumin was used as an internal control to quantify the number of genome present. Both HIV and albumin absolute copies were determined using an external standard curve. HIV clade C primer and probe sequences were Fw5′-GTATTCGCCAGACAGACGAC-3′, Rev 5′-TGATGTCGGA-TGTAGGAGG-3′, and probe 5′-RGAAGGTTTGGTGGGCGC-3′. Human albumin primer and probe sequences were Fw 5′-TGCTGACTTTGRG-3′, Rev 5′-ATGGTCGCTGTTCACCAA-3′, and probe 5′-FAM-TGACAGAATCCTACACTCAGTGTCGCCAAGAA-3′. qPCRs were performed using the Taqman Universal master mix (Life Technologies), 0.2 μM of each primer, and 0.125 μM of probe. All assays were performed on the ABI 7500 system (Life Technologies).

Statistical Analyses. All statistical analysis was performed using JMP, version 11 (SAS Institute). All bivariate continuous correlations were performed using standard linear regression. One-way comparison of means was performed using Student’s t test, and one-tailed P values are reported. Kaplan–Meier survival curves and Cox proportional hazards models were performed using an endpoint defined as a single CD4+ T-cell count reading less than 300, unless otherwise specified, and statistics reported for survival analyses were generated from the log-rank test.

PCA was performed using the JMP version 11 statistical package. Extreme positive values for each of the 16 analytes measured in plasma were Winsorized to the 90th percentile. Missing values were imputed using multiple linear regression models, and individuals for whom more than three cytokine values were missing were excluded. All 16 analytes were measured in 33 individuals were used to extract latent variables.

Depiction of cytokine loadings for PC1 and PC2 (Fig. S2) was generated using Cytoscape v3.0. The loading matrix (list of pairwise correlations between each latent variable and each of the 16 analytes tested) was extracted from the PCA and used to represent the strength of contribution of each analyte to either PC1 or PC2.
Multivariate partial-leave-squares regression portraying the independent contributions of vRC and VL to CD8+ T-cell activation phenotypes (Fig. S3D) was performed with JMP version 11, using the SIMPLS algorithm and K-fold cross validation, where K = 7.

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