HHS Public Access

Author manuscript

Genes Immun. Author manuscript; available in PMC 2012 October 01.

Published in final edited form as:

Genes Immun. 2012 April ; 13(3): 258–267. doi:10.1038/gene.2011.76.

Variations in the heme oxygenase-1 microsatellite polymorphism are associated with plasma CD14 and viral load in HIV-infected African Americans

Lillian Seu[1,2], **Trevor D. Burt**[2,3], **John S. Witte**[4,5], **Jeffrey N. Martin**[5], **Steven G. Deeks**[6], and **Joseph M. McCune**[2]

[1]Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, CA 94110

[2]Division of Experimental Medicine, Department of Medicine, University of California, San Francisco, CA 94110

[3]Division of Neonatology, Department of Pediatrics, University of California, San Francisco, CA 94143

[4]Institute for Human Genetics, University of California, San Francisco, CA 94110

[5]Department of Epidemiology and Biostatistics, University of California, San Francisco, CA 94110

[6]Positive Health Program, Department of Medicine, University of California, San Francisco, CA 94110

Abstract

Heme oxygenase-1 (HO-1) is an anti-inflammatory enzyme that maintains homeostasis during cellular stress. Given previous findings that shorter length variants of a HO-1 promoter-region GT_n microsatellite polymorphism are associated with increased HO-1 expression in cell lines, we hypothesized that shorter variants would also be associated with increased levels of HO-1 expression, less inflammation, and lower levels of inflammation-associated viral replication in HIV-infected subjects. Healthy donors (n=20) with shorter GT_n repeats had higher HO-1 mRNA transcript in peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS) (r= −0.38, p=0.05). The presence of fewer GT_n repeats in subjects with untreated HIV disease was associated with higher HO-1 mRNA levels in peripheral blood (r= −0.41, p=0.02); similar observations were made in $CD14^+$ monocytes from antiretroviral-treated subjects (r= −0.36, p=0.04). In African-Americans, but not Caucasians, greater GT_n repeats were correlated with higher soluble CD14 (sCD14) levels during highly active antiretroviral therapy (HAART) (r= 0.38, p=0.007) as well as higher mean viral load off-therapy (r= 0.24, p=0.04). These data demonstrate that the HO-1 GT_n microsatellite polymorphism is associated with higher levels of

Conflict of Interest

The authors report no conflict of interest

Supplementary information is available at the Genes and Immunity website

HO-1 expression and that this pathway may have important effects on the association between inflammation and HIV replication.

Keywords

Heme oxygenase-1; microsatellite; polymorphism; HIV; soluble CD14; monocytes

INTRODUCTION

Although host immune activation is critical for the eradication of infectious agents, the inflammatory response can also enhance pathogenesis. An example of this delicate balance is revealed by human immunodeficiency virus (HIV) disease: progressive and pathogenic infection is associated with chronic immune activation, including heightened destruction and diminished production of T lymphocytes (1), an increased frequency of T cells with an activated phenotype (2), increased T cell induction of the pro-inflammatory transcription factors, NFκB and NF-AT (3), and increased serum levels of pro-inflammatory cytokines (e.g., TNFα, IL-1 and IL-6) (4,5).

The impact of chronic inflammation on HIV (and simian immunodeficiency virus; SIV) disease progression has been well studied in both humans and non-human primates. Experiments in non-human primate models of acute SIV infection have demonstrated that increased bystander immune activation heightens viral cellular transmission by increasing the T cell target to T cell effector ratio (6). T lymphocyte activation as measured by cell surface markers has also been associated with viral load “set-point” in untreated HIV disease (7) and is associated with disease progression in both untreated and treated HIV infection (8,9).

Multiple mechanisms likely contribute to HIV-associated inflammation, including the direct effects of the virus. Recent studies have shown that circulating lipopolysaccharide (LPS) resulting from microbial translocation from the gut is a cause of systemic immune activation in chronic HIV infection (10), and is present even in HAART-suppressed subjects with undetectable viremia (11). An extension of this finding was the discovery that soluble CD14, the portion of the LPS-receptor that gets cleaved upon binding to its ligand, is elevated in HIV disease and strongly associated with enhanced mortality in chronically HIV-infected subjects (12), and is a major force driving chronic inflammation in HAART-treated patients (13).

Given the impact of immune activation/inflammation on the pace of HIV disease progression, we hypothesized that host factors controlling inflammation might be protective. In this study, we focused on heme oxygenase-1 (HO-1), the rate-limiting enzyme that initiates heme degradation and maintains cellular homeostasis during stress through its depletion of pro-oxidant heme, through generation of cytoprotective carbon monoxide and biliverdin, and through induction of ferritin by Fe^{2+} release. HO-1 is induced by a myriad of stress signals including oxidative stress, ultraviolet (UVA) radiation, and the pro-inflammatory cytokines IL-1, IL-6, TNFα (14). Furthermore, we have recently demonstrated

that the HO-1 inhibitor, tin mesoporphyrin IX (SnMP), induces activation, proliferation, and maturation of naïve human T cells via interactions with $CD14^+$ monocytes (15).

Given the important role that HO-1 has as an anti-inflammatory mediator, numerous studies have linked proximal promoter HO-1 polymorphisms to disease states that are driven by inflammation (e.g., graft versus host disease, ischemic stroke, coronary artery disease, etc.) [reviewed in (16)]. The GT_n microsatellite polymorphism is the best-characterized HO-1 genetic locus to date. Previous studies using promoter-luciferase assays in cell lines have shown an association between GT_n microsatellites with a small number of repeats and enhanced transcription of the HO-1 gene (17–20). Likewise, a recent study using primary endothelial cells isolated from newborns showed that HO-1 induction was more robust in the context of fewer ($GT_{n<23}$) than greater ($GT_{\geq 29}$) repeats (21). Although many candidate gene studies have detected associations between the short HO-1 GT_n microsatellite variant and control of inflammatory disease states, no study has examined its functional role in regulating gene expression in primary immune cells that directly influence pathogenic outcomes in HIV-infected subjects.

Here, we have studied the influence of the promoter GT_n microsatellite repeat number on HO-1 transcript expression in peripheral blood mononuclear cells (PBMCs) of healthy adults after *in vitro* stimulation and in PBMCs isolated from HIV-infected subjects on and off antiretroviral therapy. Next, we conducted a candidate genotyping study for two biomarkers of HIV disease progression: mean viral load and soluble CD14. Lastly, we determined the association between HO-1 expression and CD14 expression on primary blood $CD14^+$ monocytes from healthy donors stimulated *in vitro* with LPS. In aggregate, our results suggest that the HO-1 GT_n microsatellite polymorphism is a functional determinant of HO-1 expression in primary immune cells and is associated with important biomarkers of HIV disease outcome.

RESULTS

Heme oxygenase-1 (HO-1) promoter description across HIV-infected subjects of different ethnicities

The HO-1 promoter region analyzed extends from the nucleotide position 1876 base pairs upstream and +75 base pairs downstream of the transcription start site at position +1 (Fig. 1a). This promoter region contains the GT_n dinucleotide repeat and two common SNPs (-413AT/rs2071746 and -1195AG/rs3761439) that have been analyzed in previous studies of the HO-1 gene (19,22). Two common insertion/deletion variants (dbSNP ID rs72441698 and rs58433947) with GT_7 repeats may explain the large frequency of GT_{23} and GT_{30} variants seen across all populations of HIV-infected subjects (n=717, median age ± interquartile range = 46.2 ± 10.2, 85.5% male, 28.6% African American, 55.2% Caucasian) (Suppl. Fig. 1). The GT_n repeats in the African-Americans follow a tri-modal distribution in contrast to the bi-modal distribution seen in Caucasians (Box III in Fig. 1b). The addition of this GT_7 insertion results in greater repeat numbers in African Americans (mean 31.5 ± 0.27, n=205) than in Caucasians (mean 28.1 ± 0.14, n=396) ($p<0.001$) (Fig. 1b) and in other ethnic groups (Suppl. Fig. 1a). Allele frequencies for these SNPs were also calculated across these ethnic groups (Suppl. Fig. 1b).

