Hypermethylation at the CXCR5 gene locus limits trafficking potential of CD8+ T cells into B-cell follicles during HIV-1 infection

Funsho J. Ogunshola,1,2 Werner Smidt,1,3 Anneta F. Naidoo,4 Thandeka Nkosi,1,5 Thandekile Ngubane,6 Trevor Khaba,5 Omolara O. Baiyegunhi,1,5 Bongiwe Mahlobo,1 Sam Rasehlo,1 Namani Ngema,5 Ismail Jajbhay,6 Krista L. Dong,2 Veron Ramsuran,3 Johan Pansegrouw,6 Thumbi Ndung’u,1,2,5,7 Bruce D. Walker,2,5,8,9 Tulio de Oliveira,3 and Zaza M. Ndhlovu1,2,6

1Africa Health Research Institute, Durban, South Africa; 2Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA; 3KwaZulu-Natal Research Innovation and Sequencing Platform, University of KwaZulu-Natal, Durban, South Africa; 4HIV Vaccine Trial Network, Cape Town, South Africa; 5HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; 6Prince Mshiyeni Memorial Hospital, Durban, South Africa; 7Max Planck Institute for Infection Biology, Berlin, Germany; 8Howard Hughes Medical Institute, Chevy Chase, MD; and 9Institute for Medical Engineering and Sciences and Department of Biology, Massachusetts Institute of Technology, Cambridge MA

CD8+ T cells play an important role in HIV control. However, in human lymph nodes (LNs), only a small subset of CD8+ T cells express CXCR5, the chemokine receptor required for cell migration into B-cell follicles, which are major sanctuaries for HIV persistence in individuals on therapy. Here, we investigate the impact of HIV infection on follicular CD8+ T cell (fCD8) frequencies, trafficking patterns, and CXCR5 regulation. We show that, although HIV infection results in a marginal increase in fCD8s in LNs, the majority of HIV-specific CD8+ T cells are CXCR5− (non-fCD8s) (P < .003). Mechanistic investigations using Assay for Transposase-Accessible Chromatin using sequencing showed that non-fCD8s have closed chromatin at the CXCR5 transcriptional start site (TSS). DNA bisulfite sequencing identified DNA hypermethylation at the CXCR5 TSS as the most probable cause of closed chromatin. Transcriptional factor footprint analysis revealed enrichment of transforming growth factors (TGFs) at the TSS of fCD8s. In vitro stimulation of non-fCD8s with recombinant TGF-β resulted in a significant increase in CXCR5 expression (fCD8s). Thus, this study identifies TGF-β signaling as a viable strategy for increasing fCD8 frequencies in follicular areas of the LN where they are needed to eliminate HIV-infected cells, with implications for HIV cure strategies.

Introduction

In HIV infection, secondary lymphoid tissues serve as the major site of viral replication,1-3 whereas germinal centers (GCs) in the B-cell follicles of lymph nodes (LNs) serve as major sites of HIV persistence during suppressive antiretroviral therapy (ART).4-6 Trafficking of cytolytic CD8+ T cells to the sites of active viral replication is a major mechanism leading to elimination of infected cells.7 CXCR5 expression facilitates direct trafficking of T cells to GCs by sensing CXCL13-producing cells, which reside within LNs.8-10 However, CD8+ T cells typically lack CXCR5 expression and, therefore, are not commonly found in B-cell follicles within LNs to eliminate HIV-infected cells.11,12 which is believed to be partially responsible for HIV persistence in this compartment, particularly during ART.13,14 Development of novel
strategies for boosting HIV-specific CD8+ T-cell migration to B-cell follicles could enhance immune clearance of HIV-infected cells.

A small subset of CXCR5-expressing CD8+ T cells called follicular CD8+ T cells (fCD8s) have the capacity to infiltrate B-cell follicles and eliminate infected cells.18,16 Human and animal studies have shown inverse correlations between fCD8s and HIV or simian immunodeficiency virus (SIV) burden,17,18 suggesting that increased infiltration of fCD8s in B-cell follicles can result in enhanced immune control. Indeed, some studies demonstrated direct anti-HIV activity of fCD8s.15,18 In addition, in the SIV model, CD8+ T-cell depletion is associated with a modest increase in SIV-infected cells in B-cell follicles,12 further supporting their involvement in mediating virus replication in the follicles. Thus, a detailed understanding of the regulatory mechanisms that govern the expression of CXCR5, the chemokine receptor required for CD8+ T-cell migration to B-cell follicles, is highly relevant to the development of curative strategies for HIV.

Animal studies have investigated the transcriptional regulatory networks that distinguish fCD8s from non-fCD8s. These studies have implicated a number of transcription factors (TFs), including Blimp1 and B-cell lymphoma 6 protein (BCL6) coupled with TCF1, and inhibitor DNA binding 2 and 3 (Id2 and Id3), which they describe as a transcriptional circuit that governs fCD8 differentiation.15,19,20 Additionally, in vitro stimulation of CD8+ T cells from rhesus macaques with inflammatory cytokines, such as transforming growth factor-β (TGF-β), interleukin-12, and interleukin-23, promoted fCD8 differentiation.21 TGF-β indirectly promotes the binding of E2A to the CXCR5 loci,22 an important factor that is critical for fCD8 differentiation.15,23 Together, these studies provide an important framework for potential regulatory networks; however, the underlying molecular processes that govern fCD8 differentiation in human lymphoid tissues remain largely unknown. Moreover, it is not clear how these animal studies translate to human diseases.

