Decreased HIV Type 1 Transcription in CCR5-Δ32 Heterozygotes During Suppressive Antiretroviral Therapy

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Individuals who are heterozygous for the CCR5-Δ32 mutation provide a natural model to examine the effects of reduced CCR5 expression on human immunodeficiency virus (HIV) persistence. We evaluated the HIV reservoir in 18 CCR5-Δ32 heterozygotes and 54 CCR5 wild-type individuals during suppressive antiretroviral therapy. Cell-associated HIV RNA levels (P = .035), RNA to DNA transcriptional ratios (P = .013), and frequency of detectable HIV 2–long terminal repeat circular DNA (P = .013) were significantly lower in CD4+ T cells from CCR5-Δ32 heterozygotes. Cell-associated HIV RNA was significantly correlated with CCR5 surface expression on CD4+ T cells (r² = 0.136; P = .002). Our findings suggest that curative strategies should further explore manipulation of CCR5.

Keywords. HIV; CCR5; Δ32; coreceptor; reservoir; latency; eradication; NF-κB; HIV transcription; HIV replication.

Although antiretroviral treatment (ART) dramatically reduces the morbidity and mortality associated with human immunodeficiency virus (HIV) infection, it does not eradicate the virus [1], and residual viral replication may persist [2]. Given the challenges of delivering complex, expensive, and occasionally toxic ART over a lifetime to a large global population of HIV-infected individuals, there is growing interest in curative interventions. A key first step is defining the host factors that predict and enable HIV persistence.

Multiple host factors contribute to disease progression in the absence of ART [3]. One factor is CCR5, which is the main coreceptor used by HIV to enter target cells. A 32-base pair deletion in the CCR5 gene (CCR5-Δ32) is common in Caucasian individuals of northern European origin. CCR5-Δ32 heterozygosity is variably associated with reduced HIV viremia and delayed disease progression, while CCR5-Δ32 homozygosity protects against HIV acquisition [4, 5]. The impact of CCR5 expression during treated disease is unknown. If HIV replication persists at low levels during ART [2], then it is likely that cell surface expression of CCR5 would predict the level of HIV persistence.

We hypothesized that decreased CCR5 expression in CCR5-Δ32 heterozygous (CCR5-wt/Δ32) individuals is associated with reduced levels of HIV persistence during stable ART. To address this hypothesis, we measured the HIV reservoir in 18 CCR5-wt/Δ32 and 54 CCR5 wild-type (CCR5-wt/wt) HIV-positive individuals during suppressive ART. We also performed immunophenotyping to determine whether decreased cell surface expression of CCR5 impacts immune activation and exhaustion, which are associated with HIV persistence.

MATERIALS AND METHODS

Subject Selection

Subjects were identified from the University of California–San Francisco (UCSF)–based SCOPE and Options cohorts. CCR5 genotyping of subjects was performed using a polymerase chain reaction (PCR)–based assay, as previously described [6]. Subjects were aged ≥18 years, ART-naïve before entering the cohort, had a documented pretreatment viral load and treatment start date, and had undetectable plasma HIV RNA levels for >1 year during the study period. Subjects were allowed to have no more than 2 viral blips during the study period, defined as a viral load of >200 RNA copies/mL. Twenty million cryopreserved peripheral blood mononuclear cells (PBMCs) collected 1–2 years after ART initiation were studied.

Sample Processing and Flow Cytometry

A total of 500,000 cryopreserved PBMCs were immunophenotyped by flow cytometry. CD4+ T cells were negatively selected from the remaining cells, using the EasySep Human CD4+ T Cell Enrichment Kit (Stemcell Technologies). DNA and RNA extraction was performed with the Allprep DNA/RNA/
miRNA Universal Kit (Qiagen). Multicolor flow cytometry was performed on a LSR II flow cytometer (BD Biosciences), as previously described [7]. T-cell subsets were defined by the following populations: naive, CD45RA+CCR7+; central memory (CM), CD45RA−CCR7+; effecter/transitional memory (EM), CD45RA+CCR7−; and terminally differentiated effecter memory CD45RA− (TEMRA), CD45RA−CCR7−.