Prior studies of the HO-1 promoter region examined the association between the -413AT/rs2071746 and the -1195AG/rs3761439 SNPs with coronary artery disease outcomes and cardiac function during exercise (19,22). These SNPs were not in significant linkage disequilibrium with the most common GT_{23} and GT_{30} repeats in both African American and Caucasian populations (Fig. 1c), with r^2 values below 0.4. These results suggest limited linkage disequilibrium present at this locus.

Heme oxygenase-1 additive GT_n repeats negatively correlate with gene expression in PBMCs and $CD14^+$ monocytes from healthy donors

To extend these studies to primary cells that might be involved in the immune response to HIV, fresh peripheral blood mononuclear cells (PBMCs) (n=20) or enriched $CD14^+$ monocytes from healthy donors (n=6) (subsets of donors from those listed in Table 1) were analyzed for HO-1 relative transcript expression after stimulation with the HO-1 chemical inducers cobalt protoporphyrin IX (CoPP), hydrogen peroxide (H_2O_2), and lipopolysaccharide (LPS). Compared to the phosphate buffered saline (PBS) control, stimulation with CoPP led to a 46-fold increase in relative transcript abundance of HO-1 ($p<0.0001$, paired t-test) whereas H_2O_2 stimulation led to a 1.3-fold increase ($p=0.005$) and LPS to a 1.3-fold decrease ($p=0.007$) (Fig. 2a). Since prior studies have reported an important role of HO-1 in the function of $CD14^+$ monocytes (15,23), enriched $CD14^+$ monocytes were analyzed after *in vitro* stimulation with CoPP, H_2O_2, or LPS, which led to a 30-fold increase ($p<0.0001$), a 1.2-fold increase ($p=0.02$), and a 1.4-fold decrease ($p=0.02$), respectively (paired t-test, Fig. 2b).

Using an HO-1 promoter-reporter gene assay in HEK293T cells, the additive sum of an individual's GT_n repeat number ($GT_n^{\text{locus 1}} + GT_n^{\text{locus 2}}$) was found to be an informative variable for subsequent functional analyses of this polymorphism (i.e., the greater the repeat number the lower the level of HO-1 expression) (Suppl. Fig. 2). Healthy donors were genotyped for the HO-1 GT_n polymorphism and transcript levels in PBMCs were measured. Multiple regression analysis (controlling for self-identified ethnicity) of normalized HO-1 transcript level and additive GT_n repeats showed statistically significant negative correlations in PBMCs from healthy donors stimulated with LPS ($r= -0.38$,$\beta= -0.024$, $p=0.05$) (Fig. 2c). The same analyses in PBMCs stimulated with CoPP ($r= -0.30$, $\beta= -0.98$, $p=0.06$) and H_2O_2 ($r= -0.36$, $\beta= -0.076$, $p=0.08$) showed trends towards negative correlations (Fig. 2c). Consistent with previous findings, the HO-1 transcript levels in unstimulated PBMCs did not show a correlation with an individual's additive GT_n repeat number (20) (Suppl. Fig. 3). These results are consistent with our gene expression results in the HEK293T cell lines as well as prior reports showing that greater GT_n repeats lead to decreased gene expression (17–19).

Finally, given prior evidence that the -413AT/rs2071746 may be associated with HO-1 expression in cell lines (19), we tested whether this SNP was associated with HO-1 induction in primary PBMCs exposed to CoPP, LPS, or H_2O_2 (Suppl. Fig. 4a–c, respectively). ANOVA analysis showed no significant association between HO-1 gene expression with this SNP or another common SNP (-1195AG/rs3761439). In summary,

these results show that the GT_n microsatellite polymorphism is associated with the regulation of HO-1 gene transcription in primary immune cells.

Heme oxygenase-1 additive GT_n repeats negatively correlate with gene expression in PBMCs and $CD14^+$ monocytes from HIV-infected subjects

Prior studies have shown that HIV infection leads to up-regulation of HO-1 mRNA levels in peripheral blood cells (24). To determine whether HIV-infected subjects with a greater number of additive GT_n repeats displayed lower HO-1 expression, we harvested thawed PBMCs and measured relative HO-1 transcript abundance. Multiple regression analysis with ethnicity as a covariate was performed to determine the relationship between the number of additive GT_n repeats and HO-1 relative transcript levels in "viral non-controllers" (prototypic HIV-infected adults with plasma HIV RNA levels above 10 000 copies RNA/mL, n=34) (Table 2a) and "viral controllers" (HIV-infected adults able to fully or partially control HIV replication with plasma HIV RNA level below 1 000 copies RNA/mL in the absence of therapy, n=30) (Table 2a). Among non-controllers, there was a significant correlation between additive GT_n repeats and the HO-1 relative transcript level (Fig. 3a) ($r= -0.41$, $\beta= -3.08$, $p=0.02$). There was no association between these factors in the controllers ($r=0.03$, $\beta= -0.36$, $p=0.4$).

Further phenotyping analyses in PBMCs from HIV-infected subjects on highly active antiretroviral therapy (HAART) showed that HO-1 was expressed to the highest extent in $CD14^+$ monocytes. Among individuals with a favorable response to HAART (as defined by having a plasma viral loads with <75 copies RNA/mL) (Table 2b), there was a negative correlation in $CD14^+$ monocytes ($r= -0.36$, $\beta= -30.45$, $p=0.04$) (Fig. 3b). These results suggest that the GT_n microsatellite polymorphism is an important correlate of HO-1 expression within peripheral immune cells during the course of HIV disease, especially in those subjects with progressive disease and high viremia.

HO-1 additive GT_n repeats are correlated with levels of soluble CD14 and viremia in African American HIV-infected subjects

Given recent studies linking high levels of soluble CD14 (sCD14) with enhanced mortality in chronically HIV-infected subjects on HAART (12) and the functional role that HO-1 plays in $CD14^+$ monocyte biology, we measured levels of plasma sCD14 levels in a larger cohort of African American and Caucasian HIV-infected, HAART-suppressed subjects. Clinical and laboratory data describing these subjects is provided in Table 3a.

Greater HO-1 additive GT_n repeats correlated with higher levels of sCD14 in African American HAART subjects (n=50, $r= 0.38$, $p=0.007$) (Table 3a) (Fig. 4a). Furthermore, each unit increase in additive GT_n repeats correlated with a sCD14 level increase of $2.3 \pm 0.8 \times 10^4$ pg/mL (regression coefficient $\beta= 2.3$). Greater additive GT_n repeats also correlated with higher mean plasma HIV RNA levels in African American subjects that were during the chronic phase of infection (n=74, $r=0.24$, $p=0.04$) (Fig. 4b). Furthermore, each unit increase in additive GT_n repeats correlated with a mean viral load increase of $3\,805.13 \pm 1\,803.7$ HIV RNA copies/mL (regression coefficient $\beta= 3\,805.13$). Neither the soluble CD14 levels during suppressive HAART (n=123, $r= 0.0016$, $\beta=0.80$, $p=1.0$) nor the mean viral

load level in the absence of therapy (n=177, r= −0.02, β=3 119.26, p=0.2) were statistically different in Caucasian subjects with greater HO-1 additive GT_n repeat numbers.

In summary, these data suggest that the genetically determined level of HO-1 expression in $CD14^+$ monocytes may play a role in HIV disease outcome in African-Americans.

Higher levels of HO-1 expression in primary human $CD14^{hi}$ monocytes correlate with decreased loss of cell surface CD14 after LPS stimulation

CD14 is expressed on the cell surface of monocytes and is the receptor for LPS, a cell wall component of gram-negative bacteria. Upon binding to LPS, a cleaved form of CD14 is shed into the circulation (25,26). Given the finding that greater numbers of HO-1 additive GT_n repeats are positively correlated with higher levels of sCD14 (Fig. 4a), we tested the possibility that HO-1 expression in CD14+ monocytes predicts their ability to retain CD14 cell surface expression upon LPS stimulation. HO-1 protein levels were quantified in PBMCs from 22 healthy donors (Table 1) by multiparameter flow cytometry analysis (Fig. 5a). As previously demonstrated (15,23), HO-1 was most highly expressed at baseline in PBMCs within the $CD14^+$ blood monocyte population. Upon exposure to CoPP, HO-1 was up-regulated to a greater extent within monocytes that are $CD14^{hi}$ than in “non-classical” $CD14^{dim}CD16^+$ monocytes (55 226 vs. 13 448 in geometric mean fluorescence intensity (gMFI); $p<1\times10^{-8}$). By contrast, $CD3^+$ T cells displayed very low HO-1 expression, even after induction with 25 uM CoPP (Fig. 5a).