In this study, we investigated the trafficking pattern of CD8+ T cells in LNs following HIV infection and found that the majority of HIV-specific CD8+ T cells did not express CXCR5 and, thus, predominantly reside outside of GCs. We next studied the molecular mechanism that regulates CXCR5. Given that gene expression is mediated, in part, through epigenetic modifications, such as DNA methylation and the chromatin landscape, as well as that the epigenetic program of HIV-specific CD8+ T cells becomes less pliable with persistent HIV antigen stimulation,24-25 we investigated whether these epigenetic processes were involved in silencing the CXCR5 gene in lymphoid CD8+ T cells in the setting of HIV infection using DNA bisulfite sequencing, Assay for Transposase-Accessible Chromatin using sequencing (ATAC-Seq), and RNA sequencing (RNA-Seq) assays. Our data showed that DNA methylation and chromatin conformation are intricately involved in regulating CXCR5 expression in human CD8+ T cells. We also found that TGF-β, which has the potential to induce changes in the methylation profile of DNA,29 contributes to the expression of CXCR5 gene in CD8+ T cells and, thus, is a potential molecular target for increasing IC8D frequencies in B-cell follicles to eliminate HIV-infected cells.

### Materials and methods

**Human samples**

Fresh human inguinal LNs were obtained from participants enrolled at the Prince Memorial Mshiyeni Hospital, Umlazi, Durban, South Africa. Demographic and clinical characteristics of the study participants are summarized in Table 1. A section of the excised LN was processed for tissue imaging, and the remaining section was put through mesh to isolate LN mononuclear cells (LNMCs). LNs were homogenized using a syringe plunger and passed through a cell strainer (BD Biosciences Germany). Mononuclear cells were isolated using RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal calf serum. Extracted LNMCs were frozen for downstream experiments. Descriptions of study samples and experimental design are detailed in supplemental Information. All protocols were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Massachusetts General Hospital Institutional Review Board and were performed in accordance with the Declaration of Helsinki.

**Flow cytometry and cell sorting**

Cells were surface stained with a set of antibodies (supplemental Table 1). Cells were sorted for ATAC-Seq and RNA-Seq using a BD FACSAria. Gating strategies for sorting are detailed in supplemental Information. In all sorting experiments, the sorted cells were >95% pure (supplemental Figure 1A-C).

**Immunofluorescence staining**

Localization of CD8+ T subsets was assessed as described.4 Briefly, slides were prepared from 4-μm sections of paraffin-embedded tissue blocks and immune stained using in-house optimized protocols. For each LN, serial sections were stained singly with antibodies against BCL6, Gag-p24, CD8, and a DAB visualization kit (Envision Double Stain system; Dako) for bright-field microscopy. Alternatively, we used the Opal 4-Color Fluorescent IHC Kit (PerkinElmer) for immunofluorescence microscopy light. Slides were mounted and viewed using an Axio Observer and TissueFAXS imaging software (TissueGnostics, Vienna, Austria). Quantitative imaging analysis was conducted with TissueQuest (TissueGnostics). Medians of cell density in the GCs were used to perform statistical analyses.

**Drug treatment, ddPCR, and DNA methylation-bisulfite sequencing**

Drug treatment was performed on the same samples used for the DNA methylation assay. Briefly, an average of 100 000 non-fCD8s were sorted from the LN tissues and treated for 72 hours with 10 μM 5’aza-2-deoxycytidine (Aza). Thereafter, cells were washed and lysed, and RNA was extracted. Complementary DNA was generated from the purified RNA using (Bio-Rad, Hercules, CA). CXCR5 messenger RNA (mRNA) transcripts were measured from the generated complementary DNA using digital droplet polymerase chain reaction (ddPCR).

Specific cytosine guanine dinucleotide (CpG) within the CXCR5 gene region was measured for DNA methylation according to a protocol of Paulin et al.32 Briefly, a minimum of 500 ng of genomic DNA was treated with bisulfite and amplified using designed primers (forward-aggagagagTTTTTTTATGGGATAGGGA; reverse-aggagagagCTCTATCTAAATTTCAATCCAA) to cover 500 bp around the transcription start site (TSS). Amplified product was analyzed using a MassARRAY platform (Agena Bioscience, Berlin, Germany).
Chemosaxis assay

Chemosaxis assays were performed as previously described. Briefly, LNMCs were suspended at a density of 1 x 10^6 in RPMI 1640 medium containing L-glutamine, antibiotics, 10 mM HEPES buffer, and 0.5% fatty acid–free bovine serum albumin. Cells were cultured for 30 to 60 minutes at 37°C before being plated in Transwell inserts with a pore size of 5 μm and a diameter of 6.5 mm in 24-well plates (Corning Costar, New York). A total of 1 x 10^5 cells was added to the upper wells, and 50 ng/mL CXCL13 chemokine (PeproTech, New Jersey) was placed in the bottom wells; plates were incubated for 3 hours at 37°C in 5% CO2. Migrated cells were stained with viability dye, CD3, CD4, CD8, CXCR5, and PD-1 and counted using flow cytometry.

Sequencing and analysis

An average of 20,000 cells was used to perform ATAC-Seq and RNA-Seq. Detailed protocols on library preparation, sequencing, and analysis for ATAC-Seq and RNA-Seq are described in supplemental Information.

Statistical analysis

Data analyses were conducted using Prism software, version 6.0 (GraphPad, Inc, California). Two-tailed tests were used, and P values < .05 were considered significant. Comparisons between groups were assessed using parametric (unpaired Student t test). Categorical data were analysed using the Fisher’s exact test. Analysis of next-generation sequencing data is described in supplemental Information.

