Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections

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During HIV type-1 (HIV-1), hepatitis C virus (HCV), and hepatitis B virus (HBV) infections, altered iron balance correlates with morbidity. The liver-produced hormone hepcidin dictates systemic iron homeostasis. We measured hepcidin, iron parameters, cytokines, and inflammatory markers in three cohorts: plasma donors who developed acute HIV-1, HBV, or HCV viremia during the course of donations; HIV-1–positive individuals progressing from early to chronic infection; and chronically HIV-1–infected individuals (receiving antiretroviral therapy or untreated). Hepcidin increased and plasma iron decreased during acute HIV-1 infection, as viremia was initially detected. In patients transitioning from early to chronic HIV-1 infection, hepcidin in the first 60 d of infection positively correlated with the later plasma viral load set-point. Hepcidin remained elevated in individuals with untreated chronic HIV-1 infection and in subjects on ART. In contrast to HIV-1 infection, there was no evidence of hepcidin up-regulation or hypoferremia during the primary viremic phases of HCV or HBV infection; serum iron marginally increased during acute HBV infection. In conclusion, hepcidin induction is part of the pathogenically important systemic inflammatory cascade triggered during HIV-1 infection and may contribute to the establishment and maintenance of viral set-point, which is a strong predictor of progression to AIDS and death. However, distinct patterns of hepcidin and iron regulation occur during different viral infections that have particular tissue tropisms and elicit different systemic inflammatory responses. The hypoferremia of acute infection is therefore a pathogen-specific, not universal, phenomenon.

Disturbances in iron homeostasis commonly manifest in inflammatory and infectious diseases (1). The liver-produced hormone hepcidin regulates levels and compartmentalization of iron by inhibiting the iron exporter ferroportin (2), which is highly expressed by macrophages and duodenal enterocytes (3). Hepcidin excludes iron from serum by sequestering it in macrophages and preventing dietary uptake. In addition to its homoeostatic regulation by iron, hepcidin is an acute-phase peptide induced by interleukin (IL)-6, IL-22, and type I interferon (IFN) (4–6). Persistent exclusion of iron from serum caused by hepcidin contributes to iron-restricted erythropoiesis and the anemia of chronic inflammation.

Anemia is common during chronic HIV type-1 (HIV-1) infection (7) and is predictive of HIV-associated morbidity and mortality independently of established prognostic indicators such as CD4 count (8–11). The etiology of HIV-related anemia is complex (reviewed in ref. 10) but likely involves iron-restricted erythropoiesis (12). Iron sequestration within bone marrow macrophages, suggestive of hepcidin activity, inversely correlates with secondary infections and mortality in HIV-1 infection (13), and altered iron status (independently of anemia) correlates with HIV-associated mortality and morbidity, even after accounting for confounders including CD4 count (14–18). Hepcidin inversely correlates with CD4 counts in individuals with advanced HIV-1 (19). However, hepcidin levels in the crucial acute phase of HIV-1 infection, which dictate later events in the disease (20), are unexplored. In this early period, vast numbers of mucosal CD4+ T cells are lost (21), immune homeostasis is irreversibly perturbed, and proinflammatory cytokines are systemically elevated (22, 23).

Unusually for a systemic inflammatory state, hepcidin is suppressed during chronic hepatitis C virus (HCV) infection, contributing to pathogenic liver iron loading (24). Hepcidin can also be suppressed during hepatitis B virus (HBV)-associated cirrhosis (25), although this may not be as marked as in HCV infection (26). Nothing is known about how hepcidin behaves during acute HCV or HBV infection, in particular whether the unusual low hepcidin observed in the chronic states may occur earlier.

Significance

Altered iron levels correlate with disease progression in HIV type-1 (HIV-1) infection, and cellular iron promotes HIV-1 replication. In chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, increased liver iron levels contribute to disease. The peptide hormone hepcidin controls iron distribution. We find that hepcidin increases during the acute phase of HIV-1 infection, early hepcidin predicts later plasma viral set-point, and hepcidin remains high even in chronically infected individuals receiving antiretroviral therapy. Conversely, hepcidin is not induced, and blood iron is not decreased, during the acute response to HBV and HCV. Therefore, the nature of iron redistribution during the response to infections is a pathogen-specific phenomenon; furthermore, the deleterious effects of chronic infection on hepcidin and iron appear to be established early in infection.


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The aims of this study were to investigate the behavior of hepcidin during acute and chronic HIV-1 infection and second to compare and contrast hepcidin kinetics during acute HIV-1, HCV, and HBV infections.

Results

Hepcidin Is Up-Regulated During the Acute Phase of HIV-1 Infection. The earliest events following HIV-1 infection have a significant influence on subsequent disease pathogenesis (20). To investigate changes in hepcidin during this period, we measured hepcidin and other analytes in sequential plasma samples from plasmapheresis donors (n = 12) who developed detectable levels of HIV-1 viremia during a course of plasma donation, as described previously (22). (Fig. S1 depicts how this cohort relates to the natural history of HIV-1 infection.) These individuals were seronegative with undetectable levels of HIV-1 viremia upon commencement of donation, but during the following period became viremic and subsequently exhibited an exponential escalation in plasma HIV-1 RNA levels. Some subjects had samples that continued to the initiation of viremia control (drop in viral load). The set of samples therefore presents a rare opportunity to study the physiology of the very earliest stages of acute HIV-1 infection.