We measured HO-1 expression after LPS stimulation and saw a significant decrease in expression of HO-1 gMFI within $CD14^{hi}$ monocytes (paired t-test, p=0.04) (Fig. 5b). Since HO-1 is generally considered to be anti-inflammatory, we hypothesized that induction of HO-1 in $CD14^{hi}$ monocytes after LPS stimulation might positively correlate with the ability of these cells to retain cell surface CD14. To test this hypothesis, the relationship between the change in staining intensities for HO-1 and for CD14 (normalized ΔCD14 gMFI and ΔHO1 gMFI) were determined in $CD14^{hi}$ blood monocytes of healthy donors upon *in vitro* LPS stimulation (Fig. 5c). Multiple regression analysis (covariates included ethnicity, age, and gender) showed an association between the expression of HO-1 and retention of cell surface CD14 in LPS-stimulated $CD14^{hi}$ monocytes. (n=22, r=0.54, β=0.60, p=0.05). Notably, $CD14^{hi}$ monocytes from individuals with very high GT repeat numbers (>60) generally had less induction of HO-1 and greater loss of CD14 when stimulated with LPS (boxed data points, Fig. 5c).

These results suggest that the ability to induce HO-1 in $CD14^{hi}$ monocytes upon exposure to LPS is protective against loss of CD14 from the cell-surface, which itself is a favorable predictor of outcome in HIV disease.

Discussion

Since high levels of immune activation and inflammation predict a more rapid pace of HIV disease progression (7,9,12), host immunoregulatory factors that blunt immune activation and inflammation may contribute to delayed disease progression. Amongst such factors, HO-1 is an important anti-inflammatory enzyme that has been implicated in multiple

disorders in the past. In these studies, we have performed an assessment of HO-1 promoter polymorphism genetics, HO-1 expression, and two biomarkers associated with HIV disease outcome in a large cohort of well-characterized HIV-infected and -uninfected adults. Our data show that HO-1 GT_n microsatellite genotypes predict HO-1 expression in primary cells, and that there is a consistent association between HO-1 promoter genotype and HO-1 expression. We also show that greater additive GT_n repeats correlate with higher viral loads and higher levels of the inflammatory sCD14 biomarker in HIV-infected African Americans. Finally, we show that higher levels of HO-1 induction in monocytes correlate with retention of CD14 on the cell surface after LPS stimulation. In light of these data, we speculate that induction of the anti-inflammatory enzyme HO-1 may play a role in limiting the injurious effects of immune activation in chronic HIV disease, e.g., that caused upon microbial translocation and circulation of LPS (10,12). This is in line with evidence that HO-1 induces tolerogenic properties in monocyte-derived antigen presenting cells (15,23). By influencing monocyte maturation and activation, HO-1 may accordingly represent an anti-inflammatory control mechanism for monocyte-mediated inflammation induced by LPS.

It has long been recognized that individuals with HIV have a higher proportion of circulating $CD14^{dim}CD16^{+}$ inflammatory monocytes than do uninfected controls (27–29). Reciprocally, we have observed that initiation of suppressive HAART therapy in chronically HIV-infected subjects is associated with a decrease in the percentage of circulating $CD14^{dim}CD16^{+}$ inflammatory monocytes and an increase in the percentage of $CD14^{hi}$ ‘conventional’ monocytes (Suppl. Fig. 6). Shedding of CD14 from the surface of non-inflammatory $CD14^{hi}$ monocytes upon exposure to an activating stimulus (e.g., LPS) may represent an important part of their transition into pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes, and further studies exploring this possibility are underway.

We also demonstrate that higher copy numbers within the HO-1 GT_n repeat promoter polymorphism correlate with higher mean viral load during chronic HIV infection in African American subjects, suggesting that HO-1 activity can modulate viral load. For this analysis, we focused on individuals with high mean viral loads due to the fact that HO-1 expression is not induced to as high an extent in individuals that are able to control viral loads (Figs. 3a and 3b). HO-1 induction may limit bystander immune activation during the acute stage of HIV infection, and thereby reduce the harmful T cell target to T cell effector ratio that leads to heightened viral transmission leading into the chronic stage of infection (6).

Our data show that having fewer GT_n repeats within the microsatellite polymorphism of the HO-1 promoter region is associated with higher levels of HO-1 gene expression in primary immune cells. The alternating purine-pyrimidine repeats within the GT_n microsatellite form a left-handed helical Z-DNA conformation and may affect gene transcription through the inhibition of RNA polymerase-mediated transcription (30). The HO-1 GT_n microsatellite locus is unique in being able to influence gene transcription under multiple cellular stress conditions. Our study shows that additive GT_n repeats influence HO-1 expression upon CoPP, H_2O_2, and LPS stimulation, confirming and extending the findings of a recent study analyzing this polymorphism in primary human newborn endothelial cells (21). The central

mediator implicated in this pathway is the Nrf2 transcription factor during HO-1 induction with CoPP and H_2O_2 (31,32), and HO-1 down-regulation with LPS stimulation (33).

In this study, we report an association between greater additive GT_n repeats and plasma sCD14 in HAART-suppressed subjects and mean viral load in chronically HIV-infected, untreated African American subjects. Interestingly, this association was not observed in Caucasians. Though individuals of African ancestry have a greater average number of HO-1 GT_n repeats within this microsatellite polymorphism than do other ethnic groups (Suppl. Fig. 1), it does appear that the positive correlation between sCD14 remains consistent even in the range of 40–70 repeats, which overlaps with the number of repeats seen in Caucasians. We cannot rule out that there may be epistatic factors, whether environmental or genetic, that differentially influence HO-1 gene function in African Americans vs. Caucasians. For example, ethnicity-specific differences in the interaction of Nrf2 or Bach1 with the promoter region containing the GT_n satellite polymorphism may also influence HO-1 gene expression. Also, we cannot rule out the possibility that interactions with Toll-like receptors, such as TLR4, may influence the extent of CD14 expression on monocytes upon LPS stimulation (Figure 5c). To our knowledge, this type of regulation has not been explored in the past (25, 26), but future studies along these lines may reveal new insights about monocyte biology. To better understand the basis of the associations observed in African American subjects, it will be necessary to carry out larger cross-population comparisons of the HO-1 genetic locus as well as the up-stream mediators influencing its expression.

The prevalence of higher GT_n repeat numbers in individuals with African ancestry may reflect an interplay between HO-1 activity and the various hemolytic conditions that are prevalent in this population (e.g., about 75% of the global incidence of hemoglobinopathy is in sub-Saharan Africa) (34). Indeed, there is evidence showing positive selection for the genomic region specific to African ancestral populations, with this group showing a positive Cross Population Expression Haplotype Homozygosity (XP-EHH) coefficient of 1.8 as opposed to other populations showing negative XP-EHH coefficients (Human Genome Diversity Project: http://hgdp.uchicago.edu/) (Suppl. Fig. 5) (35).

The results of this study suggest that pharmacological augmentation of HO-1 may represent a strategy for the treatment of diseases such as HIV, in which unchecked immune activation results in deleterious clinical outcomes. Given the increased burden of HIV disease in African and African American subjects, such a strategy might prove to be particularly useful in these populations. Our study demonstrates that there are population-specific genetic variations of a GT_n dinucleotide polymorphism involved in the control of HO-1 gene expression in immune cells that have an important role in HIV disease outcome. A recent clinical study reported that the synthetic metalloporphyrin Hematin (Fe^{2+} protoporphyrin IX) (Panhematin ®, Lundbeck Inc.) can induce a 15-fold increase in HO-1 activity (36). Further investigation of the HO-1 GT_n microsatellite genetic locus may help us better understand the clinical pharmacogenetics of this type of intervention.

In summary, our data show that the HO-1 GT_n microsatellite promoter polymorphism predicts expression of HO-1 in primary immune cells that play an important role in host

interactions with HIV. We show that that greater additive GT_n repeats correlate with higher levels of the inflammatory sCD14 biomarker in African Americans on HAART and of the mean viral load in subjects off therapy. Finally, we show that the genetically-determined level of HO-1 expression in monocytes correlates with the extent of LPS-induced loss of cell-surface CD14 expression within healthy donors. This study highlights the potential role of the HO-1 GT_n genetic locus as a predictive biomarker for disease outcome in HIV-infected subjects on and off antiretroviral therapy.

