Title

Persistence of integrated HIV DNA in CXCR3+CCR6+memory CD4+ T cells in HIV-infected individuals on antiretroviral therapy

Journal

AIDS, 30(10)

ISSN

0269-9370

Authors

Khoury, Gabriela
Anderson, Jenny L
Fromentin, Rémi
et al.

Publication Date

2016-06-19

DOI

10.1097/qad.0000000000001029

Peer reviewed

HHS Public Access

Author manuscript

AIDS. Author manuscript; available in PMC 2017 June 19.

Published in final edited form as:

AIDS. 2016 June 19; 30(10): 1511–1520. doi:10.1097/QAD.0000000000001029.

Persistence of integrated HIV DNA in CXCR3+CCR6+ memory CD4+ T-cells in HIV-infected individuals on antiretroviral therapy

Gabriela Khoury[1,2,3], **Jenny L Anderson**[1,2,3], **Rémi Fromentin**[4], **Wendy Hartogensis**[5], **Miranda Z Smith**[1,3], **Peter Bacchetti**[6], **Frederick M Hecht**[5], **Nicolas Chomont**[4], **Paul U Cameron**[1,2,3], **Steven G Deeks**[5], and **Sharon R Lewin**[*,1,2,3]

[1]Department of Infectious Diseases, Monash University and Alfred Hospital, Melbourne, 3004, Australia

[2]Centre for Biomedical Research, Burnet Institute, Melbourne, 3004, Australia

[3]The Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, 3010, Australia

[4]Department of Microbiology, Infectiology and Immunology, Université de Montréal, Faculty of Medicine, and Centre de Recherche du CHUM, Montréal, Quebec, Canada

[5]Department of Medicine, University of California, San Francisco, CA

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

Abstract

Background—HIV latent infection can be established in vitro by treating resting CD4+ T-cells with chemokines (CK) that bind to chemokine receptors (CKR), CCR7, CXCR3 and CCR6, highly expressed on T-cells.

Objective—To determine if CKR identify CD4+ T-cells enriched for HIV in HIV-infected individuals receiving suppressive antiretroviral therapy (ART).

Design—A cross-sectional study of CKR expression and HIV persistence in blood from HIV-infected individuals on suppressive ART for >3 years (n=48). A subset of 20 individuals underwent leukapheresis and sorting of specific CD4+ T-cell subsets.

Methods—We used flow cytometry to quantify CCR5, CCR6, CXCR3 and CXCR5 expression on CD4+ T-cells. HIV persistence was quantified using real time PCR to detect total, integrated HIV DNA, 2-LTR circles and cell-associated HIV RNA in total CD4+ T-cells from blood or sorted T-cell subsets. Associations between CKR and HIV persistence in CD4+ T-cells in blood were determined using regression models and adjusted for current and nadir CD4+ T-cell counts.

[*]Corresponding Author: Prof Sharon R Lewin, Director – The Peter Doherty Institute for Infection and Immunity, University of Melbourne, 786-798 Elizabeth Street, Melbourne, Victoria, Australia 3010, ; Email: lewins@unimelb.edu.au, Telephone +61 3 83443159

Conflicts of interest: The authors have no conflicts of interest to declare.

Author contributions: The author contributions are as follows GK, JLA, RF, NC performed experiments. GK, JLA, RF, NC, MZS, PUC performed analysis; WH, PB statistical analysis; SGD, FMH recruitment of participants; SRL, PUC, SGD conceived, designed and oversaw the study; GK, SRL wrote manuscript; All authors reviewed and approved the manuscript.

Results—The frequency of cells harbouring integrated HIV DNA was inversely associated with current CD4+ T-cell count and positively associated with CCR5+ CD4+ T-cells, CXCR3+CCR6+ and CXCR3+CCR6- expression on total memory CD4+ T-cells ($p<0.001$, 0.048, 0.015 and 0.016 respectively). CXCR3+CCR6+ CM CD4+ T-cells contained the highest amount of integrated HIV DNA compared to all T-cell subsets examined ($p=0.001$).

Conclusion—CXCR3 and CCR6 co-expression defines a subset of CD4+ T-cells that are preferentially enriched for HIV DNA in HIV-infected individuals on ART.

Keywords

HIV reservoir; HIV latency; chemokine receptors; chemokines; CCR5; CCR6; CXCR3

Introduction

Despite the success of antiretroviral therapy (ART) in reducing HIV-related morbidity and mortality, ART is not curative and needs to be taken life-long. The main reason why ART cannot cure HIV infection is the persistence of long lived latently infected CD4+ T-cells in blood and tissue [1, 2]. HIV can persist during ART in central, transitional and effector memory CD4+ T-cells, in addition to naïve and memory stem CD4+ T-cells [3–5].

We have previously demonstrated that HIV latency can be established in vitro following direct infection of resting CD4+ T-cells pre-treated with chemokines (CK) that bind to chemokine receptors (CKR) highly expressed on these T-cells, including CCR6, CCR7, and CXCR3 [6]. Ligation of these CKR led to changes in cortical actin allowing for rapid migration of the pre-integration complex to the nucleus and efficient nuclear localisation and integration [6].

CKR also play important roles in the homing of CD4+ T-cells to tissue where they receive homeostatic survival signals or move to sites of inflammation. CCR7 expressing cells, for example, home to the lymph node (LN) [7], CXCR3+ and CCR5+ cells home to sites of inflammation [8, 9], CCR6+ cells home to the gut associated lymphoid tissue (GALT) and sites of inflammation [10, 11] and CXCR5+ cells are preferentially found in the germinal centres of lymphoid tissue [12–14]. CKR expression also defines the function of specific memory CD4+ T-cell subsets; Th1 cells express CXCR3; Th1/Th17 cells express CXCR3 and CCR6, Th17 cells express CCR4 and CCR6; Th2 cells express CCR4; and Th0 express only CCR7 [7–11, 15–17]. A percentage of all the above functional subsets express CCR5, except Th2 cells [18].