Results

Phenotypic characterization of fCD8s in HIV-infected subjects

To assess whether CD8+ T cells that have the follicular-homing phenotype (fCD8s) were indeed localized in the lymphoid tissues during HIV infection, we first used flow cytometry to measure the frequency of fCD8s in LN and in peripheral blood mononuclear cells in all 3 study groups. Consistent with previous findings, we observed a significantly higher frequency of fCD8s in LN compared with peripheral blood mononuclear cells in all groups (P < .0001 (Figure 1A). We next evaluate the effect of HIV infection and viral antigen persistence on the induction of fCD8s in LNs, comparing participants in the FRESH cohort who were uninfected with subjects who were ART suppressed, as well as untreated donors. We observed a significantly higher frequency of fCD8s as a percentage of total CD8+ T cells in treated (P = .01) and untreated (P = .008) donors compared with uninfected donors, as well as that ART limited the development of this phenotype (P = .003) (Figure 1B). These data are consistent with previous studies that suggest persistent viral infection and/or HIV-induced inflammation in ART-suppressed individuals drives the differentiation of cells, including fCD8s, during HIV infection. We next assessed whether increased fCD8s in treated and untreated individuals with HIV compared with HIV- individuals correlated with localization in GCS using multicolor immunofluorescence microscopy and TissueQuest image analysis software. This technique allows simultaneous quantitative assessment of cellular phenotype and cell localization in tissues. We defined fCD8s as CXCR5+CD8+ T cells. Active GCs were identified by BCL6 staining within B-cell follicles (supplemental Figure 1D). Image analysis revealed fCD8s localized in the GCs in HIV-infected persons, whereas there was a lack of GC fCD8s in HIV- persons (supplemental Figure 1E). Notably, we observed a significant positive correlation between the density of fCD8s localized in GCs and the frequency of fCD8s measured by flow cytometry in treated and untreated HIV infection (r = +0.87, P = .02) (supplementary Figure 1E), consistent with the notion that viral infection stimulates proliferation of fCD8s, which preferentially localize in GCs. Because CXCR5 expression by CD8+ T cells is believed to be important for migration of HIV-specific CD8+ T cells to the GCs, we next compared the proportion of HIV-specific CD8+ T cells expressing the CXCR5 receptor in the LN of treated and untreated patients with HIV. Because of the lack of class I tetramers that are capable of in situ tetramer staining of fixed human tissues, we used class I tetramers to identify HIV-specific CXCR5+CD8+ T cells using flow cytometry. The majority of HIV-specific CD8+ T cells did not express CXCR5 (Figure 1E), suggesting that the lack of CXCR5 expression by HIV-specific CD8+ T cells could limit their ability to infiltrate the GCs and clear viral reservoirs.

Lack of CXCR5 expression on CD8+ T cells attenuates their migratory capacity in response to CXCL13

Given that we could not directly assess the correlation between CXCR5 expression and migratory capacity in vivo, we assessed the direct relationship between CXCR5 expression in fCD8s and their migratory capacity toward a CXCL13 chemotaxis gradient using a Transwell assay. We observed a significantly greater chemotaxis of fCD8s toward CXCL13 compared with non-fCD8s (P = .04). GC T follicular helper cells (GCTfh’s), which express significantly greater levels of CXCR5 relative to fCD8, had the greatest migratory capacity (P = .01) (Figure 2A). Furthermore, we observed a positive correlation between CXCR5 expression levels and relative migratory

Table 1. Demographic and clinical characteristics of the study participants

| Participants | HIV- (n = 5) | HIV treated (n = 7) | HIV untreated (n = 5) |
|--------------|-------------|-------------------|---------------------|
| Females, n   | 5           | 7                 | 5                   |
| Age, y       | 21 (20.5-22)| 26 (23-36)        | 23 (18-26)          |
| CD4 counts, cells/mm³ | N/A | 642 (401-1189) | 436 (355-718) |
| Viral load, RNA copies/mL | N/A | <20               | 15068 (1200-23000) |
| Time to LN excision after HIV infection | Time to LN excision after HIV infection |

Unless otherwise noted, data are median (interquartile range).

N/A, not applicable.
capacity in response to CXCL13 ($r = +0.91$, $P = .01$) (Figure 2B), suggesting a direct link between CXCR5 expression levels and cell migration in response to CXCL13 chemokine. Next, we used immunofluorescence imaging to evaluate the localization of CD8$^+$ T cells relative to HIV-infected cells by quantifying CD8$^+$ T-cell density inside and outside of the GCs from the 3 HIV-infected individuals who began ART during hyperacute (Fiebig stage I/II) infection. Quantitative image analysis revealed greater frequencies of CD8$^+$ T cells (shown in red) outside of GCs ($P < .0001$), spatially segregated from HIV-Gag-p24 (shown in orange), which was almost exclusively localized in the GCs (Figure 2C), suggesting that exclusion of CD8$^+$ T cells from GCs contributes, in part, to HIV persistence. Together, these data demonstrate a direct link between the paucity of CXCR5-expressing HIV-specific CD8$^+$ T cells and HIV persistence in GCs, even with the early initiation of ART.

**TFs are differentially expressed between human fCD8s and GCTfh's**

To establish how CXCR5 expression is regulated in human CD8$^+$ T cells, we first investigated whether BCL6 is involved in CXCR5
expression in human CD8\(^+\) T cells. Consistent with animal studies, we found that BCL6 was highly expressed in GCTfh's but was significantly lower in fCD8s \((P = .008)\), with no significant difference between fCD8s and non-fCD8s \((P = .06)\) (Figure 3A). To gain a deeper understanding of the TFs and signaling pathways that regulate CXCR5 expression in human T cells, we performed bulk RNA-Seq on fluorescence-activated cell sorting (FACS)-sorted cells from the excised LNs of 5 HIV-infected individuals (supplemental Figure 1A).