Materials and Methods

Human subjects

Peripheral blood samples were collected from HIV-infected and -uninfected adults after written informed consent was obtained under protocols approved by the University of California at San Francisco Committee on Human Research (San Francisco, CA). Healthy adults (n=22) were recruited from the San Francisco Bay Area (Table 1). HIV-positive subjects were recruited from the San Francisco Bay Area into the UCSF-based Study of the Consequences of the Protease Inhibitor Era (SCOPE) Cohort, a clinic-based cohort of >1500 adults with chronic HIV infection (Table 2 and Table 3). Participants undergo clinical laboratory monitoring and have their biological specimens banked. HIV controllers are defined as HIV-infected subjects that are off antiretroviral therapy for at least the latest 12 month period for which follow-up is available, with at least three plasma HIV RNA levels taken during this time below 1 000 copies/mL. HIV viral non-controllers are defined as individuals with HIV RNA levels over 10 000 copies RNA/ml (by bDNA, Chiron Diagnostics, Emeryville, USA) or 20 000 copies RNA/mL (by Abbott RealTime HIV-1 PCR, Abbott Park, Illinois, USA) at some point in the past, regardless if during a period of treatment or no treatment.

Measurement of plasma CD14 in HAART-suppressed subjects and mean viral loads in HIV-infected patients off therapy

For sCD14 measurement in HAART-suppressed subjects (Table 3a), a commercially available enzyme-linked immunosorbent assay was used according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The mean viral loads measured in HIV-infected patients (Table 3b) were defined as the mean of all viral load determinations known to have been measured in the absence of therapy. All experiments were performed in a blinded manner without prior knowledge of an individual's genotype.

Primary cell isolation and culture conditions

PBMCs were isolated from whole blood drawn into sodium heparin tubes by density centrifugation using Histopaque®-1077 (Sigma Aldrich, Saint Louis, MO), and the MACS® Monocyte Isolation Kit II (Miltenyi, Auburn, CA) was used to enrich $CD14^+$ monocytes from PBMCs (mean 95% purity, as assessed by multiparameter flow cytometry). PBMCs or $CD14^+$ monocytes were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 1% penicillin/streptomycin (Mediatech, Washington, DC), and 2 mM L-glutamine (Mediatech) (hereafter referred to as R10 medium) on Upcell™ 96F MicroWell plates (Thermo Scientific,

Rochester, NY). Cobalt protoporphyrin IX (CoPP) was purchased in powdered form (Frontier Scientific, Park City, Utah), dissolved in 0.1 mM NaOH, and titrated to a pH of 7.6. Lipopolysaccharides (LPS) from Escherichia coli 055:B5 (Sigma Aldrich) were reconstituted in water to a final stock concentration of 1 mg/mL. 3% (w/w) hydrogen peroxide (H_2O_2) (Sigma Aldrich) was diluted in water for a final stock concentration of 2 mM. PBMCs or $CD14^+$ monocytes were incubated at a final concentration of 25 uM of CoPP, 100 ng/mL LPS, or 100 uM H_2O_2 in RPMI-10 at 37° for 48 hours. Adherent cells were detached from the Upcell™ plates by incubating the plates at 25°C for 20 minutes.

Cell preparation and antibody labeling

All cells were stained with a live/dead marker Amine-Aqua/Am-Cyan Live/Dead (Invitrogen) to exclude dead cells from analysis. The following fluorophore-conjugated monoclonal antibodies (mAbs) were used to detect cell surface markers: CD3-APC-Cy7 (SP34-2, BD Biosciences, Franklin Lakes, NJ), CD14-Qdot605 (Q10013, Invitrogen), and CD16-APC (3G8, Caltag-Invitrogen). The following antibodies were used for detection of intracellular antigens: HO-1 rabbit polyclonal (ab13243, unconjugated, Abcam, Cambridge, MA) and a secondary goat anti-rabbit IgG conjugate (554020, FITC, BD Biosciences). For flow cytometry analysis, cells were washed in staining buffer [PBS with 2% FBS and 2 mM ethylenediamine tetra-acetic acid – EDTA (Sigma)] and then incubated for 30 minutes at 4°C in the presence of directly-conjugated fluorescent mAbs. All cells were stained with a live/dead marker (Amine-Aqua/Am-Cyan Live/Dead; Invitrogen) so that dead cells could be excluded from analysis. The cells were washed in staining buffer, and then fixed and permeabilized in BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's protocol for intracellular staining. The cells were incubated for 1 hour at 4°C in the presence of a rabbit polyclonal antibody specific for HO-1 (ab13243, unconjugated, Abcam) and a secondary goat anti-rabbit IgG conjugate (554020, FITC, BD Biosciences). The cells were washed in staining buffer and then fixed in 1% paraformaldehyde (PFA). Data were acquired with an LSR-II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR).

DNA isolation and genotyping

DNA was isolated from the cell pellets of both healthy donors and HIV-infected subjects using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. HO-1 microsatellite genotyping was performed by the UCSF Genomics Core Facility. Primers were designed with the Primer3 algorithm http://fokker.wi.mit.edu/primer3/input.htm. The primer set was "MS-Primer1" 5′-FAM-CCAGCTTTCTGGAACCTTCTG and "MS-Primer2" 5′-GAAACAAAGTCTGGCCATAGGA. The samples were amplified on a touchdown PCR protocol. The resulting products were run on the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) and analyzed with GeneMapper (Applied Biosystems). The TaqMan® SNP Genotyping Assay was used to discriminate the allelic composition for the HO-1 SNPs at positions -1195AG/rs3761439 and -413AT/rs2071746. Unlabeled forward and reverse flanking PCR primers (900 nM final concentration) and two allele-specific probes labeled with either VIC or FAM reporter dye (200 mM final concentration) were added to 20 ng DNA in a 20-ml reaction containing TaqMan Universal PCR Mix. The

sequence for the rs2071746 SNP probe was 5′-AGTTCCTGATGTTGCCCACCAGGCT[A/T]TTGCTCTGAGCAGCGCTGCCTCCCA (Assay ID: C__15869717_10, Applied Biosystems, Carlsbad, CA) and for the rs3761439 SNP probe was 5′-CATAGGGAGACCC[T/C]GTCT (Custom assay, Applied Biosystems). Samples were run on the StepOnePlus™ Real-Time PCR system, and the results were analyzed using Step One v2.0 (Applied Biosystems)

Quantitative PCR

For quantitative PCR analysis, cells were harvested and RNA was isolated using TRIzol ® Reagent (Invitrogen, Carlsbad, CA). Total cellular RNA (0.2 μg) was used for cDNA synthesis with Oligo-dT primers and reverse transcriptase from Omniscript (Qiagen). Relative expression levels of HO-1 mRNA were measured by quantitative RT-PCR using validated Taqman® Gene Expression assay mixes for HO-1 (Assay ID: Hs00157965_m1, Applied Biosystems) and the reference gene, human hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Assay ID: Hs99999909_m1, Applied Biosystems), according to the manufacturer's protocol. The StepOnePlus™ Real-Time PCR system (Applied Biosystems) was used for amplification and detection, and the efficiency corrected calculation ($2^{-\Delta CT}$) of the threshold cycle C(t) was used to measure HO-1 gene expression relative to the HPRT gene.