We assessed changes in plasma concentrations of hepcidin, ferritin, iron, C-reactive protein (CRP), Serum Amyloid A (SAA), IL-6, IL-10, IL-18, tumor necrosis factor (TNF)-alpha, and IFN-alpha in the days before and after T0, the time point at which the plasma viral load was estimated to first exceed the lower limit of detection (LOD) (Fig. 1). To summarize changes in analytes relative to T0, for the set of 12 plasma donors, we interpolated smoothed curves for viral load and each analyte as described in SI Materials and Methods (Fig. 1, Upper panels). (Fig. S2 depicts the process in more detail for viral load.) To assess the significance of observed differences, we then binned data into 5-d windows relative to T0, fitting linear mixed-effects models on the data (Fig. 1, Lower panels).

We observed a significant increase of hepcidin shortly after the culmination of viremia, peaking around day 11 after T0 (Fig. 1A). This elevation occurred after the early elevation of the acute-phase proteins SAA (23) and CRP and the transient induction of IFN-alpha (22) (Fig. 1 B-D). Instead, the rise in hepcidin more closely matched the up-regulation of IL-10 and peaked at a similar time to IL-18, TNF-alpha, and ferritin (Fig. 1 E-H). We detected no significant change in IL-6 or IL-22 (Fig. 1 I and J). A drop in plasma iron coinciding with the escalation of viremia was observed, consistent with hepcidin activity (Fig. 1K).

Analysis of Hepcidin During Transition from Acute to Chronic HIV-1 Infection and Association of Hepcidin with Plasma Viral Load. To investigate whether hepcidin up-regulation in acute HIV-1 infection is sustained into chronic infection, we measured hepcidin longitudinally in 21 subjects from cohorts of men who have sex with men, all known to have been recently infected with HIV-1 (Fig. S1 and Tables S1 and S2). In a subset of these subjects, the first samples were obtained soon enough to catch the decline in viremia during the later stages of acute infection (Fig S3). In several of the subjects sampled within the first 2 mo of infection, hepcidin concentrations were markedly elevated at early time point(s), before stabilizing into the chronic phase of infection when set-point viremia became established (geometric mean hepcidin for day 0–60, 47.8 ng/mL (95% CI 34.1–66.8, 42 samples measured); day 89–366, 33.9 ng/mL.

Fig. 1. Summary of perturbations of plasma analytes during the acute phase of HIV-1 infection. Plasma concentrations of (A) hepcidin, (B) SAA, (C) CRP, (D) IFN-alpha, (E) ferritin, (F) IL-10, (G) IL-18, (H) TNF-alpha, (I) IL-6, (J) IL-22, and (K) plasma iron were measured in multiple samples obtained from 12 plasmapheresis donors who developed detectable levels of HIV-1 viremia during the course of plasma donations. Analyte data were plotted with reference to T0, the time at which viremia was estimated to become detectable, for each individual. (Upper panels) Analyte concentrations were interpolated as described in Materials and Methods based on the longitudinal series for each individual, and the smoothed curves were then interpolated from the mean data. (Inset) The number of individuals contributing samples on each day. (Lower panels) To assess the significance of perturbations, data were binned into time intervals; the null hypothesis that there is no difference between analyte concentrations across sets of bins was tested using the Wald test (p value stated in panels) after fitting linear mixed-effects models; specific pairwise differences between preday 5 baseline bins and other bins were examined using t tests, after accounting for subject-specific variability. Asterisks below a specific bin indicate the analyte values in that bin are significantly different from baseline values (*P < 0.05, **P < 0.01, ***P < 0.001).
Hepcidin is Elevated During Chronic HIV-1 Infection in Parallel with Other Acute-Phase Proteins and Certain Cytokines. Because established HIV-1 infection is characterized by chronic immune activation, we next investigated whether, even if not sustained at the maximal levels achieved during acute infection, hepcidin remained elevated during chronic, asymptomatic, untreated HIV-1 infection compared with uninfected controls. We measured plasma concentrations of hepcidin, ferritin, acute-phase proteins, and cytokines in a cross-sectional group of 31 HIV-1-infected males from the London cohort (Fig. S1 and Table S3; some subjects overlapping with those described above in the longitudinal analysis) who had been infected for either 6 mo (n = 20) or 3.5 y (n = 11). Although CD4 counts were significantly lower in the latter group [637 (95% CI 509, 765) vs. 354 (254, 454), P = 0.0025], all other parameters measured were similar for the two groups, and hence they were subsequently considered together as “chronically HIV-infected.”

Hepcidin Remains Elevated During ART Treatment. We next considered whether hepcidin remained elevated in HIV-infected individuals receiving antiretroviral therapy (ART). Although ART reduces viral load to undetectable levels, treated individuals often still exhibit persistent immune activation (32, 33). Consistent with this, we found that hepcidin, together with CRP, IL-18, and TNF-alpha, were higher in ART-treated HIV-infected individuals compared with the control group (although hepcidin was lower than in untreated individuals) (Table 1).