Five cell subsets were sorted using FACS from each individual LN: bulk fCD8s, non-fCD8s, naive CD8\(^+\) T cells, GCTfh's, and non-T follicular helper cells (non-Tfh's; supplemental Figure 1B). GCTfh's, which constitutively express high levels of CXCR5, and naive CD8\(^+\) T cells, which do not express CXCR5, served as positive and negative controls, respectively. Non-Tfh's were included as an additional negative control to compare with GCTfh's. Principal component analysis (PCA) of 5 biological replicates separated all experimental groups in 2-dimensional space based on quantification of mRNA transcripts (Figure 3B). Despite minimal separation between fCD8s and non-fCD8s, 607 genes (false discovery rate < 0.1) were differentially expressed between these 2 subsets (supplemental Information). Notably, gene expression in fCD8s was more similar to non-fCD8s than to GCTfh's (supplemental Figure 2A). We quantified genes that have been implicated in CXCR5 regulatory circuitry, beginning with BCL6. We observed a significant difference in BCL6 expression between fCD8s and GCTfh's \((P < .00001)\) (Figure 3C) that correlated with the difference in protein expression (Figure 3A). Notably, there was no difference in BCL6 expression between fCD8s and non-fCD8s \((P = .64)\), contrary to murine studies.\(^{15,37}\) PRDM1, a BCL6 antagonist,\(^{19}\) was significantly higher in fCD8s compared with GCTfh's \((P = .01)\), but no difference in expression was observed between fCD8s and non-fCD8s \((P = .83)\) (Figure 3D; supplemental Figure 2B).
We quantified other genes that were similarly expressed between fCD8s and GCTfh’s in mice and were reported to be part of the CXCR5 transcription circuitry, including TCF7 (gene coding for TCF-1), Id3, and Id2. Interestingly, Id3 and TCF7 were significantly downregulated in human fCD8s compared with GCTfh’s (Id3, \(P < .0001\); TCF-7, \(P < .0001\)), with no apparent difference between fCD8s and non-fCD8s (Id3, \(P = .50\); TCF-1, \(P = .90\)) (Figure 3D; supplemental Figure 2B). Notably, Id2 expression was significantly different between fCD8s and non-fCD8s (Id2:\(P = .00001\)) (supplementary Figure 2B). These data suggest that the common transcriptional regulators of CXCR5 expression in GCTfh’s and fCD8s, described in murine studies, are similar for human GCTfh’s but not for fCD8s. Therefore, additional transcription circuitry may be involved CXCR5 expression in human CD8\(^+\) T cells.

The CXCR5 gene is repressed by DNA methylation and closed chromatin structure in human lymphoid non-fCD8s

A striking finding from our transcriptional profiling analysis was the identification of 43 genes (~7% of differentially expressed genes, false discovery rate < 0.1) that encode factors regulating epigenetic processes, such as chromatin remodeling, histone modification, and DNA methylation, were upregulated in fCD8s relative to non-fCD8s (Figure 4A; supplemental Figure 3), suggesting that specific epigenetic mechanisms may be directly involved in regulating CXCR5 in human CD8\(^+\) T cells.

Given that epigenetic regulators were among the most differentially expressed genes between fCD8s and non-fCD8s, we hypothesized that distinct epigenetic mechanisms, such as changes in chromatin landscape and DNA methylation, regulate the expression of CXCR5 in human CD8\(^+\) T cells. Therefore, we performed ATAC-Seq to identify genome-wide accessible regions that precede gene expression. ATAC-Seq analysis was performed on the DNA samples isolated from the same lymphoid cell populations used for RNA-Seq studies (supplemental Figure 1B). We performed PCA on the top 10% of accessible regions, revealing a clear delineation of cell subsets based on the chromatin-accessibility profiles (Figure 4B; supplemental Figure 4A). We calculated a set of 66,514 open chromatin regions that appeared in ≥1 of the subsets. The subset separation was strikingly similar to the PCA plot for RNA-Seq data.
revealing significant overlap between accessibility and gene expression. Indeed, there was a strong association between chromatin accessibility and gene expression between fCD8s and non-fCD8s ($R^2 = 0.54$) (supplemental Figure 4B).

We next probed accessibility of the CXCR5 gene, revealing a closed chromatin conformation at the TSS of the CXCR5 gene in non-fCD8s, naive CD8$^+$ T cells, and non-Tfh’s. In contrast, fCD8s and GCTfh’s had an open chromatin conformation at the equivalent site (Figure 4C), suggesting that chromatin accessibility contributes to the repressed state of the CXCR5 gene in non-fCD8s and naive CD8$^+$ T cells. To identify epigenetic factors that may directly regulate chromatin accessibility of the CXCR5 gene, we performed a TF motif search around the CXCR5 TSS of fCD8s and GCTfh’s. We restricted the motif search to regions that were inputted to have TF footprints proximal to the TSS.\(^{41,42}\) Our analysis revealed that fCD8s and GCTfh’s shared binding motifs at the CXCR5 gene TSS for several epigenetic regulatory proteins: POU3F1, POU3F3, E2F6, and ZNF384. Interestingly, TGIF1, TGIF2, PKNOX1, E2F7, E2F8, STAT1, STAT3, and STAT4 were enriched in fCD8s but not in GCTfh’s (Figure 4D), suggesting that these factors may play a role in the regulation of CXCR5 in human CD8$^+$ T cells.