Transient transfection assay for Dual-glo gene expression assay

Transient co-transfection with the HO-1-luciferase fusion plasmid and the pRL-ef1alpha plasmid (Promega, Madison, WI, USA) was performed using the calcium phosphate method. For transfection, HEK293T cells were split 24 h before transfection and seeded in Dulbecco's Modified Eagle Medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 1% penicillin/streptomycin (Mediatech, Washington, DC), and 2 mM L-glutamine (Mediatech). The 10x co-transfection mix was made up with 1 ug of HMOX1-pGL4.20 and 0.1 ug of pRL-ef1alpha plasmid DNA in 200 uM $CaCl_2$ in Hepes buffered saline and added dropwise to the wells. Cells were harvested at 48 hours, and the transfection efficiency and cell viability was analyzed by flow cytometric analysis of %GFP+ cells (mean 80% efficiency) Luciferase activity was analyzed using the Dual-Glo ® luciferase reporter assay system (Promega, Madison, WI, USA). Renilla luciferase activity in the lysates was used to normalize the activity of HMOX1 promoter driven firefly luciferase activity on a Spectramax M2 luminometer. (Molecular Devices, Sunnyvale, CA)

Statistical and genetic analyses

Linkage disequilibrium analysis of the additive GT_n repeats and the SNPs -1195A/G (rs3761439) and -413A/T (rs2071746) was carried out using the "pwld" function (http://www.gene.cimr.cam.ac.uk/clayton). HO-1 GT_n repeat differences between African Americans and Caucasians (Fig. 1b), HO-1 relative qPCR levels before and after each stimulation condition using paired Student's T-test analysis (Fig. 2ab), and HO-1 gMFI levels in $CD14^{hi}$ monocytes before and after each stimulation condition using paired Student's T-test analysis (Fig. 5b) were all performed using GraphPad Prism v5.0d (Graphpad Software, La Jolla, CA, USA). We performed multiple linear regression of

healthy donor HO-1 additive GT_n repeats and the HO-1 relative qPCR levels upon different stimulation conditions (Fig. 2c) of HIV-infected subject additive GT_n repeats and the HO-1 relative qPCR levels (Figs. 3a and 3b), for additive GT_n repeats compared to mean viral load and post-HAART soluble CD14, and for comparing the normalized delta CD14 gMFI to normalized delta HO-1 gMFI in $CD14^{hi}$ monocytes (Fig. 5c). For all correlation analyses, the Pearson rcorrelation and its p values are reported using GraphPad Prism v5.0d (Graphpad Software, La Jolla, CA, USA). For all multiple regression analyses, the regression coefficient β was calculated and adjusted for ethnicity, age, and gender when noted. All regression analyses were undertaken using STATA v11.2 (StataCorp LP, College Station, TX).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank all participants from the San Francisco General Hospital and San Francisco Veteran Affairs Medical centers. We would like to thank Dr. David Williamson for his help in setting up the HO-1 SNP assay as well as curation of DNA samples. L.S. was supported by a pre-doctoral dissertation California HIV Research Program grant (#D09-SF-313). During the period in which this work was carried out, T.D.B. was a fellow of the Pediatric Scientist Development Program and was supported by the American Pediatric Society, the American Academy of Pediatrics, and the March of Dimes. J.M.M. was supported by NIH Awards U01 AI43641 and R37 AI40312, and is the recipient of the National Institutes of Health Director's Pioneer Award, part of the National Institutes of Health Roadmap for Medical Research, through Grant DPI OD00329. The SCOPE cohort was supported by the Centers for AIDS Research at UCSF (PO AI27763), CFAR Network of Integrated Systems (R24 AI067039), the UCSF CTSI (UL1 RR024131), NIAID (RO1 AI087145, K24AI069994, AI-76174, amfAR, and the Ragon Institute. The authors would also like to thank Christopher R. Gignoux for critical discussion during the preparation of the manuscript.

References

1. Hellerstein M, Hanley MB, Cesar D, Siler S, Papageorgopoulos C, Wieder E, et al. Directly measured kinetics of circulating T lymphocytes in normal and hiv-1-infected humans. Nat Med. 1999 Jan; 5(1):83–9. [PubMed: 9883844]
2. Hazenberg MD, Stuart JW, Otto SA, Borleffs JC, Boucher CA, de Boer RJ, et al. T-Cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: A longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). Blood. 2000 Jan 1; 95(1):249–55. [PubMed: 10607709]
3. Kinoshita S, Su L, Amano M, Timmerman LA, Kaneshima H, Nolan GP. The T cell activation factor nf-atc positively regulates HIV-1 replication and gene expression in T cells. Immunity. 1997; 6(3):235–44. [PubMed: 9075924]
4. Matsuyama T, Kobayashi N, Yamamoto N. Cytokines and HIV infection: Is AIDS a tumor necrosis factor disease? AIDS. 1991; 5(12):1405. [PubMed: 1814326]
5. Poli G, FAUCI AS. The effect of cytokines and pharmacologic agents on chronic HIV infection. AIDS Res Hum Retroviruses. 1992; 8(2):191–7. [PubMed: 1540407]
6. Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. Annu Rev Med. 2010 Jan 27.
7. Deeks SG, Kitchen CM, Liu L, Guo H, Gascon R, Narváez AB, et al. Immune activation set point during early HIV infection predicts subsequent CD4+ t-cell changes independent of viral load. Blood. 2004 Aug 15; 104(4):942–7. [PubMed: 15117761]
8. Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, Deeks SG. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with

sustained viral suppression during antiretroviral therapy. J Infect Dis. 2003 May 15; 187(10):1534–43. [PubMed: 12721933]

9. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. J Infect Dis. 1999; 179:859–70. [PubMed: 10068581]
10. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006 Dec; 12(12):1365–71. [PubMed: 17115046]
11. Baroncelli S, Galluzzo CM, Pirillo MF, Mancini MG, Weimer LE, Andreotti M, et al. Microbial translocation is associated with residual viral replication in haart-treated HIV+ subjects with <50copies/ml HIV-1 RNA. J Clin Virol. 2009 Dec; 46(4):367–70. [PubMed: 19782638]
12. Sandler NG, Wand H, Roque A, Law M, Nason MC, Nixon DE, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. J Infect Dis. 2011 Jan 20.
13. Cassol E, Malfeld S, Mahasha P, van der Merwe S, Cassol S, Seebregts C, et al. Persistent microbial translocation and immune activation in hiv-1-infected south africans receiving combination antiretroviral therapy. J Infect Dis. 2010 Sep 1; 202(5):723–33. [PubMed: 20629534]
14. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: From basic science to therapeutic applications. Physiol Rev. 2006 Apr; 86(2):583–650. [PubMed: 16601269]
15. Burt TD, Seu L, Mold JE, Kappas A, McCune JM. Naive human T cells are activated and proliferate in response to the heme oxygenase-1 inhibitor tin mesoporphyrin. J Immunol. 2010 Nov 1; 185(9):5279–88. [PubMed: 20921523]
16. Exner M, Minar E, Wagner O, Schillinger M. The role of heme oxygenase-1 promoter polymorphisms in human disease. Free Radic Biol Med. 2004 Oct 15; 37(8):1097–104. [PubMed: 15451051]
17. Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet. 2000 Jan; 66(1):187–95. [PubMed: 10631150]
18. Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, et al. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet. 2002 Jul; 111(1):1–8. [PubMed: 12136229]
19. Ono K, Mannami T, Iwai N. Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women. J Hypertens. 2003 Aug; 21(8):1497–503. [PubMed: 12872043]
20. Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, et al. Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. Blood. 2003 Sep 1; 102(5):1619–21. [PubMed: 12730098]
21. Taha H, Skrzypek K, Guevara I, Nigisch A, Mustafa S, Grochot-Przeczek A, et al. Role of heme oxygenase-1 in human endothelial cells: Lesson from the promoter allelic variants. Arterioscler Thromb Vasc Biol. 2010 Aug; 30(8):1634–41. [PubMed: 20508205]
22. He Z, Hu Y, Feng L, Lu Y, Bao D, Xi Y, et al. Association between HMOX-1 genotype and cardiac function during exercise. Appl Physiol Nutr Metab. 2008 Jun; 33(3):450–60. [PubMed: 18461097]
23. Chauveau C, Rémy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood. 2005 Sep 1; 106(5):1694–702. [PubMed: 15920011]
24. Levere RD, Staudinger R, Loewy G, Kappas A, Shibahara S, Abraham NG. Elevated levels of heme oxygenase-1 activity and mrna in peripheral blood adherent cells of acquired immunodeficiency syndrome patients. Am J Hematol. 1993 May; 43(1):19–23. [PubMed: 8317458]
25. Hiki N, Berger D, Prigl C, Boelke E, Wiedeck H, Seidelmann M, et al. Endotoxin binding and elimination by monocytes: Secretion of soluble CD14 represents an inducible mechanism counteracting reduced expression of membrane CD14 in patients with sepsis and in a patient with

paroxysmal nocturnal hemoglobinuria. Infect Immun. 1998 Mar; 66(3):1135–41. [PubMed: 9488406]

26. Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol. 1991 Sep 1; 147(5):1567–74. [PubMed: 1880416]
27. Locher C, Vanham G, Kestens L, Kruger M, Ceuppens JL, Vingerhoets J, Gigase P. Expression patterns of fc gamma receptors, HLA-DR and selected adhesion molecules on monocytes from normal and hiv-infected individuals. Clin Exp Immunol. 1994 Oct.98(1):115. [PubMed: 7923868]
28. Thieblemont N, Weiss L, Sadeghi HM, Estcourt C, Haeffner-Cavaillon N. Cd14Lowcd16High: A cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. Eur J Immunol. 1995 Dec; 25(12):3418–24. [PubMed: 8566032]
29. Pulliam L, Gascon R, Stubblebine M, McGuire D, McGrath MS. Unique monocyte subset in patients with AIDS dementia. Lancet. 1997 Mar 8; 349(9053):692–5. [PubMed: 9078201]
30. Herbert A, Rich A. The biology of left-handed Z-DNA. J Biol Chem. 1996; 271(20):11595. [PubMed: 8662853]
31. Ashino T, Yamanaka R, Yamamoto M, Shimokawa H, Sekikawa K, Iwakura Y, et al. Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages. Mol Immunol. 2008 Apr; 45(7):2106–15. [PubMed: 18022235]
32. Brunt KR, Fenrich KK, Kiani G, Tse MY, Pang SC, Ward CA, Melo LG. Protection of human vascular smooth muscle cells from h2o2-induced apoptosis through functional codependence between HO-1 and AKT. Arterioscler Thromb Vasc Biol. 2006 Sep.26(9):2027. [PubMed: 16840713]
33. Miyazaki T, Kirino Y, Takeno M, Samukawa S, Hama M, Tanaka M, et al. Expression of heme oxygenase-1 in human leukemic cells and its regulation by transcriptional repressor bach1. Cancer Sci. 2010 Jun; 101(6):1409–16. [PubMed: 20345481]
34. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: An increasing global health problem. Bull World Health Organ. 2001; 79(8):704–12. [PubMed: 11545326]
35. Sabeti PC, Varilly P, Fry B, Lohmueller J, Hostetter E, Cotsapas C, et al. Genome-Wide detection and characterization of positive selection in human populations. Nature. 2007 Oct 18; 449(7164): 913–8. [PubMed: 17943131]
36. Bharucha AE, Kulkarni A, Choi KM, Camilleri M, Lempke M, Brunn GJ, et al. First-InHuman study demonstrating pharmacological activation of heme oxygenase-1 in humans. Clin Pharmacol Ther. 2010 Feb; 87(2):187–90. [PubMed: 19956091]

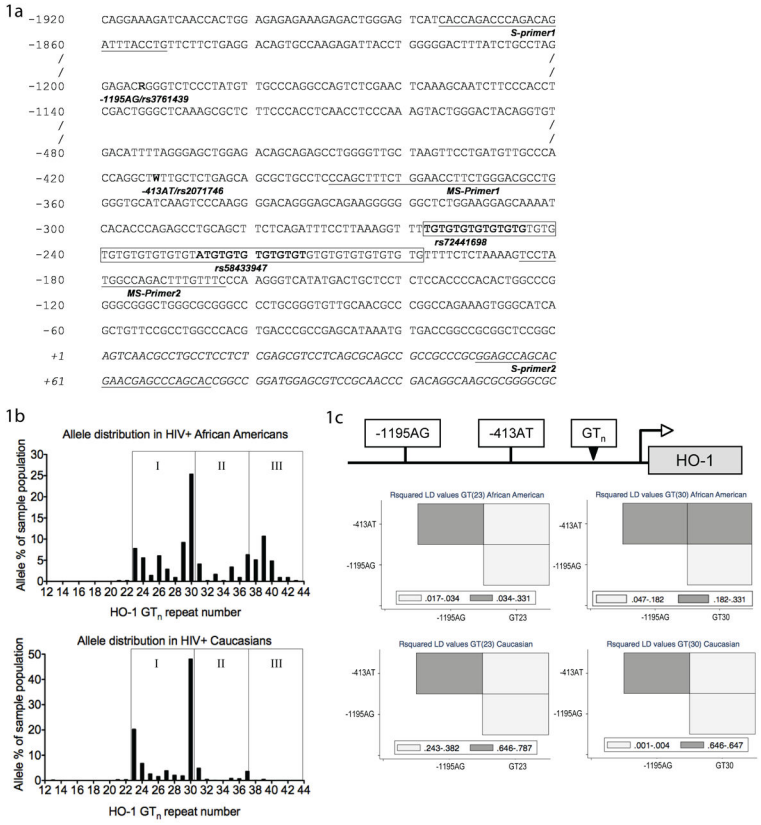

Figure 1. Heme oxygenase-1 (HO-1) promoter description and variation across HIV-infected subjects of different ethnicities

(a) HO-1 promoter region (−1876 to +75) encompassing subcloning region, GT_n repeat, sequencing primers, and transcription start site (TSS) at position +1. The GT_n dinucleotide repeat is shown in the boxed area (extending from 260 base pairs to 200 base pairs upstream of the TSS) and two common SNPs (-413AT/rs2071746 and -1195AG/rs3761439) are shown. Two common insertion deletion variations (dbSNP ID rs72441698 and rs58433947) with GT_7 repeats are shown in bold font within the GT_n repeat. Microsatellite sequencing primers for capillary electrophoresis are denoted by “MS-Primer1” and “MS-Primer2.” Subcloning primers for the promoter-reporter gene expression assay are denoted by “S-primer1” and “S-primer2.” **(b)** GT_n allele frequencies within HIV-infected patients reported as (mean ± s.e.m.): African Americans (31.5 ± 0.27, n=205) and Caucasians (28.1 ± 0.13, n=396) (difference in mean length between ethnic groups, $p<0.0001$, Student’s unpaired T-test). Boxes I, II, and III represent the distribution of the additive GT_n repeats with peaks at GT_{23}, GT_{30}, and GT_{37}. **(c)** The GT_n microsatellite repeats that are most represented in all populations (n=23 and 30) are not in linkage disequilibrium (LD) with -413AT/rs2071746 and -1195AG/rs3761439 in either Caucasians (n=396) or African Americans (n=205). The top two heat maps represent the pair-wise LD results for African American patients with GT_{23} (left) and GT_{30} (right), with the values in the legend corresponding to the r^2 values for each pair-wise comparison between the SNPs -413AT/rs2071746, -1195AG/rs3761439, and the GT_n microsatellite repeat. The bottom two heat maps represent the same for Caucasian patients.

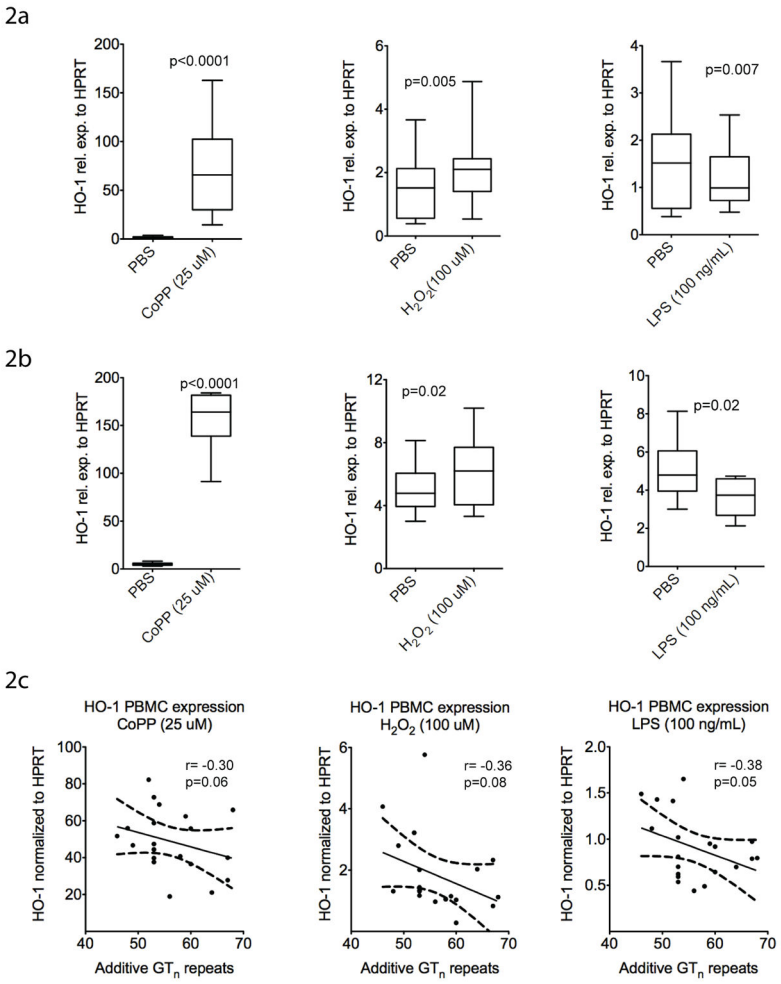

Figure 2. Heme oxygenase-1 additive GT_n repeats negatively correlate with relative gene expression in PBMCs and $CD14^+$ monocytes from healthy donors