In this dataset, there was no significant association between hepcidin and plasma viral load measured contemporaneously (r = 0.195, P = 0.312). Within the whole dataset, and among HIV-infected individuals alone, SAA levels did correlate significantly with hepcidin (Fig. S5A). Similarly, hepcidin correlated significantly with ferritin in the control group alone (r = 0.546, P = 0.0235) as previously observed in healthy individuals (31), in the HIV-infected group alone (r = 0.367, P = 0.0422), or when considering both groups together (r = 0.467, P = 0.0008) (Fig. S5B). Together, the data suggest that the increase in hepcidin during chronic HIV is driven by inflammation.

No Hepcidin Up-Regulation During Acute HBV or HCV Infection. Next, we examined whether hepcidin was also up-regulated during acute HBV and HCV infections, using longitudinal samples from HBV- and HCV-infected plasmapheresis donors (n = 10 for both infections), analogous to those described above for HIV-1 in Fig. 1. Plasma viral loads rapidly escalated following T0 in both infections and remained at high levels longer than in HIV-1 infection (Fig. S2). In contrast to acute HIV-1 infection, there was no significant hepcidin up-regulation after T0 in acute HBV infection (Fig. S4). There was evidence of hepcidin down-regulation at the time of peak viremia in HCV infection (Fig. 3A), although the sample was not powered to demonstrate this. This lack of evidence for significant alterations in hepcidin was consistent with the lack of significant changes in ferritin and plasma iron at this time, and the lack of induction of other inflammatory cytokines (22); a modest and later increase in IL-18 was detected, but no significant changes in IFN-alpha or IL-6 were found (Fig. 3B–F).

Similarly, we failed to detect any significant changes in hepcidin or ferritin during the acute phase of HBV infection (Fig. 4A and B). However, there was a small but significant increase in plasma iron at the highest point of viral load (Fig. 4C). We detected no significant perturbations in IFN-alpha, IL-6, or IL-18 during the first 3 wk post-T0 (Fig. 4D–F). Thus, these liver-trophic infections behave differently to acute HIV-1 infection in terms of inflammatory response, hepcidin, and plasma iron.

Discussion

Events in the first few weeks following HIV-1 infection establish disturbances in immune homeostasis that likely have a major impact during the later chronic phases of the infection (20). Several factors are implicated in initiating a long-term state of immune activation, including direct effects of HIV replication, coinfections, and the drastic loss of CD4+ T cells from the...
intestinal mucosae during acute infection, which serves to increase microbial translocation through the gut, contributing to systemic inflammation (32). The extent of immune activation predicts HIV-1 disease progression rates (34) independently of viral load or CD4 count. Moreover, this immune activation is relatively unresponsive to ART, even though viral loads are reduced below detectable levels (32, 33).

Typically, acute-phase proteins were among the first plasma proteins to be detectably increased during HIV-1 infection (23). In addition, SAA, CRP, and ferritin are elevated during chronic HIV-1 infection and predict HIV-associated mortality (14, 18, 35). Here we demonstrate that hepcidin—a liver-produced acute-phase peptide—was significantly up-regulated between 10–15 d after the first detection of viremia during acute HIV-1 infection. A significant decrease in plasma iron, consistent with hepcidin activity, was also observed, although the decline in iron slightly preceded peak hepcidin up-regulation. Relative to elevation of SAA, CRP, and IFN-alpha, hepcidin up-regulation was slightly delayed, peaking more in line with viral load, ferritin, and cytokines such IL-10, IL-18, and TNF-alpha. The rapid and transient induction of IFN-alpha was previously noted as a feature of acute HIV-1 infection (22). IFN-alpha induces hepcidin mRNA in vitro and in mice; pegylated IFN-alpha administration to humans infected with HCV also induces hepcidin (6). IFN-alpha may therefore contribute to hepcidin up-regulation during acute HIV-1 infection, especially as we did not detect significantly elevated levels of IL-6 or IL-22.

Hepcidin levels during acute infection wane as viremia is controlled, but hepcidin remains significantly up-regulated (by roughly twofold) during chronic HIV-1 infection, in concert with a range of other acute-phase proteins (CRP and SAA) and inflammatory cytokines (IL-10, IL-18, and TNF-alpha). In addition, hepcidin levels during the early phase of infection positively correlated with subsequent set-point viral load, which is itself the strongest single indicator of times to AIDS and death (29, 30). Furthermore, there was a relationship between contemporaneous hepcidin and set-point viral load during initial chronic infection. The increased hepcidin may reflect greater levels of inflammation in individuals who are failing to control viral replication as efficiently. Hepcidin, like CRP, IL-18, and TNF-alpha, remained elevated in ART-treated, HIV-infected individuals despite reduction of viral loads to below detectable levels.

Deregulated iron status has been linked with morbidity and mortality in HIV infection (14–16, 18). Three mechanisms by