To determine whether methylation was responsible for CXCR5 gene silencing, we first performed a nonspecific DNA methylation assay by culturing FACS-sorted non-fCD8s with Aza, which inhibits the enzymatic activity of DNA methyl transferases.\(^{43}\) After 72 hours of incubation, we measured CXCR5 mRNA transcript levels by ddPCR. We found that Aza treatment significantly increased CXCR5 mRNA levels ($P = 0.002$) (Figure 5A). We confirmed these data by measuring DNA methylation levels proximal to CXCR5 from the same cell subsets used in the Aza drug treatment assay, using loci-specific bisulfite-treated DNA sequencing. We FACS-sorted GCTfh’s, fCD8s, non-fCD8s, and naive CD8$^+$ T cells from LNs and

(see Figure 3A)
Figure 4. Closed chromatin impacts CXCR5 gene expression. (A) Ranked expression of selected epigenetic modifiers. Epigenetic modifiers were grouped according to functional attributes (ie, chromatin remodeling, histone chaperone, histone modification) and by transcription activity. (B) PCA plots obtained from the ATAC-Seq cut count data.
sequenced them. DNA methylation levels were measured in CpG islands within 300 bp upstream to 200 bp downstream of the CXCR5 TSS. We observed significantly higher methylation levels proximal to the CXCR5 promoter region in naive CD8+ T cells compared with GCTfh's (P = .001) and fCD8s (P = .001). Interestingly, methylation levels were comparable between naive CD8+ T cells and non-fCD8s at the equivalent sites (P = .10) (Figure 5B-C). Taken together, these data identify locus-specific DNA methylation and close chromatin conformation as distinct epigenetic mechanisms repressing the CXCR5 gene in human non-fCD8s.

TGF-β was shown to induce CXCR5 in CD8+ T cells in rhesus macaques21 and was identified in our analysis as a TF footprint at the TSS of fCD8s (Figure 4D), suggesting that TGF-β signaling is involved in demethylation of the CXCR5 TSS, resulting in increased gene expression. To test this, we cultured CD8+ T cells isolated from LN in vitro in the presence or absence of TGF-β. We observed a significant increase in CXCR5 expression in the presence of TGF-β compared with the control condition (Figure 4D), consistent with previous work.21 This suggests that TGF-β stimulation can reverse CXCR5 gene silencing in human CD8+ T cells.

Discussion

Understanding the regulation of CD8+ T-cell trafficking to B-cell follicles has far-reaching implications for developing strategies to eradicate HIV-infected cells in B-cell follicles. This study investigated how trafficking patterns of HIV-specific CD8+ T cells impact HIV persistence in LNs, and elucidated molecular mechanisms that regulate the expression of CXCR5 in human CD8+ T cells. We found that the majority of HIV-specific CD8+ T cells exhibit a non-fCD8 phenotype and, therefore, are devoid of the molecular machinery required to traffic to GCs, where HIV persists during suppressive therapy. ATAC-Seq analysis and DNA methylation measurements in purified fCD8s and non-fCD8s showed that the closed chromatin of CXCR5 TSS and DNA hypermethylation at this loci contribute to the repressed state of CXCR5 expression in non-fCD8s. Our data also suggest that TGF-β signaling may mediate the reversal of this phenotype. This study highlights the need for additional exploration of TGF-β signaling as a potential molecular target for enhancing CXCR5 expression on human CD8+ T cells within lymphoid tissues.

Conceptualization of this study was motivated by 3 studies in mice that described a subset of CXCR5-expressing CD8+ T cells that are termed fCD8s because of their ability to accumulate in B-cell follicles.7,15,16 Murine models of lymphocytic choriomeningitis virus infection showed that the transcriptional profile of fCD8s is similar to that of GCTfh's but not non-fCD8s. More importantly, they showed that, following lymphocytic choriomeningitis virus infection, fCD8s readily accumulated in B-cell follicles and were able to eradicate infected cells.15,16 Additionally, subsequent SIV studies in rhesus macaques showed similar results.21 Thus, we asked whether fCD8s were also increased in HIV infection in human LNs and whether their differentiation profile was similar to that described in mice. Indeed, our data showed an increased frequency of fCD8s in LNs of HIV-infected individuals, but this increased frequency was not sufficient to eliminate HIV reservoirs. Initiation of antiviral therapy mitigated fCD8 differentiation, suggesting that ICDB induction is antigen driven, as described in animal studies.7

CXCR5 is not typically expressed on CD8+ T cells, but it is required for their localization in B-cell follicles during viral infection.21,44 Although the role of CXCR5 on chemotaxis for follicular localization is a well-established phenomenon,45-47 the mechanisms that limit the expression of CXCR5 on human CD8+ T cells remain largely unknown. Our data show that, during HIV infection, virus-specific fCD8s exist at significantly reduced frequencies compared with non-fCD8s. We analyzed purified non-fCD8s, fCD8s, and GCTfh's to identify molecular processes that impede CXCR5 expression on non-fCD8s. Purified ICDBs and non-fCD8s were phenotypically indistinguishable, with the exception of CXCR5 expression.