Cells from healthy donors (n=20) were stimulated with CoPP (25 μM), H_2O_2 (100 uM), or LPS (100 ng/mL), and then harvested 48 hours later for HO-1 transcript analysis. Paired T-test analyses yield statistically significant increases in HO-1 relative transcript abundance normalized to HPRT in **(a)** PBMCs and **(b)** $CD14^+$ monocytes upon stimulation with CoPP or H_2O_2 and decreases upon stimulation with LPS. **(c)** PBMCs from healthy donors were stimulated with CoPP (25 uM), H_2O_2 (100 uM) or LPS (100 ng/mL) and genotyped for their HO-1 GT_n repeat polymorphism. Multiple regression and correlation analyses with ethnicity as a covariate showed a significant decline in normalized HO-1 transcript levels within LPS-stimulated cells as the additive GT_n repeats increased ($r= -0.38$, $\beta= -0.024$, $p=0.05$), and trends in PBMCs stimulated with CoPP ($r= -0.30$, $\beta= -0.98$, $p=0.06$) and H_2O_2 ($r= -0.36$, $\beta= -0.076$, $p=0.08$). The solid line represents a fitted linear regression line and dashed lines represent the 95% confidence interval band.

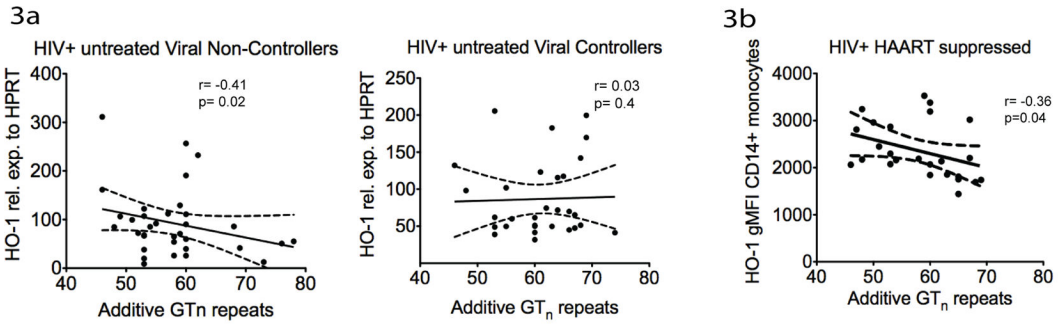

Figure 3. Heme oxygenase-1 additive GT_n repeats negatively correlate with gene expression in PBMCs and $CD14^+$ monocytes from HIV-infected subjects

PBMCs from HIV-infected subjects were analyzed for HO-1 transcript analysis. **(a)** Total mRNA was harvested from thawed PBMCs of HIV viral non-controllers and multiple regression analysis with ethnicity as a covariate yields a significant decline in normalized HO-1 transcript levels as the additive GT_n repeats increase (n=34, r= −0.41, β= −3.08, p=0.02). Total mRNA was harvested from thawed PBMCs of HIV viral controllers and multiple regression analysis with ethnicity as a covariate does not lead to a significant decline in normalized HO-1 transcript levels as the additive GT_n repeats increase (n=30, r=0.03, β= −0.36, p=0.4). **(b)** Thawed PBMCs from HAART-suppressed subjects were analyzed by multiparameter flow cytometry for HO-1 expression within $CD14^+$ monocytes. Multiple regression analysis with ethnicity as a covariate yields a significant decline in normalized HO-1 gMFI as the additive GT_n repeats increase within $CD14^+$ monocytes (n=25, r= −0.36, β= −30.45, p=0.04). The solid line represents a fitted linear regression line and dashed lines represent the 95% confidence interval band.

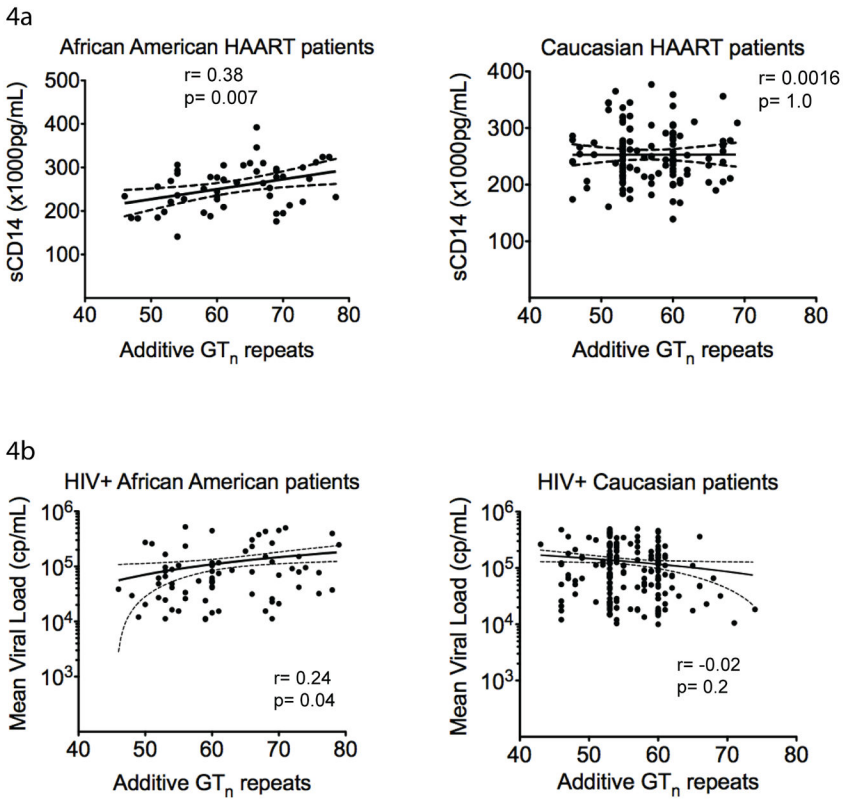

Figure 4. Increased levels of soluble CD14 during HAART and mean viral load are detected in African American HIV-infected subjects with higher HO-1 additive GT_n repeats

Two important clinical parameters associated with accelerated HIV disease (high levels of circulating soluble CD14 and a mean viral load) were measured. **(a)** Plasma soluble CD14 levels were measured by ELISA in African American and Caucasian HAART-suppressed HIV-infected subjects. Greater HO-1 additive GT_n repeats correlated with higher levels of sCD14 in African American (n=50, r= 0.38, β= 2.3, p=0.007) but not Caucasian HIV-infected HAART subjects (n=123, r= 0.0016, β=0.80, p=1.0); this correlation remained after adjusting for gender as a covariate. **(b)** Mean viral loads were measured in African American and Caucasian subjects and in the chronic stage of infection before they were placed on HAART. Greater HO-1 additive GT_n repeats correlated with higher mean viral loads in African American but not Caucasian HIV-infected HAART subjects (n=74, r=0.24, β= 3 805.13, p=0.04), and stayed significantly associated after adjusting for gender as a covariate. The mean viral load level in the absence of therapy was not statistically associated with the additive GT_n repeat numbers in Caucasian HIV subjects (n=177, r= −0.02, β=3 119.26, p=0.2). Mean viral loads are shown in logarithmic base 10 scale. The solid line represents a fitted linear regression line, and dashed lines represent the 95% confidence interval band.