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Control, n = 20</th>
<th>HIV-infected, n = 31</th>
<th>P value, vs. control</th>
<th>ART-treated HIV, n = 20</th>
<th>P value, vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>Years</td>
<td>33.01 (29.82, 36.20)</td>
<td>36.85 (33.85, 39.86)</td>
<td>0.0874</td>
<td>41.20 (37.64, 44.75)</td>
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</tr>
<tr>
<td>Hepcidin†</td>
<td>ng/mL</td>
<td>8.25 (5.33, 13.07)</td>
<td>19.13 (12.68, 28.87)</td>
<td>0.0089</td>
<td>17.51 (11.13, 27.53)</td>
<td>0.0198</td>
</tr>
<tr>
<td>Ferritin‡</td>
<td>µg/L</td>
<td>63.47 (45.49, 88.54)</td>
<td>89.95 (68.93, 117.38)</td>
<td>0.1053</td>
<td>82.14 (55.73, 121.07)</td>
<td>0.3053</td>
</tr>
<tr>
<td>CRP†</td>
<td>mg/L</td>
<td>0.27 (0.14, 0.50)</td>
<td>1.41 (0.80, 2.48)</td>
<td>0.0003</td>
<td>1.73 (1.00, 3.02)</td>
<td>&lt;0.0001</td>
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<tr>
<td>SAA†</td>
<td>ng/mL</td>
<td>1,553 (961, 2511)</td>
<td>3,488 (2484, 4887)</td>
<td>0.0052</td>
<td>2,337 (1533, 3562)</td>
<td>0.1890</td>
</tr>
<tr>
<td>IL-1beta†</td>
<td>pg/mL</td>
<td>0.36 (0.21, 0.61)</td>
<td>0.30 (0.20, 0.46)</td>
<td>0.6000</td>
<td>0.31 (0.18, 0.52)</td>
<td>0.6857</td>
</tr>
<tr>
<td>IL-2‡</td>
<td>pg/mL</td>
<td>0.84 (0.42, 1.67)</td>
<td>1.20 (0.73, 1.97)</td>
<td>0.3751</td>
<td>0.95 (0.49, 1.84)</td>
<td>0.7878</td>
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<tr>
<td>IL-6†</td>
<td>pg/mL</td>
<td>1.47 (0.82, 2.64)</td>
<td>2.14 (1.27, 3.61)</td>
<td>0.3484</td>
<td>0.98 (0.55, 1.74)</td>
<td>0.3003</td>
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<tr>
<td>IL-7‡</td>
<td>pg/mL</td>
<td>2.89 (1.80, 4.69)</td>
<td>3.17 (2.66, 3.79)</td>
<td>0.8393</td>
<td>2.44 (1.38, 4.31)</td>
<td>0.6435</td>
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<tr>
<td>IL-8†</td>
<td>pg/mL</td>
<td>4.35 (2.84, 6.65)</td>
<td>5.62 (4.40, 7.36)</td>
<td>0.1056</td>
<td>4.55 (3.51, 5.89)</td>
<td>0.8431</td>
</tr>
<tr>
<td>IL-10†</td>
<td>pg/mL</td>
<td>9.14 (6.05, 13.79)</td>
<td>12.02 (7.34, 19.56)</td>
<td>0.0007</td>
<td>12.02 (5.34, 27.03)</td>
<td>0.5451</td>
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<tr>
<td>IL-12§</td>
<td>pg/mL</td>
<td>2.21 (0.88, 5.52)</td>
<td>2.96 (1.53, 5.75)</td>
<td>0.5856</td>
<td>2.07 (1.12, 3.81)</td>
<td>0.8995</td>
</tr>
<tr>
<td>IL-18§</td>
<td>pg/mL</td>
<td>337.3 (267.0, 426.2)</td>
<td>744.3 (640.2, 865.3)</td>
<td>&lt;0.0001</td>
<td>605.4 (476.3, 769.6)</td>
<td>0.0008</td>
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<tr>
<td>IFN-gamma†</td>
<td>pg/mL</td>
<td>5.23 (3.06, 8.92)</td>
<td>3.40 (2.09, 5.53)</td>
<td>0.2470</td>
<td>4.52 (2.47, 8.28)</td>
<td>0.7114</td>
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<tr>
<td>TNF-alpha†</td>
<td>pg/mL</td>
<td>3.08 (2.51, 3.77)</td>
<td>6.60 (5.21, 8.36)</td>
<td>&lt;0.0001</td>
<td>5.13 (3.74, 7.05)</td>
<td>0.0087</td>
</tr>
</tbody>
</table>

*P value from unpaired t test, P < 0.05 shown in italics. P values compare values of HIV-infected versus control, or ART-treated, HIV-infected versus control.

†Arithmetic mean.

‡Geometric mean; parentheses indicate 95% confidence interval of the mean.

§Data available for 17 individuals.

Data available for 18 individuals.

Data available for 19 individuals.

Data available for 30 individuals.

Fig 3. Summary of perturbations of plasma analytes during the acute phase of HCV infection. Plasma concentrations of (A) hepcidin, (B) ferritin, (C) iron, (D) IFN-alpha, (E) IL-6, and (F) IL-18 were measured in multiple samples obtained from 10 plasmapheresis donors who developed detectable HCV viremia during the course of plasma donations. Data were plotted with reference to T0, the time when viremia was estimated to become detectable, as described in Fig. 1. The P value within panels refers to Wald test, and asterisks below a specific bin indicate the analytic values in that bin are significantly different from baseline values (*P < 0.05, **P < 0.01, ***P < 0.001).
which altered iron status resulting from hepcidin activity could influence HIV-1 pathogenesis can be proposed. Hepcidin down-regulates surface expression of ferroportin in both lymphocytes and macrophages (36, 37), increasing iron availability in key cellular sites of HIV-1 replication. HIV-1 replication is iron dependent (27); accordingly, HIV-1 replication is enhanced, in parallel with decreased ferroportin and increased cellular iron following treatment of promonocytic THP1 cells, primary human macrophages, and primary CD4+ lymphocytes with hepcidin (28). Thus, elevated hepcidin, as well as reflecting inflammation, may also contribute to a cellular environment favoring HIV-1 replication. This relationship could underlie the observed associations between hepcidin and plasma viral load, so that hepcidin might play a role in the establishment and maintenance of viral load set-point.