Quantification of HIV DNA and RNA

Cellular HIV pol and 2-LTR circular DNA were quantified by droplet digital PCR assay, using the Bio-Rad QX-100, as previously described [8]. Samples with undetectable HIV DNA droplets were reported as the limit of detection. Total cellular HIV RNA transcripts were measured by a real-time quantitative PCR (qPCR) assay with single copy sensitivity, as previously described [9]. Samples with undetectable HIV DNA droplets were excluded from analyses using the ratio of HIV RNA to DNA. The RNA:DNA ratio was defined as the number of copies of cell-associated HIV RNA per million CD4+ T cells divided by the number of copies of cellular HIV pol DNA per million CD4+ T cells.

Gene Expression Analysis for Nuclear Factor κ Light Chain Enhancer of Activated B Cell (NF-κB) Target Genes by Real-Time qPCR

The expression of nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α (NFκBIA), interleukin 6 (IL 6), and tumor necrosis factor (TNF) was assayed by real-time qPCR from cellular RNA. Complementary DNA (cDNA) was synthesized from RNA, using the SuperScript VILO cDNA synthesis kit (Invitrogen). The reaction mixture contained 10 μL of 2X Universal Master Mix II with UNG (Applied Biosciences), 1 μL of 1X TaqMan primer and probe mixes (Applied Biosciences), and 9 μL of cDNA. The following assay identification numbers were used for primer and probe mixes: Hs00153283_m1, Hs99999905_m1, Hs00174131_m1, and Hs99999043_m1. Relative messenger RNA (mRNA) copy numbers for NF-κB targets were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA.

Statistics

Two-tailed Mann–Whitney U, Spearman rank correlation coefficient, and Fisher exact tests were performed in GraphPad Prism (version 6.0c).

RESULTS

Eighteen CCR5-wt/Δ32 and 54 CCR5-wt/wt individuals were selected for this study. Subject characteristics are presented in Supplementary Table 1. No difference was observed in the nadir CD4+ T-cell count (P = .39), pretreatment viral load (P = .81), CD4+ T-cell count (P = .17; Supplementary Figure 1), or percentage of CD4+ T cells in peripheral blood (P = .56; Figure 1A) between the 2 genotype groups. Of 18 CCR5-wt/Δ32 individuals, 6 initiated ART during acute/early infection (defined as <1 year after infection; range, 0.11–0.58 years), and 12 subjects initiated during chronic infection (defined as >1 year after infection). Of 54 CCR5-wt/wt individuals, 17 and 37 initiated ART during early and chronic infection, respectively. The groups initiating treatment during early and chronic infection were similar in terms of age, sex, ethnicity, and treatment duration between CCR5 genotypes (Supplementary Table 1).

We initially examined CCR5 surface expression in both genotype groups. CCR5-wt/Δ32 individuals had significantly lower CCR5 mean fluorescence intensities (MFI), compared with CCR5-wt/wt individuals (1.26-fold decrease; P = .03; data not shown) and significantly lower percentages of CD4+ T cells expressing CCR5 (1.49-fold decrease; P = .01; Figure 1B). There was no significant difference in the frequency of CD4+ T cell subsets between the 2 genotypes (Figure 1C). The significant decrease in CCR5 surface expression in CCR5-wt/Δ32 individuals was observed across all CD4+ T cell subsets (naive, 1.77-fold [P = .04]; CM, 1.86-fold [P < .0001]; EM, 2.28-fold [P < .0001]; Figure 1D). CCR5 MFI on CD4+ T cells correlated with the percentage of CCR5+ CD4+ T cells (p = 0.91; P < .0001; data not shown), and further analyses were conducted with CCR5 MFI exclusively. Additionally, cell surface expression of CCR5 was examined on CD8+ T cells and CD8+ T-cell subsets, and significantly lower expression was observed in CCR5-wt/Δ32 subjects (P < .0001; data not shown).