Given the differential penetration of ART in certain tissue sites [19, 20] and the higher frequency of infected cells in tissues such as the gastrointestinal (GI) tract [21–23] and in germinal centres of the LN [24], we hypothesised that resting CD4+ T-cells that express CKR that home to either GALT or LN may be potential targets for direct infection and establishment of latent infection, and that expression of these CKR will be associated with HIV persistence in individuals on suppressive ART. The aims of this study were: 1. to assess the relationship between CKR expression on CD4+ T-cells and CK concentration of their respective ligands and virus persistence and 2. to determine if there was enrichment of HIV

in central memory CD4+ T-cells that expressed CXCR3 or CCR6; in individuals receiving long-term suppressive ART.

Materials & Methods

Participant recruitment

Forty-eight HIV-infected individuals receiving suppressive ART were recruited at the University of California San Francisco (UCSF), San Francisco, CA. Inclusion criteria included receiving ART for >3 years, CD4+ T-cell count >350 cells/μl and viral load <40 copies/ml (Abbott real time HIV-1 PCR) for ≥3 years. A subset of 20 individuals underwent leukapheresis for CD4+ T-cell subsets sorting. The study was approved by the institutional review boards at UCSF, Monash University, and the Alfred Hospital, Melbourne, Australia. All participants provided informed consent.

Flow cytometry

CKR expression was measured on CD4+ T-cells in whole blood as both the peripheral blood mononuclear cells (PBMC) isolation and freezing process can alter the expression of certain CKR. The panel of fluorescently labelled antibodies included CD3-Alexa700, CD4-QDot605, CD45RA-APC-H7, CD27-QDot655, CCR7-PE-Cy7, CCR5-V450, CCR6-PE, CXCR5-Alexa488, CXCR3-PECy5 and CD14/19 AmCyan (Becton Dickinson, Franklin Lakes, NJ). Subsets were identified by CD27, CD45RA, and CCR7 expression described in [3], (Supplementary Figure 1).

Quantification of total and integrated HIV DNA, 2-LTR circles and cell associated unspliced HIV RNA

CD4+ T-cells were isolated from cryopreserved PBMC using magnetic negative selection as per manufacturer's protocol (Stem Cell Technologies, Vancouver, Canada). Quantification of HIV DNA (total, integrated and 2-LTR circles) and cell-associated unspliced HIV RNA (CA-US HIV RNA) were performed using quantitative real time nested PCR described in [25, 26].

Quantification of chemokines in plasma

CK levels were measured in frozen plasma isolated from blood collected in EDTA tubes using multiplex Luminex assays (Millipore, Billerica, MA and Life Technologies).

Statistical Analysis

Data distributions were assessed through descriptive statistics and scatter plots. Spearman rank correlations were used to assess the relationship between measures of HIV persistence with each CKR (measured as the percent of total and memory CD4+ T cells expressing each marker) and CK concentration within the plasma.

Negative binomial regression models were run for comparisons between viral reservoir measures (outcome, dependent variable) and CKR expression on memory and total CD4+ T-cells (predictor, independent variable). The PCR assays for HIV persistence produce results with similar structure and were therefore analyzed by similar statistical methods. In each

case, we modelled copies/(input to the assay) by applying negative binomial regression with copies as the outcome variable and the measure of input as an “exposure” variable, and using robust standard errors. In this type of regression, the outcome is modelled on the log scale (technically, using a log “link function”), and log (exposure) is included in the regression equation with a fixed coefficient of 1, so that multiplicative effects of covariates on copies/input are modelled. For example, the CA-US HIV RNA assay uses a measure of 18s RNA as the input total RNA, indicating the number of cells, so our models estimated covariate effects on copies per million cells. This regression method has some advantages over approaches that first calculate log (copies/input) for each person and then apply standard methods such as correlation, t-tests, or linear regression. Notably, the methods we have used take into account that copies/input is measured with less precision when the number of copies is lower and when the amount of input is lower; this was important because there were cases of low or no copies and there was some variation in input total RNA. The methods also permit proper quantitative use of instances where zero copies were present in the specimen assayed, without a need for ad hoc modifications to permit taking logarithms. For some of the assays, there were instances of signal detected, but below the amount corresponding to a single copy. We treated these as having an intermediate value of 0.5 copies to reflect uncertainty about whether or not a copy was actually present. In the negative binomial models, absolute current and nadir CD4+ T-cell counts were included in multivariate models to adjust for their effects. To mitigate skewness, the percentage of CCR5+ CD4+ T-cells were log2 transformed.

To compare integrated HIV DNA and CA-US HIV RNA across sorted CD4+ T-cell subsets, we fit negative binomial models with cell subset as a predictor variable and with a random person effect to account for within-person correlation. These models did not use robust standard errors, because of the smaller sample size [27]. To compare HIV RNA:DNA ratios between T-cell subsets, we fit similar models to a data set that included separate observations for RNA and DNA. Predictor variables included an indicator of whether the observation was for RNA (vs DNA), cell subset, and an interaction of RNA and subset, which quantified and tested the difference in RNA:DNA ratio between subsets. These models included random intercept and RNA terms in order to produce matched, within-person comparisons. Analyses were run in Stata version 12 (Stata Corp, College Station, TX).

Results

Current CD4+ T-cell count and expression of chemokine receptors are strongly associated

The clinical characteristics of the study participants are summarised in Table 1. Given previous studies have shown an inverse association between HIV DNA and current CD4+ T-cell count [3, 28], we first examined this relationship. Strong statistically significant negative correlations were identified between integrated HIV DNA and current CD4+ T-cell count ($r=-0.54$, $p<0.001$) and between 2-LTR circles and nadir CD4+ T-cell count ($r=-0.45$, $p=0.002$; Figure 1A and 1B).

Next, we quantified the expression of CKR on total CD4+ T-cells. The proportion of CCR7+ CD4+ T-cells was highest, followed by CXCR3 and CCR6, then CCR5 and CXCR5 at

similarly low proportions (Supplementary Figure 2A). There was a statistically significant positive correlation between the frequency of CD4+ T cell expressing CCR7 and current CD4+ T-cell count (r= +0.59, p value <0.001). In contrast, current CD4+ T-cell count was negatively correlated with the frequency of CD4+ T cells expressing CCR5 (r= -0.50, p value <0.001), CCR6 (r= -0.42, p value= 0.003) and CXCR3 (r= -0.57, p value <0.001), (Figure 1C). There was no statistically significant relationship between the frequency of CD4+ T cells expressing CXCR5 and current CD4+ T-cell count (r= -0.15, p value >0.05, data not shown). When similar analyses were performed using nadir CD4+ T-cell count, none of the correlations were statistically significant. In addition, there was no substantial relationship between the concentration of CK quantified in plasma (Supplementary Figure 3) with current or nadir CD4+ T-cell counts (data not shown).