Second, chronically elevated hepcidin may be directly involved in the pathogenesis of HIV-1–associated anemia (7). Although the etiology of this anemia is likely multifactorial, it commonly carries the hallmarks of an inflammatory anemia with iron-restricted erythropoiesis, consistent with persistently raised hepcidin activity (e.g., bone marrow macrophage iron accumulation) (13). Anemia is an important comorbidity factor during HIV-1 infection in the developing world, associating with elevated risk of mortality (8, 11). Interestingly, declines in hemoglobin and serum iron have been observed during the first months following HIV-1 infection in South African women (38). This is consistent with our findings of hepcidin induction during acute infection and raised hepcidin in individuals with recently established asymptomatic chronic infection.

Third, iron loading in macrophages caused by hepcidin may inhibit immunological macrophage functions (39) and favor growth of macrophage-tropic infections (e.g., Mycobacterium tuberculosis) (40). Iron redistribution at enrollment into a Gambian HIV cohort was predictive of incident tuberculosis (17), and common secondary infections (Mycobacterium, Candida, and Pneumocystis) were more common in HIV-1–infected individuals with a high degree of macrophage iron loading (13).

In contrast to acute HIV-1 infection, hepcidin up-regulation was not observed during the acute viremic phase of HCV or HBV infections. The acute viremic phases of HCV and HBV are not accompanied by the high magnitude systemic “cytokine storm” that is seen in acute HIV-1 infection (22) (although local production of some IFN may occur in the infected liver; ref. 41). This is likely because HIV-1 triggers rapid activation of plasmacytoid dendritic cells (pDCs) to produce IFN-alpha and other cytokines and chemokines (42), whereas HCV and HBV virions are weak pDC activators (43). These differences may reflect HIV-1 evolution to drive high levels of immune activation that promotes its replication, whereas HBV/HBV use “stealth” strategies to allow viral replication to proceed before induction of inflammatory cytokines. Our data are consistent with this pattern, with hepcidin induction in the inflammatory acute HIV-1 infection, and no evident hepcidin response in the liver-tropic infections that are quieter during the acute phase (although hepcidin might fluctuate later in HBV infection, when a vigorous cytokine response occurs around days 45–90 postfirst viremia) (44). The infections also differ in terms of iron status: hepcidin is suppressed during chronic HCV and (possibly) HBV infections, and increased serum iron occurs in HBV (45). Hepcidin was suppressed (not significantly) during acute HCV, and serum iron was raised in acute HBV in our study. It is conceivable that the mechanisms that suppress hepcidin in chronic disease are active early in infection.

In conclusion, distinct patterns of hepcidin regulation are apparent in three important viral infections. Acute HIV-1 infection causes an increase in hepcidin, accompanied by decreased serum iron. Similar hepcidin induction was not detected in the acute viremic phases of HCV and HBV infections. The hypoferrremia of infection is therefore not a universal occurrence during all infections, but varies with the tropism of the infectious agent and the host hepcidin response. For all three viruses, hepcidin changes during acute infection are consistent with later iron distribution patterns—macrophage iron accumulation in chronic HIV-1 and liver iron loading in chronic HCV and HBV. In turn, iron redistribution may contribute to subsequent disease progression: establishment of viral load set-point, susceptibility to macrophage-tropic infections and likelihood of anemia in HIV-1/AIDS, and liver damage and dysfunction in chronic viral hepatitis.

Materials and Methods

Study Subjects. We performed studies on a cohort of plasmapherisis donors who became infected with HIV-1, HBV, or HCV during the course of donations; a cohort of HIV-1 patients caught early in infection and followed into chronic infection; and a cross-sectional analysis of chronically HIV-1–infected individuals who were untreated or receiving ART. Details of the cohorts can be found in SI Materials and Methods, and see Fig. 51 for a scheme of the three cohorts in relation to the natural history of HIV-1 infection.

Quantification of Iron Indices and Plasma Cytokines. Plasma ferritin (Architect Ferritin Assay) was quantified using the Abbott Architect 2000R automated analyzer (Abbott Laboratories); CRP (MULTIGENT CRP Vario Kit, with high sensitivity calibrators) and plasma iron (MULTIGENT Iron Kit) were quantified using an analyzer (Abbott Laboratories); CRP was quantified using Advia 2400 Chemistry (Siemens). Levels of IL-1beta, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12(p70), TNF-alpha, and IFN-gamma were measured as described previously (22). The value of the most dilute standard was defined as the LOD. Colorimetric ELISAs were used to quantify plasma levels of IL-18 (Invirotgen), IL-22 (R&D Systems), and SAA (Abazyme) according to the manufacturer’s protocols. For each analyte, samples returning values below the LOD were reported as LOD/2.