The frequency of HLA-DR ̶ CD38+ cells did not significantly differ between the 2 genotypes (P = .47; Figure 1E). The patterns observed in total CD4+ T cells were observed in both CM and EM CD4+ T-cell subsets; the frequency of HLA-DR ̶ CD38+ cells was not significantly different between both genotypes (CM, P = .19; EM, P = .33; data not shown). CCR5 cell surface expression was lower in CCR5-wt/Δ32 individuals, compared with CCR5-wt/wt subjects on both activated HLA-DR ̶ CD38+ cells (P < .0001) and nonactivated HLA-DR ̶ CD38+ cells (P = .01; Figure 1F). There was no difference in the frequency of PD-1 expression between genotype groups (P = .36; data not shown).

To test the hypothesis that decreased cell surface expression of CCR5 limits infection and results in a smaller HIV reservoir, levels of HIV pol and 2–long terminal repeat (2-LTR) circular DNA were measured in CD4+ enriched PBMCs, using droplet digital PCR [8]. Given the well-established effect that duration of infection has on the size of the reservoir during ART, we analyzed each group separately [10]. There was no difference in HIV pol DNA between CCR5 genotypes in either early (P = .58) or chronic treatment initiators (P = .26; Figure 2A). There was a near-significant reduction in 2-LTR circular DNA, a possible marker of ongoing replication, observed in CCR5-wt/Δ32 individuals who initiated treatment during
chronic infection ($P = .07$; Figure 2B). Notably, significantly fewer CCR5-wt/Δ32 subjects had detectable 2-LTR circular DNA ($P = .01$; Figure 2C). In subjects who initiated ART during chronic infection, cell-associated HIV RNA levels were 3.21-fold lower in CCR5-wt/Δ32 subjects, compared with CCR5-wt/wt individuals ($P = .04$; Figure 2D). Transcriptional activity was also significantly decreased in CCR5-wt/Δ32 subjects (2.35-fold decrease; $P = .01$; Figure 2E). Cell-associated HIV RNA levels were significantly correlated with CCR5 MFI on CD4+ T cells ($\rho = 0.36$; $P = .002$; Figure 2F). In contrast, HIV DNA
Figure 2. Human immunodeficiency virus (HIV) reservoir measurements in CD4+ T cells. HIV reservoir size was evaluated by measuring the numbers of copies of HIV pol DNA (A), copies of 2–long terminal repeat (2-LTR) circular DNA (B), detectable 2-LTR circular DNA in subjects who initiated treatment during chronic infection (C), and copies of cell-associated HIV RNA (D). E, HIV transcription was measured as the HIV RNA:DNA ratio. Subjects with undetectable HIV DNA by droplet digital polymerase chain reaction are indicated in red, reported as the limit of detection, and omitted from analysis of the HIV RNA:DNA ratio. Lines and error bars represent medians and interquartile ranges. F, The Spearman correlation between cell-associated HIV RNA and CCR5 mean fluorescence intensity (MFI) on CD4+ T cells. Subjects who initiated antiretroviral therapy during early infection and chronic infection are represented by open and closed circles, respectively.
levels were not correlated with CCR5 MFI on CD4+ T cells (pol DNA, \( \rho = 0.12 \) [\( P = .33 \)]; 2-LTR circular DNA, \( \rho = 0.16 \) [\( P = .19 \)]; data not shown). Cell-associated RNA levels were not significantly correlated with pre-ART viral load (\( \rho = 0.16 \); \( P = .18 \); data not shown), excluding pretreatment viremia as a potential confounder.

To explore the hypothesis that decreased cell-associated HIV RNA in CCR5-wt/Δ32 individuals resulted from decreased CCR5-mediated NF-κB–dependent HIV transcription, RNA levels of genes encoding 3 molecules in the NF-κB pathway (NFKBIA, IL 6, and TNF) were analyzed by reverse-transcription qPCR. None of the 3 genes were differentially transcribed between CCR5-wt/wt and CCR5-wt/Δ32 individuals (NFKBIA, \( P = .77 \); IL 6, \( P = .66 \); TNF, \( P = .06 \); Supplementary Figure 2A–C). In addition, none of the 3 genes correlated with CCR5 MFI on CD4+ T cells or cell-associated HIV RNA levels (Supplementary Figure 2D–I).