Relationship between CKR expression and integrated HIV DNA

Next the relationship between the expression of single CKR on total CD4+ T-cells and HIV persistence was assessed. There were no substantial correlations observed between CKR expression and total HIV DNA, US HIV RNA or 2-LTR circles (Supplementary Table 1). Only CCR5 had a statistically significant positive, but relatively weak correlation with integrated HIV DNA (r=+0.29, p value= 0.048), (Figure 2A; Supplementary Table 1). Additional post-hoc sensitivity testing was performed on the relationship between CCR5 expression on total CD4+ T-cells with integrated HIV DNA and two outliers were removed for subsequent analysis. Of note, the association remained after removal of these outliers (r= +0.32, p value=0.031).

Given the strong relationships between CKR expression and integrated HIV DNA with current CD4+ T-cell count; and 2-LTR circles with nadir CD4+ T-cell count (Figure 1), we then used a negative binomial regression model that adjusted for either absolute current or nadir CD4+ T-cell counts to examine the relationships between virus persistence and CKR expression. After adjusting for current CD4+ T-cell count, the association between CCR5 and integrated HIV DNA largely disappeared (Table 2).

We next assessed the relationship between HIV persistence and the co-expression of CXCR3 and CCR6 on total memory CD4+ T-cells (Figure 2B), this mixed population includes central, transitional and effector memory CD4+ T-cell subsets ie all cells that express CXCR3 and CCR6 (Summarised in Supplementary Table 2). There was a statistically significant positive association between integrated HIV DNA with memory CD4+ T-cells that co-expressed, CXCR3+CCR6+ (r=+0.35, p value=0.015) and memory CD4+ T-cells that were CXCR3+CCR6- (r=+0.34, p value=0.016) (Figure 2C), but again this was no longer statistically significant after adjusting for current or nadir CD4+ T-cell count (Supplementary Table 3). Despite a strong correlation between integrated and total HIV DNA (r=+0.73 p value <0.001), all associations observed with total HIV DNA were consistently weaker compared to those observed between CKR expression with integrated HIV DNA (data not shown). In addition, there were no substantial associations between US HIV RNA and individual CKR expressed on total CD4+ T-cells or the memory CD4+ T-cell subsets when using negative binomial regression analysis (data not shown).

Relationship between CKR expression and 2-LTR circles

Using a negative binomial regression model, we observed statistically significant inverse associations between the frequency of 2-LTR circles with CD4+ T-cells that expressed either CCR5 (0.61 fold-change in 2-LTR [95% CI 0.39 to 0.95], p=0.030) or CXCR3 (0.58 fold-change in 2-LTR [95% CI 0.42 to 0.79], p<0.001) which both remained statistically significant after adjusting for nadir or current CD4+ T-cell counts (Summarised in Table 2 and Supplementary Figure 2B). A weak inverse association between CCR6+ CD4+ T-cells with 2-LTR circles was detected after adjusting for the effect of nadir CD4+ T-cell count, but this did not reach statistical significance (0.80 fold-change in 2-LTR circles for 10 unit increase in percentage CCR6, [95% CI 0.63 to 1.02]) p value =0.074). Interestingly, we also found a positive association between 2-LTR circles and the percentage of CCR7+ CD4+ T-cells (1. 36 fold-change in 2-LTR for 10 unit increase in percentage CCR7 [95% CI 1.04 to 1.76] p value= 0.023) which was statistically significant after adjusting for the effects of nadir CD4+ T-cell count.

Consistent with the analyses of single CKR expression, we observed a negative association between 2-LTR circles and the proportion of CXCR3+CCR6- memory CD4+ T-cells after adjusting for the effect of nadir CD4+ T-cell count (0.46 fold-change in 2-LTR circles for each 10 unit change in percentage CXCR3+CCR6- cells [95% CI 0.28 to 0.76] p value =0.002), (Supplementary Table 3).

With the exception of an inverse correlation between 2-LTR circles and the ligand for CCR7, CCL21 (r=-0.31, p=0.042), there were no substantial relationships between HIV persistence and CK levels measured in plasma. Taken together, after adjusting for current and nadir CD4+ T-cell counts, the only statistically significant association between the expression of a CKR and HIV persistence was an inverse association between 2-LTR circles and expression of CCR5 and CXCR3 found on activated and Th1 polarised CD4+ T-cells respectively.

Central memory CD4+ T-cells co-expressing chemokine receptors CXCR3 and CCR6 are enriched with HIV DNA in HIV-infected participants on suppressive ART

To determine if CD4+ T-cells that express CXCR3 and CCR6 were preferentially infected in individuals receiving suppressive ART, we recruited 20 individuals who underwent leukapheresis (Table 1).

As central memory CD4+ T-cells are the greatest contributor to the pool of latently infected CD4+ T-cells during ART [3], we sorted central memory CD4+ T-cells (all expressing CCR7) into four different subpopulations based on the expression of CCR6 and CXCR3 and quantified integrated HIV-DNA and CA-US HIV RNA using real-time PCR. Using regression analysis we found a statistically significant higher concentration of integrated HIV-DNA in central memory CD4+ T-cells that co-expressed both CXCR3 and CCR6 compared to all other central memory CD4+ T-cell subsets (p value <0.001). Integrated HIV DNA in CXCR3+CCR6+ central memory CD4+ T-cells was also higher than transitional memory CD4+ T-cells (p value= 0.014) and naïve CD4+ T-cell subsets (p value <0.001), (Figure 3A).

When CA-US HIV RNA was assessed (Figure 3B), all memory CD4+ T-cell subsets had higher levels of CA-US HIV RNA compared to naïve CD4+ T-cells (p value <0.001) while transitional memory CD4+ T-cells had higher levels of CA-US HIV RNA compared to the CXCR3-CCR6+ and the CXCR3-CCR6- central memory CD4+ T-cell subsets (p value= 0.002 and 0.001 respectively). CXCR3+CCR6- central memory CD4+ T-cells had a higher amount of CA-US HIV RNA compared to central memory CD4+ T-cells that were negative for both CKR (p value= 0.027).