Quantification of Hepcidin. Hepcidin was quantified using the Human Hepcidin-25 EIA Kit (Bachem) according to the manufacturer’s protocol, modified to

![Graphs and images related to the text]
use a 9-point, twofold serial dilution standard curve (25 ng/mL to 0.05 ng/mL), reporting the mean of samples (diluted 1 in 12 before the assay) run in triplicate (plasmin/serinesinonorphors and cross-sectional study) or duplicate (early-to-chronic transition study). The lower LOD of the assay (1.12 ng/mL after adjustment for dilution) was calculated by subtracting three times the normalized mean SD (across the whole set of assays run in the study) from the normalized OD450 of the “no hepcidin blank” for each plate. Samples returning a reading below LOD were assigned the value LOD/2 = 0.56 ng/mL; samples returning a value above the upper limit of the standard curve were assigned the value of the highest standard (300 ng/mL after adjustment for dilution).

Full details of statistical analyses are given in SI Materials and Methods. 


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Supporting Information

Armitage et al. 10.1073/pnas.1402351111

SI Materials and Methods

Study Subjects. Plasma donor acute virus infection study. Cryopreserved sequential plasma samples were obtained (via Zeptometrix Corporation and SeraCare Life Sciences) from plasmaapheresis donors who were infected with HIV type-1 (HIV-1) (n = 12), hepatitis B virus (HBV) (n = 10), or hepatitis C virus (HCV) (n = 10) upon sample screening at the end of a period of plasma donation, as described previously (1, 2). All donors had been seronegative for each virus at the initiation of donation, and donated plasma typically every 2–5 d over a 1–3 mo period; therefore, infection likely occurred during the course of plasma donation. No further data concerning patient characteristics were available for these subjects. Viral loads were established retrospectively, as described previously (2).

Early-to-chronic HIV-1 time course study. Details of study participants and the number of samples analyzed are given in Tables S1 and S2.

i) In the London cohort, HIV-1–infected male subjects, mostly of Caucasian ethnicity, who had presented with symptoms of acute retroviral infection, were recruited by the Mortimer Market Center for Sexual Health and HIV Research (London, United Kingdom) and gave written informed consent as part of a study approved by the National Health Service Camden and Islington local research ethics committee (LREC 98/60), as previously described (3). Some study subjects initiated antiretroviral therapy (ART) in early infection, whereas others chose to remain untreated; only samples taken before initiation of ART were included in this time course study. Plasma samples separated from EDTA blood were collected longitudinally following diagnosis.

ii) In the Beijing cohort, Chinese male subjects diagnosed with acute HIV-1 infection during 2007/2008 from a prospective cohort study of men who have sex with men were included. The study was approved by Beijing Youan Hospital of Capital Medical University research ethics committee (no. 20061109V1), and all participants provided written informed consent. Plasma was separated from EDTA blood samples taken at 1, 2, 4, 8, and 12 wk postseroconversion, and every 3 mo thereafter, as previously described (4, 5).

Cross-sectional study. This study included samples from an overlapping set of subjects from the London cohort described above. For comparison, plasma was separated from control blood samples [1U Na-heparin (LEO Laboratories Ltd) per mL blood] collected from 20 healthy male volunteers from Oxford, the majority of whom were also of Caucasian ethnicity and of similar age to the HIV-infected subjects at baseline. All blood was taken with written informed consent in accordance with the Declaration of Helsinki. Details of study participants are summarized in Table S3.

Statistical Analyses. Plasma donor acute virus infection study. All statistical analyses were performed using R statistical language (6) and the packages fields (7), nlme (8), and lme4 (9).

i) Regarding estimation of time origin, for each plasma donor panel, longitudinal series were normalized to a time origin, T0, estimated as described previously (2). Briefly, T0 was defined as the time point at which the plasma viral load first exceeded the lower limit of detection (100 copies per mL for HIV, 200 copies per mL for HBV, and 600 copies per mL for HCV) and was estimated for each donor using linear mixed-effects models.

ii) Regarding interpolation curves, cubic spline interpolation was performed for each subject for each analyte to estimate values for the days where sample measurements were not available, using the spline of Forsythe et al. (10). Average measurements across all subjects for each day were then calculated. A smoothing spline regression was then applied to these data, where the amount of smoothness was estimated from the data by generalized cross-validation (GCV) (11). For viral loads, we calculated average measurements across all samples for each day before applying a smoothing spline regression, again estimating the amount of smoothness from the data by GCV.

iii) Regarding assessment of the effect of time relative to T0 on analyte concentrations, to investigate the behavior of each analyte over time, all longitudinal data were grouped into bins from day −5 to T0 and then in 5-d interval bins post-T0. The range of bins was restricted to the latest point for which bins contained data from 70% or more of individuals; all data from before day −5 were grouped into a single bin to give a baseline estimate against which parameter values were subsequently compared. Linear mixed-effects models were fitted on these data following a described model formulation (12) and computational framework (13). Linear mixed-effects models describe a relationship between a response variable (in this case the analyte of interest) and covariates measured or observed along with the response. They incorporate both fixed-effects parameters and random effects: here, the fixed categorical variables (i.e., the 5-d bins) are modeled by fixed effects, whereas random effects are used to capture interindividual variability. The Wald test was used to test the null hypothesis that there is no significant difference in analyte levels across the set of time-related bins analyzed; specific pairwise comparisons between analyte levels at baseline and other time points were then tested by t tests, after accounting for subject-specific variability.

Early-to-chronic HIV-1 time course study. For the London cohort, the date of onset of symptoms was recorded. The date of infection was estimated as 17 d before day of symptom onset, which we assumed to occur in Fiebig stage 2 (14). For the Beijing cohort, dates of infection were defined as 17 d before an HIV-1 RNA positive test occurring before seroconversion, 30 d before the date of an indeterminate Western blot, or the midpoint between negative and positive antibody tests.