We first investigated TFs that were implicated in CXCR5 regulation in murine fCD8s and GCTfh's: BCL6, Id3, Id2, PRDM1, and TCF-1.15,16,19 RNA-Seq analysis of sorted LN fCD8s, non-fCD8s, and GCTfh's from HIV-infected individuals revealed that TF expression profiles in human GCTfh's were similar to those reported in mice.37 However, we found significant differences in TF expression profiles between human GCTfh's and fCD8s that were not found in mice. The TF expression profiles in human fCD8s were more similar to non-fCD8s than to GCTfh's. Interestingly, we found low-level expression of BCL6 in fCD8s and non-fCD8s. Conversely, PRDM1, a BCL6 antagonist,19,48,49 was upregulated in fCD8s and non-fCD8s, suggesting that BCL6 may not be sufficient to initiate CXCR5 expression but, instead, works in tandem with other epigenetic factors.

Our RNA-Seq analysis identified a number of epigenetic regulatory genes that were differentially expressed between fCD8s and non-fCD8s. Using ATAC-Seq and loci-specific bisulfite-treated DNA sequencing analysis, we found closed chromatin conformation at the CXCR5 TSS in non-fCD8s relative to fCD8s and GCTfh's, as well as increased levels of methylation at the TSS of non-fCD8s. It is well-known that DNA methylation increases nucleosome compaction and rigidity.50 Therefore, the greater DNA methylation observed in non-fCD8s is the most probable cause of the observed condensed chromatin at the CXCR5 TSS and the corollary silencing of the CXCR5 gene in non-fCD8s. A motif search at the TSS for potential TFs that regulate CXCR5 expression revealed enrichment of TGFs as 1 of the dominant factors in the CXCR5 TSS. Indeed, in vitro stimulation of non-fCD8s with recombinant TGF-β resulted in increased CXCR5 expression, suggesting that TGF-β could play a major role in changing the methylation profile at the CXCR5 gene loci in human CD8+ T cells, opening up the gene for transcription.

Figure 4 (continued) The top 10% of ATAC-Seq peaks (merged between subsets) were used to create the PCA plot. (C) Overview of the ATAC-Seq signal around the CXCR5 gene loci. The ATAC-Seq signal is shown for different loci (marked in gray) at which differential binding was detected in ≥1 sample. The black box shows the TSS region where there is clear equivalence between ICDB and GCTfh ATAC-Seq signals, whereas very low signals were observed for non-fCD8s and naive CD8+ T cells. (D) Regions of predicted transcription factor footprints in respective cell subsets. The pie charts show the relative Wellington bootstrap scores for each subset against all others acting as a proxy for the relative TF activity observed in that region. The bars indicate the extent of the predicted TF footprint, with colors assigned to each subset. Footprints with unassigned TFs are also included.
Figure 5. Impact of DNA methylation on CXCR5 gene transcription. (A) Non-CD8s were sorted by FACS and treated for 72 hours with 10 μM Aza, a DNA methyltransferase inhibitor that causes hypomethylation of DNA. Fold change relative to the B2M housekeeping control indicated a significant increase in CXCR5 expression levels after treatment (n = 8). (B) Methylation levels for each subset analyzed across the 15 CpG sites for 3 biological replicates. (C) Representative plot of the quantitative measurement of DNA methylation levels within specific cell subsets (GCTfh’s, fCD8s, non-CD8s, and naive CD8+ T cells), as determined using EpiTYPER DNA Methylation Analysis. Methylation levels were measured from bisulfite-treated genomic DNA, followed by PCR amplification of a 500-bp fragment containing 15 CpG sites (red letters). The naive and non-CD8 cells show higher levels of methylation within several sites (darker circles), whereas the GCTfh’s and fCD8s show lower levels of methylation (lighter circles) (n = 3). The position of CpG sites are shown relative to the TSS. (D) Analysis of CXCR5 induction using recombinant TGF-β. CXCR5 expression was profiled at baseline and after the sorted cells were cultured in the presence and absence of TGF-β. A significant increase was observed after LNMCs were cultured in the presence of TGF-β (n = 5) for 7 days. Stim/stim, stimulation.
Previous studies showed that TGF-β levels increase significantly following SIV and HIV infections. Increased CD8 frequency during HIV infection could be driven by TGF-β, as suggested by previous studies. Further investigation into early immune signatures of acute HIV infection within the microenvironment of secondary lymphoid tissues will help to better describe the molecular mechanisms that influence in vivo generation of CD8s.

To our knowledge, this is the first study to elucidate the epigenetic processes that regulate CXCR5 expression in human CD8 T cells. However, because of insufficient samples we could not validate the histone modification pattern around the CXCR5 gene in CD8s and non-CD8s using chromatin immunoprecipitation sequencing. Nonetheless, chromatin immunoprecipitation sequencing data in a B-cell line that expresses higher levels of CXCR5 shows H3K4me2 at the gene TSS, which denotes open chromatin within the accessible regions around the CXCR5 gene (GM12787 [ENCODE Project Consortium 2012]), consistent with our findings.

In summary, our data show that only a small subset of HIV-specific CD8 T cells expresses CXCR5 (CD8s). Importantly, we identify key epigenetic and transcriptional processes that intricately orchestrate the regulation of the CXCR5 gene in human CD8 T cells, different from what have been described in mice studies. We also identify a putative TGF-β signaling along with epigenetic mechanisms, including DNA methylation and chromatin structure, as potential targets for inducing CXCR5 expression on human CD8 T cells. Manipulation of these processes has the potential to enhance trafficking of CD8 T cells to B-cell follicles where they are needed to eradicate HIV-infected cells.

Acknowledgments

The authors thank the study participants, the laboratory staff at the HIV Pathogenesis Programme, and the clinic staff at Prince Mshiyeni Hospital (Durban, South Africa). They also thank John E. Wherry, John L. Johnson, and Sasikanth Manne (Institute of Immunology, Philadelphia, PA) for training on ATAC-Seq and technical guidance on data analysis. Additionally, they thank Erica Andersen-Nissen and Julie McElrath at the HIV Vaccine Trial Network, Cape Town for access to its sequencing platform and Daniel Muema for technical support with the generation of RNA sequencing libraries.