Finally we examined the ratio of CA-US HIV RNA to integrated HIV DNA (CA-US HIV RNA:DNA) to assess the average level of transcription per infected cell [29]. While the majority of T-cell subsets had a ratio of CA-US HIV RNA to DNA less than 1, with the exception of CXCR3-CCR6+ central memory CD4+ T-cells (2.1 fold-change [95% CI 0.97 to 4.5] p value= 0.059) all CD4+ T-cell subsets had statistically significant higher CA-US HIV RNA:DNA ratios when compared to CXCR3+CCR6+ central memory CD4+ T-cells (CXCR3+CCR6- p value= 0.004; CXCR3-CCR6- p value= 0.04; Naïve p value<0.001; Transitional p value= 0.015; Figure 3C). This data is consistent with greater transcriptional silencing in the CXCR3+CCR6+ central memory CD4+ T-cells which would favour long term persistence.

Discussion

The overall goal of this study was to assess the relationship between CKR and CK expression with markers of viral persistence and to determine if central memory CD4+ T-cells expressing CKR CXCR3 and CCR6 were enriched for HIV in vivo in HIV-infected individuals receiving suppressive ART. We found that 1) central memory CD4+ T-cells that co-expressed CXCR3 and CCR6 were enriched with integrated HIV-DNA and had a lower ratio of CA-US HIV RNA to DNA than any other T-cell subset, consistent with latency; 2) strong relationships between current CD4+ T-cell count and integrated DNA and nadir CD4+ T-cell count and 2-LTR circles and 3) an inverse association between the frequency of 2-LTR circles and the proportion of CD4+ T-cells expressing CCR5 or CXCR3 that remained significant even after accounting for both current and nadir CD4+ T-cell counts.

There are several potential explanations for the enrichment of HIV DNA within CXCR3+CCR6+ central memory CD4+ T-cells in HIV-infected individuals receiving ART. First this may occur as a result of direct infection of resting CD4+ T-cells that express these CKR, as we have demonstrated in vitro [6]. Tissue compartments such as the GI tract which are highly enriched for CCR6+ cells would be an ideal environment for ongoing infection of resting CD4+ T-cells to occur given the high expression of the CCR6 ligand, CCL20 [10] and potentially suboptimal penetration of ARVs [20]. Second, CXCR3+CCR6+ cells are a subset of Th17 cells, which have been previously shown to undergo self-renewal and homeostatic proliferation [30]. Since Th17 cells are long lived and retain a stem cell-like molecular signature [30], it is possible that self-renewal favours long lived viral persistence in these cells through proliferation [31]. Third, CXCR3+CCR6+ central memory CD4+ T-cells are preferentially infected during productive infection as previously demonstrated in vitro and in vivo [32–36] and if latency is established through survival and reversion of these infected cells to a resting state, then one might expect a higher proportion of latently

infected cells expressing these CKR. Finally, CXCR3+CCR6+ or Th1Th17 cells express high levels of CCR5 making them more susceptible to HIV infection compared to CXCR3+ Th1 cells, at least in vitro [32, 33, 35, 36]. Therefore, any or a combination of these explanations could explain the enrichment of HIV in this subset.

In our cross sectional analysis of the proportion of cells that expressed a single CKR, CCR5 expression was positively associated with integrated DNA, but this was largely due to the effect of current CD4+ T-cell count. Our findings with CCR5 are consistent with other previous studies that demonstrated a higher concentration of HIV DNA in activated CD4+ T-cells [37] and a positive correlation between HIV DNA and markers of T-cells activation, including CCR5 [28]. Given that CKR guide T-cells to different anatomical sites or sites of inflammation, it is possible that identifying a relationship between CKR expression and HIV persistence requires examination of CD4+ T-cells from tissue sites rather than blood.

It is generally thought that the detection of 2-LTR circles represents a recently infected cell [38-41] although it remains controversial how stable these episomal forms of DNA are [41-43]. We observed that nadir CD4+ T-cell count had an inverse relationship with 2-LTR circles within total CD4+ T-cells. This might suggest that ongoing viral replication may occur more frequently in individuals who initiate ART late. Using regression models we observed an inverse association between the frequency of 2-LTR circles and the expression of CCR5 and CXCR3 on total CD4+ T-cells. One potential explanation is that 2-LTR circles are diluted in cells that proliferate – such as transitional and effector memory CD4+ T-cells which express both CXCR3 and CCR5 [3]. Taken together with our observation of an inverse association between CCR5 and CXCR3 expression with current CD4+T-cell count, it is possible that individuals with lower current and nadir CD4+ T-cell counts have higher levels of T-cell proliferation and this proliferation is potentially occurring in CD4+ T-cells that express higher levels of these specific chemokine receptors.

Like previous studies, we detected relatively low frequencies of CXCR5-expressing CD4+ T-cells within the blood as opposed to high expression of CXCR5 in the germinal centres of lymph nodes, where follicular helper T-cells (TFH) that express CXCR5 are abundant [12, 14, 44, 45]. There is currently high interest in these cells as a potential reservoir for HIV infection on ART [24, 46]. Here we found no substantial relationship between CXCR5-expressing CD4+ T-cells in blood with any markers of virus persistence. However, we didn't examine co-expression of CXCR5 with PD-1, which is the generally accepted strategy for identifying TFH cells [24, 45]. Again, it is possible that a relationship between CXCR5 and viral persistence will only be observed in the lymph node where CXCR5+ CD4+ T-cells are enriched [14, 45].