Set-point viral load was defined as the mean of any log10 viral load measurements from samples taken between 3 and 12 mo (day 89–366) of infection for an individual; across the study, the mean number of plasma viral load measurements taken within this time window and contributing to the calculation of set-point plasma viral load was 3.6 (95% CI 3.3–3.9) (Table S2).

Because hepcidin data were not normally distributed, hepcidin values were log10-transformed before analysis. “Early” hepcidin was calculated by taking the mean of log10 hepcidin values from any samples for an individual taken on day 60 postinfection or earlier. “Set-point” hepcidin was calculated by taking the mean of log10 hepcidin values from samples taken between 3 and 12 mo postinfection (day 89–366); samples with a coincident C-reactive protein (CRP) > 5 mg/L were excluded as they likely reflect an inflammatory episode, potentially unrelated to HIV-1
infection. Univariate associations with set-point viral load were tested using Pearson correlations. 

**Cross-sectional study.** Statistical analysis was carried out using Stata11 (StataCorp). Figures were produced using Prism6 (GraphPad Software, Inc). Indices that were not normally distributed were log10-transformed before analysis. Pairwise comparisons were made using Student t test. P < 0.05 was considered significant. Univariate associations were tested using Pearson’s correlations.

3. Turnbull EL, et al. (2011) Escape is a more common mechanism than avidity reduction for evasion of CD8+ T cell responses in primary human immunodeficiency virus type 1 infection. Retrovirology 8:41.

**Fig. S1.** Scheme representing the different cohorts involved in this study. Plasma viral load of HIV-1 (represented by the black line) typically peaks between 10 and 15 d postdetection of viremia, before immune control then decreases plasma viral load to a stable threshold (set-point) that varies between individuals. The level of this set-point is a useful prognostic indicator of progression to AIDS, with higher set-points being associated with more rapid progression. (1) The kinetics of hepcidin during the acute phase of infection were investigated using a cohort of US plasmapheresis donors. (2) Cohorts of subjects from Beijing and London who were identified shortly after HIV-1 infection and followed longitudinally were used to investigate the behavior of hepcidin during the transition from early to chronic infection and the relationship between hepcidin and plasma viral load set-point. (3) Cross-sectional samples from an overlapping set of subjects from the London cohort were then used to investigate hepcidin and other iron/inflammatory markers compared with uninfected controls during the chronic phase of infection.

**Fig. S2.** Interpolated curves summarizing changes in viral load data relative to T0 for (A) HIV-1, (B) HCV, and (C) HBV. Each point represents the mean value of data on each day derived from each panel of plasma donor samples; smoothed curves were interpolated for each dataset as described in Materials and Methods.
Time courses of hepcidin and plasma viral load during the transition from acute to chronic HIV-1 infection. Hepcidin was measured in longitudinal samples obtained from males recently infected with HIV-1 from cohorts in London and Beijing. For a subset of samples, CRP was measured to assess coincidence of proinflammatory episodes: a, CRP > 5 mg/L; b, CRP < 5 mg/L; u, sample above upper limit of assay and therefore assigned upper limit value; NA, clarifies that sample was not available for CRP measurement. CRP was not measured for points where there is no annotation on the hepcidin curve.
Fig. S4. Levels of plasma analytes in healthy controls and individuals with chronic untreated HIV-1 infection. (A) Hepcidin. (B) Ferritin. (C) CRP (hsCRP). (D) Serum Amyloid A (SAA). (E) IL-10. (F) IL-18. (G) TNF-alpha. (H) IL-6. Statistics (unpaired t test) are based on log-transformed data.

Fig. S5. Pearson correlations of hepcidin with (A) SAA and (B) Ferritin in HIV-infected and uninfected control individuals. Statistics performed on log-transformed data. (A) Correlation of log-hepcidin with log-SAA: all individuals together, r = 0.473, P = 0.0005; HIV-infected individuals only, r = 0.423, P = 0.0427; controls only, not significant, P = 0.13. (B) Correlation of log-hepcidin with log-ferritin: all individuals together, r = 0.467, P = 0.0008; uninfected controls only, r = 0.546, P = 0.0235; HIV-infected individuals only, r = 0.367, P = 0.0422.
### Table S1. Study participants/subjects with early-to-chronic time courses, as presented in Fig. S3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acute-to-chronic time course, London</th>
<th>Acute-to-chronic time course, Beijing</th>
<th>Acute-to-chronic time course, all</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Sex, male, %</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Age at enrolment (95% CI)</td>
<td>34.3 (23.8–44.9), n = 6</td>
<td>33.4 (26.7–40.1)</td>
<td>33.7 (28.6–38.8), n = 20</td>
</tr>
<tr>
<td>Set-point log plasma viral load (95% CI) copies per mL*</td>
<td>4.30 (3.15–5.46)</td>
<td>4.31 (3.81–4.82)</td>
<td>4.31 (3.86–4.76)</td>
</tr>
<tr>
<td>“Early” hepcidin, day 0–60 (95% CI) ng/mL†</td>
<td>35.3 (16.8–74.4)</td>
<td>58.3 (40.8–83.2), n = 10</td>
<td>47.4 (33.6–66.9), n = 17</td>
</tr>
<tr>
<td>Set-point hepcidin, day 89–366 (95% CI) ng/mL‡</td>
<td>30.6 (12.0–77.9)</td>
<td>37.5 (27.3–51.6)</td>
<td>35.1 (25.3–48.6)</td>
</tr>
<tr>
<td>Day postinfection of first hepcidin reading (95% CI)*</td>
<td>31.9 (27.0–36.7)</td>
<td>49.0 (39.7–58.3)</td>
<td>43.3 (36.2–50.4)</td>
</tr>
<tr>
<td>Day postinfection of final hepcidin reading (95% CI)</td>
<td>359.3 (114.5–604.1)</td>
<td>384.9 (324.9–445.0)</td>
<td>376.4 (299.9–452.8)</td>
</tr>
<tr>
<td>Mean number of samples in time course per individual (95% CI)</td>
<td>7.4 (5.6–9.3)</td>
<td>5.7 (5.1–6.3)</td>
<td>6.3 (5.6–7.0)</td>
</tr>
</tbody>
</table>