This work was supported by an Howard Hughes Medical Research Institute International Research Scholar Award (55008743 [Z.M.N.]), National Institutes of Health National Institute of Allergic and Infectious Diseases (grants R01 AI145305 [Z.M.N.] and R37 AI67073 [B.D.W.]), a Dan and Marjorie Sullivan Research Scholar Award (224910 [Z.M.N.], the Mark and Lisa Schwartz Foundation, the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative (UKZNRSA1001), the Victor Daitz Foundation, and the Sub-Saharan African Network for TB/HIV Research Excellence. The DELTAS Africa Initiative (grant DEL-15-006) provided a doctoral fellowship to F.J.O. (2016-2020). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences’ Alliance for Accelerating Excellence in Science in Africa and is supported by the New Partnership for Africa’s Development Planning and Coordinating Agency with funding from the Wellcome Trust (grant 107752/Z/15/Z) and the UK government.

The views expressed in this publication are those of the authors and not necessarily those of the African Academy of Sciences, the New Partnership for Africa’s Development Planning and Coordinating Agency, the Wellcome Trust, or the UK government.

DNA methylation assays using bisulfite treatment and sequencing were performed by Inquaba Biotec.

Authorship

Contribution: Z.M.N. conceived the project; Z.M.N. and F.J.O. designed the experiments; I.J., J.P., and T. Ngubane performed the LN biopsies; T. Nkosi processed the LN samples; F.J.O. performed the majority of the experiments under the supervision of Z.M.N. with technical assistance from V.R. on the drug treatment assay; O.O.B. performed tissue staining and analysis with technical support from T.K.; F.J.O. and A.F.N. performed next-generation sequencing; W.S. performed next-generation sequencing analysis (ATAC-Seq and RNA-Seq) under the supervision of T.d.O.; F.J.O. analyzed other data reported in this article; F.J.O., W.S., and Z.M.N. wrote the manuscript; Z.M.N., B.D.W., and T. Ndung’u critically reviewed the manuscript; and all authors reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: W.S., 0000-0002-6197-4103; O.O.B., 0000-0001-8361-5534; S.R., 0000-0002-2324-8362; K.L.D., 0000-0002-2038-3518; V.R., 0000-0003-1590-9893; B.D.W., 0000-0001-6122-9245; T.d.O., 0000-0002-3027-5254; Z.M.N., 0000-0002-2708-3315.

Correspondence: Zaza M. Ndhlovu, Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, 400 Technology Square, Cambridge, MA 02139; e-mail: zndhlovu@mgh.harvard.edu.

References

1. Pantaleo G, Graziosi C, Demarest JF, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature*. 1993;362(6418):355-358.

2. Folkvord JM, Armon C, Connick E. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. *AIDS Res Hum Retroviruses*. 2005;21(5):363-370.

3. Horiike M, Iwami S, Kodama M, et al. Lymph nodes harbor viral reservoirs that cause rebound of plasma viremia in SIV-infected macaques upon cessation of combined antiretroviral therapy. *Virology*. 2012;423(2):107-118.

4. Banga R, Procopio FA, Noto A, et al. PD-1(+) and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals. *Nat Med*. 2016;22(7):754-761.
5. Perreau M, Savoye AL, De Crignis E, 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(1):143-156.

6. Kohler SL, Pham MN, Folkvord JM, et al. Germinal center T follicular helper cells are highly permissive to HIV-1 and alter their phenotype during virus replication. J Immunol. 2016;196(6):2711-2722.

7. Petrovas C, Ferrando-Martinez S, Germer MY, et al. Follicular CD8 T cells accumulate in HIV infection and can kill infected cells in vitro via bispecific antibodies. Sci Transl Med. 2017;9(373):eaag2289.

8. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science. 1999;286(5447):2098-2102.

9. Moser B, Ebert L. Lymphocyte traffic control by chemokines: follicular B helper T cells. Immunol Lett. 2003;85(2):105-112.

10. Hansell CA, Simpson CV, Nibbs RJ. Chemokine sequestration by atypical chemokine receptors. Biochem Soc Trans. 2006;34(Pt 6):1009-1013.

11. Connick E, Mattila T, Folkvord JM, et al. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. J Immunol. 2007;178(11):6975-6983.

12. Fukazawa Y, Lum R, Okoye AA, et al. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. Nat Med. 2015;21(2):132-139.

13. Streeck H. AIDS virus seeks refuge in B cell follicles. Nat Med. 2015;21(2):111-112.

14. Velu V, Mylvaganam G, Ibegbu C, Amara RR. Tfh1 cells in germinal centers during chronic HIV/SIV infection. Front Immunol. 2019;9:1272.

15. Leong YA, Chen Y, Ong HS, et al. CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles. Nat Immunol. 2016;17(10):1187-1196.

16. He R, Hou S, Liu C, et al. Follicular CXCR5-expressing CD8(+) T cells curtail chronic viral infection [published correction appears in Nature. 2016;540(7633):470]. Nature. 2016;537(7620):412-428.

17. Reuter MA, Del Rio Estrada PM, Buggert M, et al. HIV-specific CD8(+) T cells exhibit reduced and differentially regulated cytolytic activity in lymphoid tissue. Cell Rep. 2017;21(12):3458-3470.