We observed a strong inverse relationship between current CD4+ T-cell count and integrated HIV DNA, although unlike previous studies we did not observe a statistically significant relationship with total HIV DNA [28, 47, 48]. This observation was probably related to the long duration of ART in our cohort potentially leading to a loss of unintegrated HIV DNA [49]. Another clear difference between our study and previous studies was that the median current CD4+ T-count in our study was high with close to all patients (81%) having a CD4+>500 cells/μl. We show here that even with a “normalised” CD4+ T-cell count, there

was an inverse relationship between current CD4+ T-cell count and the number of latently infected cells. This inverse relationship could potentially be related to the proportion of naïve CD4+ T-cells, which have a lower frequency of infection compared to memory CD4+ T-cells, in HIV-infected patients on ART as we have previously shown [4]. It is possible that with effective immune reconstitution and expansion of naïve CD4+ T-cells (which have a lower frequency of infection) via either homeostatic proliferation or enhanced thymic output there is a “dilution” of latently infected memory CD4+ T-cells leading to a lower overall frequency of infected cells. Similarly, within our study we also found that associations with total HIV DNA and CKR were consistently weaker than integrated HIV DNA. Again, it is possible that the differences in observations between integrated and total HIV DNA were related to the duration on cART in our cohort, median 8.5 years (5.0-12.4), potentially leading to a loss of unintegrated HIV DNA forms in some individuals.

There were several limitations of our study. First, this study was cross-sectional and therefore participants were receiving ART for different durations and initiated ART at different nadir CD4+ T-cell counts, but we accounted for this variation by adjusting for both current and nadir CD4+ T-cell counts using regression models. Second, we examined the relationship between CKR and CK with markers of virus persistence in total CD4+ T-cells within the blood and not in tissue where there is preferential enrichment of T-cells that express specific CKR. These studies in rectal tissue and lymph node are ongoing. Third, given limitations in cell numbers, we only quantified virus persistence using PCR based assays and quantification of replication competent virus is required to truly demonstrate a significant viral reservoir on ART.

In conclusion, we found that CXCR3+CCR6+ central memory CD4+ T-cell from individuals receiving suppressive ART were enriched with HIV DNA and had a lower ratio of CA-US HIV RNA:HIV DNA. Targeting CCR6 and/or CXCR3 with chemokine or chemokine receptor antagonists could provide a potential novel strategy to reduce virus persistence on ART.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank all the patient participants who have generously donated samples to this study. We are grateful to Dr Christina Chang, Dr Megan Crane (The Peter Doherty Institute for Infection and Immunity, Australia); Kylie Goy, Amanda Brass and A/Prof Rose Ffrench (Burnet Institute ImmunoMonitoring Facility, Australia) for advice on the luminex assays; Ms Surekha Tennakoon and Ms Ajantha Solomon (The Peter Doherty Institute for Infection and Immunity, Australia) for technical assistance; A/Prof Peter Hunt for careful review of the manuscript and helpful discussions; Dr Elizabeth Sinclair and the flow cytometry core facility, plus the Clinical Core at the University of California San Francisco, for specimen collection and processing.

Funding: This work was supported by the National Institutes of Health Delaney AIDS Research Enterprise (Grant U19 AI096109). G.K is a recipient of a National Health and Medical Research Council of Australia (NHMRC) Dora Lush biomedical post-graduate scholarship (579719). M.Z.S. is a recipient of an NHMRC Overseas Biomedical Research Fellowship (490988). S.R.L is an NHMRC practitioner fellow.

References

1. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med. 1999; 5:512–517. [PubMed: 10229227]
2. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. 1997; 387:183–188. [PubMed: 9144289]
3. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med. 2009; 15:893–900. [PubMed: 19543283]
4. Wightman F, Solomon A, Khoury G, Green JA, Gray L, Gorry PR, et al. Both CD31(+) and CD31(-) naive CD4(+) T cells are persistent HIV type 1-infected reservoirs in individuals receiving antiretroviral therapy. J Infect Dis. 2010; 202:1738–1748. [PubMed: 20979453]
5. Buzon MJ, Sun H, Li C, Shaw A, Seiss K, Ouyang Z, et al. HIV-1 persistence in CD4+ T cells with stem cell-like properties. Nat Med. 2014; 20:139–142. [PubMed: 24412925]
6. Cameron PU, Saleh S, Sallmann G, Solomon A, Wightman F, Evans VA, et al. Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton. Proc Natl Acad Sci U S A. 2010; 107:16934–16939. [PubMed: 20837531]
7. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999; 401:708–712. [PubMed: 10537110]
8. Mikhak Z, Fleming CM, Medoff BD, Thomas SY, Tager AM, Campanella GS, et al. STAT1 in peripheral tissue differentially regulates homing of antigen-specific Th1 and Th2 cells. J Immunol. 2006; 176:4959–4967. [PubMed: 16585592]
9. Sundrud MS, Grill SM, Ni D, Nagata K, Alkan SS, Subramaniam A, et al. Genetic reprogramming of primary human T cells reveals functional plasticity in Th cell differentiation. J Immunol. 2003; 171:3542–3549. [PubMed: 14500650]
10. Wang C, Kang SG, Lee J, Sun Z, Kim CH. The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. Mucosal Immunol. 2009; 2:173–183. [PubMed: 19129757]
11. Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol. 2008; 180:214–221. [PubMed: 18097022]
12. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med. 2000; 192:1545–1552. [PubMed: 11104797]
13. Kim JM, Oh YK, Kim YJ, Oh HB, Cho YJ. Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to Bacteroides fragilis enterotoxin: NF-kappa B plays a major role in the regulation of IL-8 expression. Clin Exp Immunol. 2001; 123:421–427. [PubMed: 11298129]
14. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med. 2000; 192:1553–1562. [PubMed: 11104798]
15. Andrew DP, Ruffing N, Kim CH, Miao W, Heath H, Li Y, et al. C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. J Immunol. 2001; 166:103–111. [PubMed: 11123282]
16. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol. 2007; 8:639–646. [PubMed: 17486092]
17. Rivino L, Messi M, Jarrossay D, Lanzavecchia A, Sallusto F, Geginat J. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. J Exp Med. 2004; 200:725–735. [PubMed: 15381728]

18. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, et al. Rules of chemokine receptor association with T cell polarization in vivo. J Clin Invest. 2001; 108:1331–1339. [PubMed: 11696578]
19. Patterson KB, Prince HA, Stevens T, Shaheen NJ, Dellon ES, Madanick RD, et al. Differential penetration of raltegravir throughout gastrointestinal tissue: implications for eradication and cure. AIDS. 2013; 27:1413–1419. [PubMed: 23945503]
20. Fletcher CV, Staskus K, Wietgrefe SW, Rothenberger M, Reilly C, Chipman JG, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A. 2014; 111:2307–2312. [PubMed: 24469825]
21. Yukl SA, Gianella S, Sinclair E, Epling L, Li Q, Duan L, et al. Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. J Infect Dis. 2010; 202:1553–1561. [PubMed: 20939732]
22. Yukl SA, Sinclair E, Somsouk M, Hunt PW, Epling L, Killian M, et al. A comparison of methods for measuring rectal HIV levels suggests that HIV DNA resides in cells other than CD4+ T cells, including myeloid cells. AIDS. 2014; 28:439–442. [PubMed: 24322272]
23. Chun TW, Nickle DC, Justement JS, Meyers JH, Roby G, Hallahan CW, et al. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. J Infect Dis. 2008; 197:714–720. [PubMed: 18260759]
24. Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, et al. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. J Exp Med. 2013; 210:143–156. [PubMed: 23254284]
25. Vandergeeten C, Fromentin R, Merlini E, Bramah-Lawani M, DaFonseca S, Bakeman W, et al. Cross-clade Ultrasensitive PCR-based Assays to Measure HIV Persistence in Large Cohort Studies. J Virol. 2014
26. Elliott JH, Wightman F, Solomon A, Ghneim K, Ahlers J, Cameron MJ, et al. Activation of HIV Transcription with Short-Course Vorinostat in HIV-Infected Patients on Suppressive Antiretroviral Therapy. PLoS Pathog. 2014; 10:e1004473. [PubMed: 25393648]
27. Imbens GW, Kolesar M. Robust Standard Errors in Small Samples: Some Practical Advice. The National Bureau of Economic Research. 2012
28. Hatano H, Jain V, Hunt PW, Lee TH, Sinclair E, Do TD, et al. Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells. J Infect Dis. 2013; 208:50–56. [PubMed: 23089590]
29. Yukl SA, Shergill AK, Ho T, Killian M, Girling V, Epling L, et al. The distribution of HIV DNA and RNA in cell subsets differs in gut and blood of HIV-positive patients on ART: implications for viral persistence. J Infect Dis. 2013; 208:1212–1220. [PubMed: 23852128]
30. Muranski P, Borman ZA, Kerkar SP, Klebanoff CA, Ji Y, Sanchez-Perez L, et al. Th17 cells are long lived and retain a stem cell-like molecular signature. Immunity. 2011; 35:972–985. [PubMed: 22177921]
31. Lichterfeld, M. Conference on Retroviruses and Opportunistic Infections. Boston, USA: 2014. T Memory Stem Cells: The Stem Cell Reservoir for HIV?.
32. Bernier A, Cleret-Buhot A, Zhang Y, Goulet JP, Monteiro P, Gosselin A, et al. Transcriptional profiling reveals molecular signatures associated with HIV permissiveness in Th1Th17 cells and identifies peroxisome proliferator-activated receptor gamma as an intrinsic negative regulator of viral replication. Retrovirology. 2013; 10:160. [PubMed: 24359430]
33. Alvarez Y, Tuen M, Shen G, Nawaz F, Arthos J, Wolff MJ, et al. Preferential HIV infection of CCR6+ Th17 cells is associated with higher levels of virus receptor expression and lack of CCR5 ligands. J Virol. 2013; 87:10843–10854. [PubMed: 23903844]
34. Monteiro P, Gosselin A, Wacleche VS, El-Far M, Said EA, Kared H, et al. Memory CCR6+CD4+ T cells are preferential targets for productive HIV type 1 infection regardless of their expression of integrin beta7. J Immunol. 2011; 186:4618–4630. [PubMed: 21398606]
35. Gosselin A, Monteiro P, Chomont N, Diaz-Griffero F, Said EA, Fonseca S, et al. Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection. J Immunol. 2010; 184:1604–1616. [PubMed: 20042588]

36. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, et al. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. J Infect Dis. 2010; 201:843–854. [PubMed: 20144043]
37. Chun TW, Nickle DC, Justement JS, Large D, Semerjian A, Curlin ME, et al. HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J Clin Invest. 2005; 115:3250–3255. [PubMed: 16276421]
38. Murray JM, McBride K, Boesecke C, Bailey M, Amin J, Suzuki K, et al. Integrated HIV DNA accumulates prior to treatment while episomal HIV DNA records ongoing transmission afterwards. AIDS. 2012; 26:543–550. [PubMed: 22410637]
39. Sharkey M, Triques K, Kuritzkes DR, Stevenson M. In vivo evidence for instability of episomal human immunodeficiency virus type 1 cDNA. J Virol. 2005; 79:5203–5210. [PubMed: 15795303]
40. Sharkey ME, Teo I, Greenough T, Sharova N, Luzuriaga K, Sullivan JL, et al. Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. Nat Med. 2000; 6:76–81. [PubMed: 10613828]
41. Pierson TC, Kieffer TL, Ruff CT, Buck C, Gange SJ, Siliciano RF. Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. J Virol. 2002; 76:4138–4144. [PubMed: 11907256]
42. Pace MJ, Graf EH, O'Doherty U. HIV 2-long terminal repeat circular DNA is stable in primary CD4+T Cells. Virology. 2013; 441:18–21. [PubMed: 23537959]
43. Zhu W, Jiao Y, Lei R, Hua W, Wang R, Ji Y, et al. Rapid turnover of 2-LTR HIV-1 DNA during early stage of highly active antiretroviral therapy. PLoS One. 2011; 6:e21081. [PubMed: 21687638]
44. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity. 2013; 39:770–781. [PubMed: 24138884]
45. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. J Clin Invest. 2012; 122:3271–3280. [PubMed: 22922259]
46. Fukazawa Y, Lum R, Okoye AA, Park H, Matsuda K, Bae JY, et al. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. Nat Med. 2015; 21:132–139. [PubMed: 25599132]
47. Sheth PM, Chege D, Shin LY, Huibner S, Yue FY, Loutfy M, et al. Immune reconstitution in the sigmoid colon after long-term HIV therapy. Mucosal Immunol. 2008; 1:382–388. [PubMed: 19079202]
48. Chun TW, Justement JS, Pandya P, Hallahan CW, McLaughlin M, Liu S, et al. Relationship between the size of the human immunodeficiency virus type 1 (HIV-1) reservoir in peripheral blood CD4+ T cells and CD4+:CD8+ T cell ratios in aviremic HIV-1-infected individuals receiving long-term highly active antiretroviral therapy. J Infect Dis. 2002; 185:1672–1676. [PubMed: 12023777]
49. Koelsch KK, Liu L, Haubrich R, May S, Havlir D, Gunthard HF, et al. Dynamics of total, linear nonintegrated, and integrated HIV-1 DNA in vivo and in vitro. J Infect Dis. 2008; 197:411–419. [PubMed: 18248304]