*Arithmetic mean.
†Calculated as the mean of log_{10} plasma viral loads measured in samples taken between 3 and 12 mo postinfection (day 89–366).
‡Geometric mean; (n = x) refers to number of individuals for whom data were available, if < N.
§“Early” hepcidin calculated as the geometric mean of hepcidin measurements from any sample taken between day 0 and 60 postinfection.
¶Set-point hepcidin calculated as the geometric mean of hepcidin measurements from any sample taken between day 89 and 366 postinfection.

### Table S2. Study participants/subjects from Beijing and London early-to-chronic cohorts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acute-to-chronic pVL correlation study, London</th>
<th>Acute-to-chronic pVL correlation study, Beijing</th>
<th>Acute-to-chronic pVL correlation study, all</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>32</td>
<td>46</td>
</tr>
<tr>
<td>Sex, male, %</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Age at enrolment (95% CI), years</td>
<td>33.5 (28.5–38.6), n = 13</td>
<td>32.2 (28.7–35.7)</td>
<td>32.6 (29.8–35.4)</td>
</tr>
<tr>
<td>Set-point log plasma viral load (95% CI) copies per mL*</td>
<td>3.97 (3.33–4.61)</td>
<td>4.33 (4.08–4.59)</td>
<td>4.22 (3.97–4.48)</td>
</tr>
<tr>
<td>Number of samples contributing to set-point pVL calculation</td>
<td>3.2 (2.6–3.8)</td>
<td>3.8 (3.5–4.2)</td>
<td>3.6 (3.3–3.9)</td>
</tr>
<tr>
<td>“Early” hepcidin, day 0–60 (95% CI) ng/mL†</td>
<td>35.9 (17.5–73.8), n = 7</td>
<td>58.3 (43.7–77.7), n = 12</td>
<td>48.8 (36.2–65.8), n = 19</td>
</tr>
<tr>
<td>Set-point hepcidin, day 89–366 (95% CI) ng/mL‡</td>
<td>25.3 (15.4–41.6)</td>
<td>31.4 (24.1–40.9), n = 27</td>
<td>29.2 (23.1–36.8), n = 41</td>
</tr>
</tbody>
</table>

*Calculated as the mean of log_{10} plasma viral loads measured in samples taken between 3 and 12 mo postinfection (day 89–366).
†Geometric mean; (n = x) refers to number of individuals for whom data were available, if < N.
‡“Early” hepcidin calculated as the geometric mean of hepcidin measurements from any sample taken between day 0 and 60 postinfection.
§Set-point hepcidin calculated as the geometric mean of hepcidin measurements from any sample taken between day 89 and 366 postinfection.

### Table S3. Study participants/subjects involved in London cross-sectional study

<table>
<thead>
<tr>
<th>Group</th>
<th>Control, n = 20</th>
<th>Untreated HIV, n = 31</th>
<th>ART-treated HIV, n = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex male, %</td>
<td>100 (39.2–62.3)</td>
<td>100 (39.2–62.3)</td>
<td>100 (39.2–62.3)</td>
</tr>
<tr>
<td>Age, years (95% CI)</td>
<td>33.01 (29.82, 36.20)</td>
<td>36.85 (33.85, 39.86)</td>
<td>41.2 (37.64, 44.75)</td>
</tr>
<tr>
<td>Plasma viral load (95% CI) cells per mL*</td>
<td>NA</td>
<td>36,996 (16,544, 82,731), n = 29</td>
<td>50 (50)</td>
</tr>
<tr>
<td>CD4 count, (95% CI) cells per μL</td>
<td>ND</td>
<td>528 (428, 628), n = 26</td>
<td>582 (486, 678), n = 18</td>
</tr>
<tr>
<td>Hemoglobin, g/dL†</td>
<td>ND</td>
<td>14.3 (13.6, 15.0)</td>
<td>14.8 (14.4, 15.3)</td>
</tr>
<tr>
<td>Anemia (Hb &lt; 13.0 g/dL) % of group</td>
<td>ND</td>
<td>10.3, n = 29</td>
<td>0, n = 20</td>
</tr>
<tr>
<td>Time on ART, years (95% CI)</td>
<td>NA</td>
<td>1.83 (1.41, 2.24)</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not done. (n = x) refers to number of individuals for whom data were available. All data refer to the date on which the cross-sectional sample was taken.
*Geometric mean.
†Arithmetic mean.