**DISCUSSION**

The host mechanisms that determine levels of HIV persistence during effective antiretroviral therapy are largely unknown. Given the critical role of CCR5 in HIV pathogenesis, we hypothesized that CCR5-Δ32 heterozygosity is associated with lower reservoir size. In our study, no difference was observed between CCR5 genotypes in the frequency of HIV pol DNA in CD4+ T cells. However, cell-associated HIV RNA, RNA:DNA transcriptional ratios, and frequency of detectable 2-LTR circular DNA were significantly lower in CD4+ T cells from CCR5-wt/Δ32 individuals. Correspondingly, a correlation was observed between HIV RNA and CCR5 surface expression in CD4+ T cells. Collectively, these data suggest that CCR5 expression during ART is an important determinant of reservoir size. Two principal hypotheses may explain the observed decrease in cell-associated HIV RNA in CCR5 wt/Δ32 individuals: (1) decreased CCR5 cell surface expression leads to decreased HIV transcription through loss of CCR5-mediated signaling, and/or (2) decreased CCR5 cell surface expression leads to a reduction in ongoing residual replication.

In CCR5-wt/Δ32 individuals, reduced CCR5 cell surface expression may translate to decreased CCR5 signaling on a per cell basis. ART intensification with maraviroc, a CCR5 antagonist, increased NF-κB activity in resting CD4+ T cells [11]. This was hypothesized to contribute to a lower reservoir size via a so-called shock-and-kill mechanism. Increases in CCR5 signaling may elevate levels of cell-associated HIV RNA through enhanced NF-κB–mediated transcription of the HIV LTR. To explore whether NF-κB activity was modulated by CCR5 cell surface density, we used real-time qPCR to quantify the expression of genes encoding IkBα, IL 6, and TNF. While measurement of nuclear protein is the most definitive way to measure NF-κB activity, NFKBIA, IL 6, and TNF mRNA expression levels are reliable proxies for NF-κB activity [12]. Our study showed no evidence of decreased NF-κB signaling in CCR5-wt/Δ32 individuals, suggesting that NF-κB is unlikely to be the driver of decreased cell-associated HIV RNA observed in CCR5-wt/Δ32 subjects.

Recent data suggest that ongoing HIV replication likely persists during suppressive ART [2]. Therefore, the decreased availability of CCR5 in CCR5-wt/Δ32 individuals may limit ongoing replication and replenishment of the viral reservoir, accelerating clearance and decay of the latent pool. Cell-associated HIV RNA has been previously shown to be a measure of the functional reservoir and ongoing viral replication during suppressive ART [13]. Additionally, although controversial, 2-LTR circles have commonly been used as a marker of ongoing replication [14].

Our findings do not directly evaluate decreased ongoing replication as a potential explanation of the decreased cell-associated RNA level and frequency of detectable 2-LTR circles in CCR5-wt/Δ32 subjects. In vitro models may be used to explore the connection between coreceptor expression levels and residual viral replication in the presence of ART.

The magnitude of the decrease in cell-associated HIV RNA and 2-LTR circular DNA associated with CCR5-Δ32 heterozygosity was partially obscured in individuals who initiated ART during early infection. This pattern is intuitive, as the timing of treatment initiation is known to impact cellular HIV DNA and RNA levels [10]. Beneficial effects of early treatment initiation may override the impact of host genetic variables on the HIV reservoir. Reduced cellular activation levels in early treated patients would intuitively support less viral replication during ART [10].

This is the first study to describe the impact of CCR5 expression on HIV persistence during ART. The effects of CCR5 expression should be further studied in lymphoid tissues to clarify its role in HIV persistence and ongoing viral replication. Manipulation of CCR5 cell surface expression by use of techniques such as zinc-finger nuclease silencing [15] should be explored to advance eradication strategies and generalizability to the wider CCR5 wild-type population.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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Potential conflicts of interest. D. D. R. has consulted for Chimerix, BMS, Gilead, Gen-Probe, Monogram, Sirenas, and Prism. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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