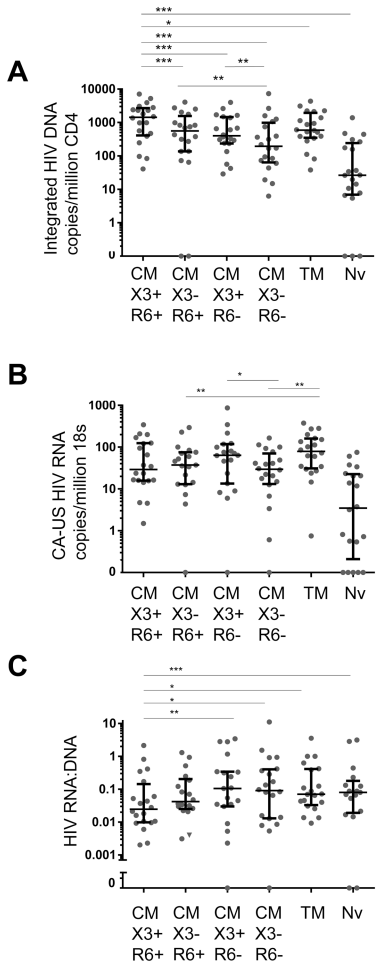

Figure 1.

The relationship between A. current absolute CD4+ T-cell count with integrated HIV DNA; and B. absolute nadir CD4+ T-cell count with 2-LTR circles, Spearman Rho correlations are shown. C. Spearman Rho correlations between CKR expression on total CD4+ T-cells and absolute current CD4+ T-cell count, in HIV-infected individuals receiving long-term suppressive ART (n=48). Within the HIV persistence measures, zero data points were incorporated into the statistical analysis refer to methods section for further details.

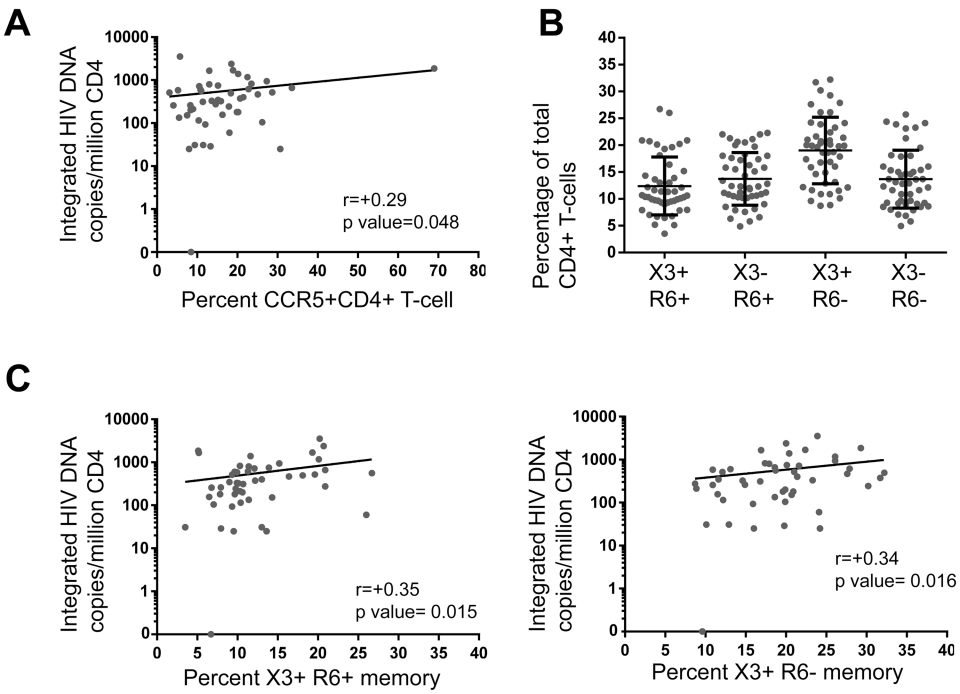

Figure 2.

The relationship between integrated HIV DNA and CKR expression including A. CCR5 expression on CD4+ T-cells, represented as a Spearman Rho correlation, (n=48). B. The expression of CCR6 and CXCR3 on total memory CD4+ T-cells, (n=48). C. Correlations between CCR6 and CXCR3 expression on total memory CD4+ T-cells and integrated HIV DNA, Spearman Rho correlations are represented, (n=48). Within the HIV persistence measures, zero data points were incorporated into the statistical analysis refer to methods section for further details.

