CCR5-Δ32 Heterozygosity, HIV-1 Reservoir Size, and Lymphocyte Activation in Individuals Receiving Long-termSuppressive Antiretroviral Therapy

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We conducted a case-controlled study of the associations of CCR5-Δ32 heterozygosity with human immunodeficiency virus type 1 (HIV-1) reservoir size, lymphocyte activation, and CCR5 expression in 114 CCR5Δ32/WT and 177 wild-type CCR5 AIDS Clinical Trials Group participants receiving suppressive antiretroviral therapy. Overall, no significant differences were found between groups for any of these parameters. However, higher levels of CCR5 expression correlated with lower amounts of cell-associated HIV-1 RNA. The relationship between CCR5-Δ32 heterozygosity, CCR5 expression, and markers of HIV-1 persistence is likely to be complex and may be influenced by factors such as the duration of ART.

Keywords. human immunodeficiency virus; CCR5-Δ32; HIV persistence; HIV reactivation; HIV latency; antiretroviral therapy.

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METHODS

Samples were obtained from participants in 4 ACTG trials of ART-naïve individuals (ACTG 384, A5095, A5142, and A5202) and the ACTG Longitudinal Linked Randomized Trials (ALLRT) cohort, members of which had existing CCR5 genotypic data from a prior genome-wide association study of HIV-1 progression and host factors [9]. Samples from participants who did not achieve viral suppression by 24 weeks after ART initiation or who had subsequent persistent low-level viremia (defined as ≥3 consecutive measurements of ≥50 copies/mL), a confirmed viral load of ≥200 copies/mL, or viral blips of ≥1000 copies/mL were excluded. Tests were performed on samples collected from 114 CCR5Δ32/WT cases and 177 WT controls frequency matched as 1:1.5 ratio by sex, initial ART regimen, and time of study. DNA and RNA were extracted from cryopreserved PBMCs by using the AllPrep kit (Qiagen, Valencia, California) and were quantified using a real-time polymerase chain reaction (PCR) assay as described previously [10, 11]. This assay targets a conserved LTR/gag region that is specific to nearly all HIV-1
group M sequences; each sample was tested in triplicate [10, 11]. Unspliced cell-associated RNA was quantified by a real-time PCR assay, using the same primers and probes as used for HIV-1 DNA; HIV-1 DNA and RNA levels were normalized to cell count, using a conserved region of the CCR5 gene not influenced by the Δ32 mutation, as described elsewhere [10, 11]. The 2-LTR circles were quantified from PBMC DNA by real-time quantitative PCR as described previously [11]. A 149-base pair sequence of the human mitochondrial gene ND4 was quantified to estimate the efficiency of episome extraction [11]; the results of 2-LTR amplification were normalized to the number of input PBMCs.

Thawed, cryopreserved PBMCs were stained with Fixable Blue Dead Cell Stain, anti-CD3 (Invitrogen, Eugene, Oregon), anti-CD4 (eBioscience, San Diego, California), anti-CCR5 (BioLegend, San Diego, California), and anti-CD8, anti-CD38, anti-CD4 (eBioscience, San Diego, California), anti-CCR5 (Biosciences, San Jose, California). Following surface staining, cells were fixed, permeabilized, and stained for intracellular anti-Ki-67 (BD). Selected samples were stained for intracellular CCR5 to verify the efficiency of antibody binding. Samples were analyzed on a BD LSRII flow cytometer, using FACSDiva software (BD). Cytometer settings were kept consistent by tracking laser voltages, using UltraRainbow Fluorescent Particles (Spherotech, Lake Forest, Illinois). Compensation settings were assessed using CompBead particles (BD). Samples were analyzed using FlowJo (Tree Star, Ashland, Oregon). Only live cells were included in the flow cytometry analyses.