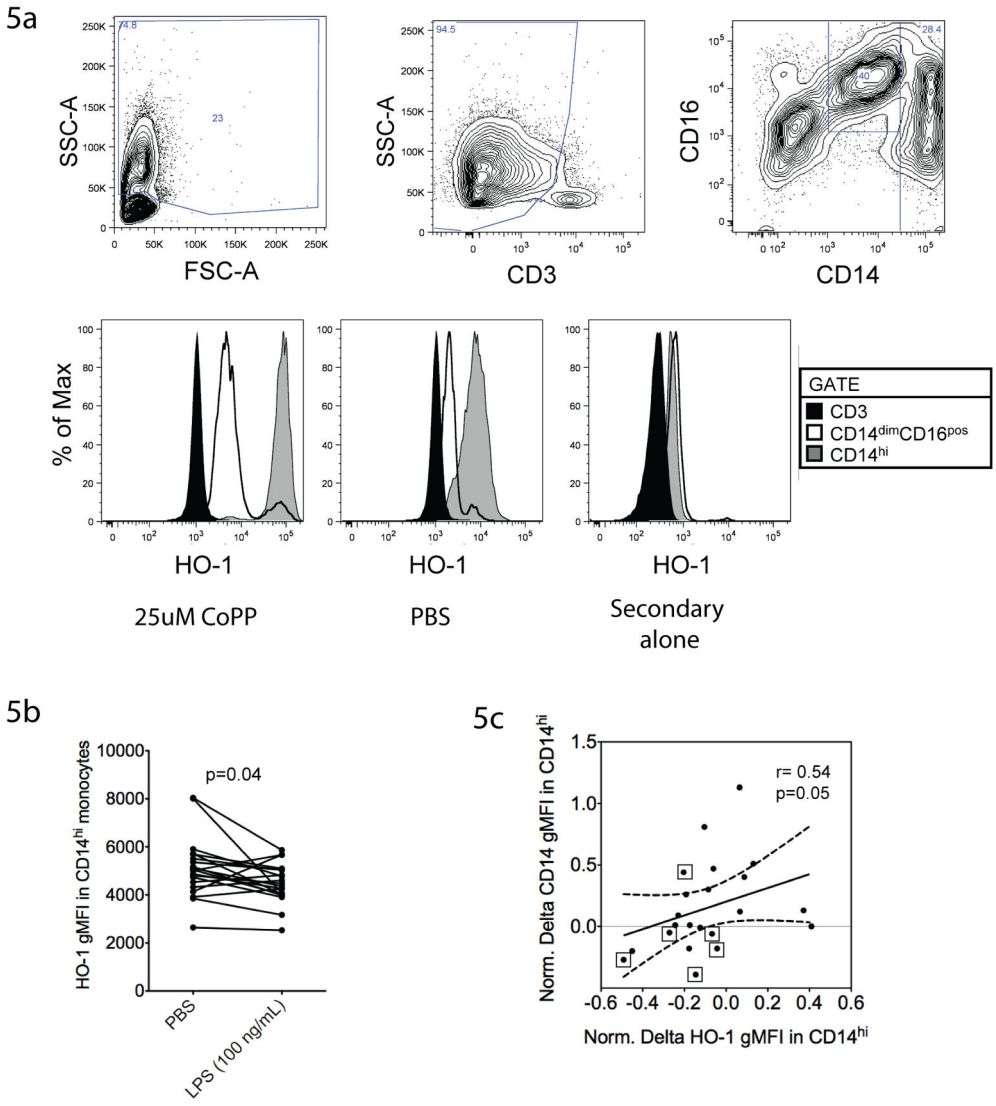

Figure 5. A higher level of HO-1 expression in primary human CD14^{hi} monocytes correlates with decreased loss of cell-surface CD14 after LPS stimulation

(a) Fresh PBMCs from healthy donors were stimulated with either 25 uM CoPP or PBS for 48 hours. Analysis was performed by sequentially gating on live cells, singlets (FSC-A/FSC-H), non-lymphocyte (SSC-A high/FSC-A high), and CD3^{-} populations. Monocyte populations were further gated on CD14 and CD16. HO-1 was induced to a greater extent within CD14^{hi} monocytes upon stimulation with 25uM CoPP as compared to CD14^{dim}CD16^{pos} monocytes (55 226 3 ± 916.9 vs. 13 448 ± 2 458.4 in gMFI; unpaired Student's T-test $p<1\times10^{-8}$). Histograms are shown depicting the intensity of HO-1 staining in CD3+ T cells, CD14^{dim}CD16^{+} monocytes, or CD14^{hi} monocytes (25 uM CoPP or PBS) as well as with the secondary antibody (goat anti-rabbit-FITC). **(b)** PBMCs were stimulated with either LPS (100 ng/mL) or PBS for 48 hours. HO-1 gMFI within CD14^{hi} monocytes is reduced upon LPS stimulation (5 068 ± 256.5 vs. 4 481 ± 196.2 gMFI, p=0.04) paired Student's T-test). **(c)** Multiple linear regression with ethnicity, age, and gender as covariates showed that normalized delta HO-1 gMFI in CD14^{hi} monocytes correlates with the

normalized delta CD14 gMFI upon LPS stimulation (n=22, r=0.54, β=0.60, p=0.05). The solid line represents a fitted linear regression line and dashed lines represent the 95% confidence interval band. Boxed data points refer to individuals with $GT_{>60}$ repeats.

Table 1

Healthy donors

Characteristics	Healthy donors for HO-1 phenotyping studies (n=22)
Age (median ± IQR)	33 ± 13
Gender (% Male)	45.4%
Ethnicity	
Caucasian	77.3%
African	4.5%
Asian	18.2%

Table 2a

Clinical characteristics of HIV-infected subjects off HAART

Characteristics (median ± IQR)	Viral controllers (n=30)	Viral non-controllers (n=34)
Age	45 ± 9	44 ± 8
Gender (% male)	68.0%	91.2%
Plasma viral load (copies/mL)	75 ± 98	84 142 ± 112 100
CD4 (cells/uL)	745 ± 433	317 ± 264
Ethnicity (%African American)	40%	29.4%
HLA-DR+CD38+% of CD4+ T cells	3.12 ± 1.5	11 ± 11.4
HLA-DR+CD38+% of CD8+ T cells	8.4 ± 10.8	23.5 ± 11.4

Table 2b

Clinical characteristics of HIV-infected subjects on HAART

Characteristics (median ± IQR)	HAART patients (n=25)
Age	45 ± 7
Gender (% male)	84.0%
Months on HAART	20.1 ± 6.7
Plasma viral load (copies/mL)	75 ± 25
CD4 (cells/uL)	300 ± 151
Ethnicity (% African American)	24.0%
HLA-DR+CD38+% of CD4+ T cells	9.7 ± 7.7
HLA-DR+CD38+% of CD8+ T cells	41.2 ± 26.4

Table 3a

Clinical characteristics of HIV-infected HAART suppressed subjects

Characteristics (median ± IQR)	African American (n=50)	Caucasian (n=123)
Age	44.0 ± 8.8	43.8 ± 10.0
Gender (% male)	62.0%	88.6%
Soluble CD14 (median ± IQR)	260 ± 72	249 ± 66
Months on HAART	21.2 ± 27.0	23.0 ± 32.8
Pre-HAART VL (copies/mL)	29 809 ± 64 571	64 649 ± 149 040
Post-HAART VL (copies/mL)	75 ± 28	75 ± 25
Pre-CD4 T cell count (cells/uL)	162 ± 182	192 ± 242
Post-CD4 T cell count (cells/uL)	310.5 ± 398	430 ± 374

Table 3b

Clinical characteristics of HIV-infected subjects off therapy

Characteristics (median ± IQR)	African American (n=74)	Caucasian (n=177)
Age	32.2 ± 9.0	46.8 ± 11.3
Gender (% male)	60.0%	89.8%
Mean VL (copies/mL)	63 935 ± 511 768	87 485 ± 161 380
CD4 T cell count (cells/uL)	330 ± 401.5	448 ± 344