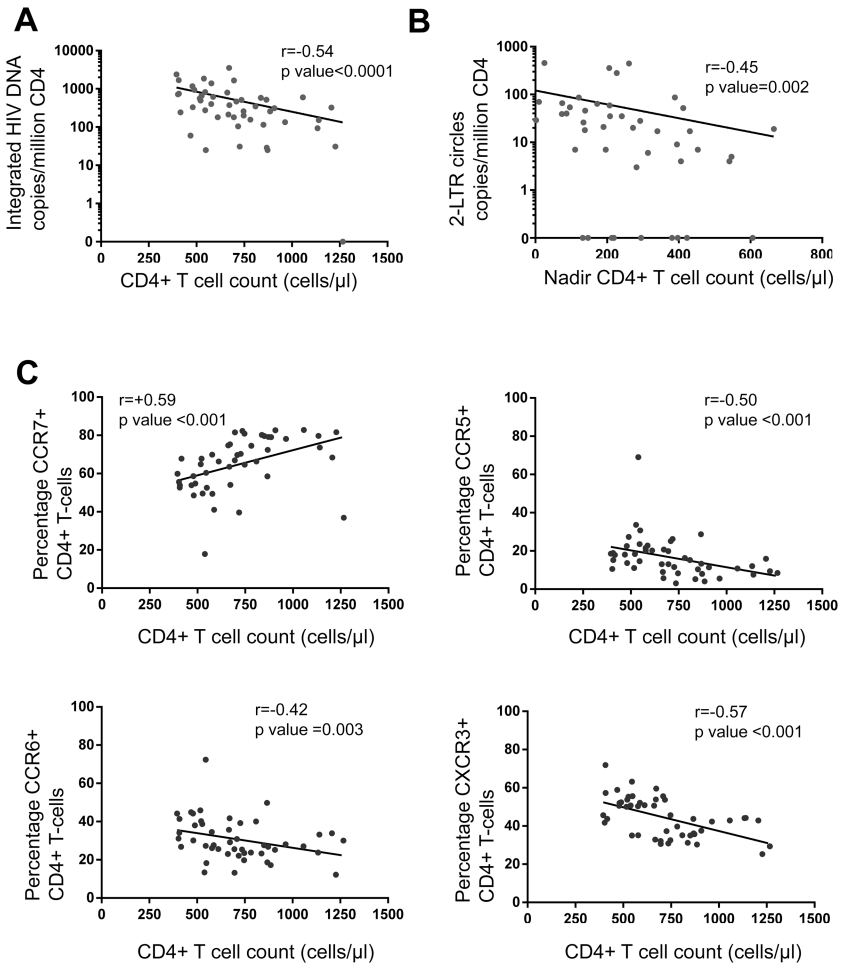

Figure 3.

The quantification of A. integrated HIV DNA, B. CA-US HIV RNA and C. CA-US HIV RNA:DNA ratio within sorted CD4+ T-cell subsets isolated from HIV-infected individuals receiving suppressive ART (n=20). Central memory (CM) CD4+ T-cell subsets were sorted into four subsets based on the expression of CXCR3 (X3) and CCR6 (R6), in addition to transitional memory (TM) and naïve (Nv) CD4+ T-cells using FACS ARIA sorter (Becton Dickinson). The median and interquartile ranges are shown. *p value <0.05; **p value <0.01; ***p value <0.001. In A and B, all comparisons with naïve CD4+ T-cells were statistically significant (p values <0.001) but are not marked to avoid visual clutter. In E, the triangle symbol was used for a sample that had no integrated HIV DNA detected and therefore the lower limit of detection was used to calculate the ratio. The true value of this ratio is considered to be >0.004.

Table 1

Clinical demographics of cohort.

Parameter	Total cohort (N=48)	Leukapharesis (N=20)
Gender, n (%)		
Male	46 (96%)	20 (100%)
Female	1 (2%)	0 (0%)
Transgender	1 (2%)	0 (0%)
Age, years, median (IQR)	57 (51-62)	57 (50-62)
Nadir CD4+ T-cell count, cells/μl, median (IQR)	216 (133-385)	137 (78-376)
Current CD4+ T-cell count, cells/μl, median (IQR)	684 (533-858)	639 (496-733)
Current CD4+ T-cell percentage, median (IQR)	32 (26-41)	30 (23-35)
Years on ART, median (IQR)	8.5 (5.0-12.4)	11.1 (5.3-13.1)
HCV co-infection, n (%)	5 (10%)	3 (15%)
HBV (HBsAg+), n (%)	2 (4%)	1 (5%)

IQR= Interquartile range; HCV= Hepatitis C virus; HBV= Hepatitis B virus; HBsAg+= Hepatitis B surface antigen positive.

Table 2

Negative binomial regression models of CKR expression on total CD4+ T-cells and integrated HIV DNA and 2-LTR circles

	Integrated HIV DNA		2-LTR circles	
	Result (95% CI)	**p value**	**Result (95% CI)**	**p value**
Unadjusted				
CCR5[1]	1.40 (1.06 to 1.86)	**0.020**	0.61 (0.39 to 0.95)	**0.030**
CXCR3[2]	1.32 (0.97 to 1.79)	0.078	0.58 (0.42 to 0.79)	**<0.001**
CCR6[2]	1.16 (0.82 to 1.64)	0.41	0.78 (0.60 to 1.03)	0.078
CCR7[2]	0.88 (0.72 to 1.09)	0.24	1.29 (0.87 to 1.92)	0.21
Adjusted- CD4 current				
CCR5[1]	1.07 (0.85 to 1.34)	0.58	0.57 (0.37 to 0.90)	**0.015**
CXCR3[2]	1.09 (0.79 to 1.52)	0.59	0.56 (0.36 to 0.89)	**0.013**
CCR6[2]	1.05 (0.76 to 1.45)	0.78	0.81 (0.57 to 1.15)	0.24
CCR7[2]	1.07 (0.84 to 1.38)	0.58	1.38 (0.96 to 1.99)	0.078
Adjusted- CD4 nadir				
CCR5[1]	1.37 (1.07 to 1.77)	**0.014**	0.60 (0.43 to 0.85)	**0.004**
CXCR3[2]	1.31 (0.98 to 1.76)	0.063	0.55 (0.43 to 0.71)	**<0.001**
CCR6[2]	1.13 (0.81 to 1.58)	0.47	0.80 (0.63 to 1.02)	0.074
CCR7[2]	0.87 (0.72 to 1.05)	0.14	1.36 (1.04 to 1.76)	**0.023**

CD4 current= Current CD4+ T-cell count; CD4 nadir= nadir CD4+ T-cell count. Percentage of CCR5 was log2 transformed. Post sensitivity analysis was used and two outliers were removed when examining the relationship between integrated HIV DNA and CCR5. Statistically significant p values are <0.05 and in bold type.

[1]Results arepresented as fold-change in the outcomeie integrated HIV DNA or 2-LTR circles for each doubling of the predictor iepercentage of CCR5+ CD4+ T-cells.

[2]Results are presented as fold-change in the outcomeie integrated HIV DNA or 2-LTR circles for each 10 unit increase in the predictor, iepercentage of CXCR3+ CD4+ T-cells.