Conditional logistic regression models appropriate for the case-control design were used to compare measures of reservoir size, CCR5 expression, and lymphocyte activation between cases and control design were used to compare measures of reservoir size, live cells were included in the

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differences in markers of lymphocyte activation or proliferation between CCR5Δ32/WT and WT individuals after long-term ART. However, CCR5 expression as measured by MFI was significantly lower on CD8+ T cells from CCR5Δ32/WT individuals. Expression of CCR5 has been shown to decline for 36 months after ART initiation [12] and because of PBMC cryopreservation. We observed a low frequency of surface CCR5-expressing T cells in both CCR5Δ32/WT and WT individuals. The low numbers of CCR5-expressing cells may explain the lack of observed significant differences in the frequency of CCR5 expressing CD4+ T cells between the CCR5Δ32/WT and WT groups.

HIV-1 reservoirs, as measured by low-level, residual plasma viremia and cell-associated HIV-1 DNA, demonstrate biphasic decay after ART initiation [13, 14], with a faster HIV-1 DNA decay rate in the first year and slower decay phase thereafter [13]. It is possible that differences in HIV-1 cell-associated RNA levels between CCR5Δ32/WT and WT individuals are greater during the initial 1–2 years following ART initiation and become less pronounced after longer-term ART. Of note, odds ratios in our regression models for cell-associated RNA levels, RNA/DNA ratio, and the frequency of LTR circles were <1.0, consistent with the possibility of a protective effect of
CCR5-Δ32 heterozygosity and HIV-1 reservoir size. However, none of the odds ratios reached statistical significance.

Interestingly, higher levels of CCR5 expression on PBMCs and CD4+ T cells from both CCR5Δ32/WT and WT individuals were associated with lower levels of HIV-1 cell-associated RNA. This finding is counterintuitive as it has been postulated that decreased CCR5 expression may lead to a reduction in the level of residual replication in the setting of ART [8]. Maraviroc intensification therapy has also been shown to increase HIV-1 reactivation through higher CCR5-ligand expression and upregulation of the nuclear factor κB pathway [15]. However, a prior study of CCR5Δ32/WT individuals did not find any evidence of decreased nuclear factor κB signaling in Δ32 heterozygous individuals [8]. We also observed a significant positive correlation between CD4+ T-cell markers of activation and CCR5 expression but not between activation markers and HIV-1 transcription. None of our study participants were exposed to maraviroc therapy.

Our findings are in contrast with a smaller, prior study of 18 CCR5Δ32/WT patients in whom cell-associated RNA levels were significantly correlated with the level of CCR5 expression on CD4+ T cells [8]. In that study, CD4+ T cells from CCR5Δ32/WT patients had significantly lower cell-associated RNA levels, RNA/DNA ratios, and frequency of detectable 2-LTR circles as compared to WT individuals [8]. The reasons for divergent results between our investigation and the prior study may be explained, in part, by differences in the quantitative PCR assays used, the makeup of the patient cohort, the duration of ART, and the study design. For example, the prior study used samples from patients receiving ART for 1.4–1.8 years, whereas our patients were all treated with suppressive ART for >2.6 years, allowing them to reach stabler HIV-1 cell-associated RNA and DNA levels.

It is also possible that early ART initiation influences the relationship between reservoir size and CCR5 expression. However, the prior study described above identified significant differences between CCR5Δ32/WT and WT individuals treated during chronic infection. Although we lacked information regarding the timing of ART initiation, a large majority of our participants were likely treated during chronic infection, given the low median pretreatment CD4+ T-cell counts observed. A limitation of our study is that HIV-1 nucleic acids were extracted from frozen PBMC pellets or cryopreserved PBMCs. Therefore, we did not measure HIV-1 reservoirs from purified CD4+ T cells; direct measures of these parameters in purified CD4+ T cells may have yielded different results. In addition, low levels of CCR5 expression secondary to cryopreservation may have led to the lack of observed differences between our case and control groups. However, the prior positive study also relied on cryopreserved cells.

In summary, the relationship between CCR5-Δ32 heterozygosity, CCR5 expression, and markers of HIV-1 persistence is likely to be complex and may be influenced by factors such as duration of ART. Further longitudinal studies are needed to fully understand the impact of CCR5Δ32/WT on viral transcription and reservoir size.

**Supplementary Data**

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted
materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases (NIAID) or the National Institutes of Health (NIH).

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