18. Byrne EH, Anarth MN, Cohen KE, et al. Association between injectable progestin-only contraceptives and HIV acquisition and HIV target cell frequency in the female genital tract in South African women: a prospective cohort study. Lancet Infect Dis. 2016;16(4):441-448.

19. Johnston RJ, Pooleke AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006-1010.

20. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. Science. 2009;325(5943):1001-1005.

21. Mylvaganam GH, Rios D, Abdelaal HM, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection [published correction appears in Proc Natl Acad Sci USA. 2017;114(16):E3366]. Proc Natl Acad Sci USA. 2017;114(8):1976-1981.

22. Manzanares J, Li J, Salleras L, et al. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. Nat Immunol. 2011;12(1):86-95.

23. Perdomo-Celis F, Feria MG, Taborda NA, Rugeles MT. Induction of follicular-like CXCR5+ CD8(+) T cells by TGF-β1/IL-23 is limited during HIV infection. Viral Immunol. 2019;32(7):278-288.

24. Abdelsamed HA, Moustaki A, Fan Y, et al. Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis. J Exp Med. 2017;214(6):1593-1606.

25. Henning AN, Roychoudhuri R, Restifo NP. Epigenetic control of CD8(+) T cell differentiation. Nat Rev Immunol. 2018;18(5):340-356.

26. Iwafuchi-Doi M, Zaret KS. Pioneer transcription factors in cell reprogramming. Proc Natl Acad Sci USA. 2017;114(16):E3366. Proc Natl Acad Sci USA. 2017;114(8):E3366.

27. Reuter MA, Del Rio Estrada PM, Buggert M, et al. HIV-specific CD8(+) T cells exhibit reduced and differentially regulated cytolytic activity in lymphoid tissue. Cell Rep. 2017;21(12):3458-3470.

28. Byrne EH, Anahar MN, Cohen KE, et al. Association between injectable progestin-only contraceptives and HIV acquisition and HIV target cell frequency in the female genital tract in South African women: a prospective cohort study. Lancet Infect Dis. 2016;16(4):441-448.

29. Johnston RJ, Phooleke AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006-1010.

30. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. Science. 2009;325(5943):1001-1005.

31. Mylvaganam GH, Rios D, Abdelaal HM, et al. Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection [published correction appears in Proc Natl Acad Sci USA. 2017;114(16):E3366]. Proc Natl Acad Sci USA. 2017;114(8):1976-1981.

32. Manzanares J, Li J, Salleras L, et al. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. Nat Immunol. 2011;12(1):86-95.

33. Perdomo-Celis F, Feria MG, Taborda NA, Rugeles MT. Induction of follicular-like CXCR5+ CD8(+) T cells by TGF-β1/IL-23 is limited during HIV infection. Viral Immunol. 2019;32(7):278-288.

34. Abdelsamed HA, Moustaki A, Fan Y, et al. Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis. J Exp Med. 2017;214(6):1593-1606.

35. Henning AN, Roychoudhuri R, Restifo NP. Epigenetic control of CD8(+) T cell differentiation. Nat Rev Immunol. 2018;18(5):340-356.

36. Iwafuchi-Doi M, Zaret KS. Pioneer transcription factors in cell reprogramming. Genes Dev. 2014;28(24):2679-2692.

37. Yu B, Zhang K, Milner JJ, et al. Erratum: epigenetic landscapes reveal transcription factors that regulate CD8(+) T cell differentiation. Nat Immunol. 2017;18(6):705.

38. Youngblood B, Hale JS, Ahmed R. T-cell memory differentiation: insights from transcriptional signatures and epigenetics. Immunology. 2013;139(3):277-284.

39. Youngblood B, Noto A, Porichis F, et al. Cutting edge: prolonged exposure to HIV reinforces a poised epigenetic program for PD-1 expression in virus-specific CD8 T cells. J Immunol. 2013;191(2):540-544.

40. Cardenas H, Vieth E, Lee J, et al. TGF-β induces global changes in DNA methylation during the epithelial-to-mesenchymal transition in ovarian cancer cells. Epigenetics. 2014;9(11):1461-1472.

41. Bai J, Xi Q. Crosstalk between TGF-β signaling and epigenome. Acta Biochim Biophys Sin (Shanghai). 2018;50(1):60-67.

42. Paulin R, Grigg GW, Davey MW, Piper AA. Urea improves efficiency of bisulphite-mediated sequencing of 5'-methylcytosine in genomic DNA. Nucleic Acids Res. 1998;26(21):5009-5010.

43. Allen CD, Ansell KM, Law C, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol. 2004;5(9):943-952.

44. Buggert M, Nguyen S, Salgado-Montes de Oca G, et al. Identification and characterization of HIV-specific resident memory CD8(+) T cells in human lymphoid tissue. Sci Immunol. 2018;3(24):eaar4526.

45. Germer MY, Kastenmuller W, Ihrig I, Kabat J, Germain RN. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. Immunity. 2012;37(2):364-376.
36. Fukazawa Y, Park H, Cameron MJ, et al. Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines. *Nat Med.* 2012;18(11):1673-1681.

37. Im SJ, Hashimoto M, Gerner MY, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature.* 2016;537(7620):417-421.

38. Medvedeva YA, Lennartsson A, Ehsani R, et al. EpiFactors: a comprehensive database of human epigenetic factors and complexes. *Database (Oxford).* 2015;2015:bav067.

39. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods.* 2013;10(12):1213-1218.

40. Winter DR, Jung S, Amit I. Making the case for chromatin profiling: a new tool to investigate the immune-regulatory landscape. *Nat Rev Immunol.* 2015;15(9):585-594.

41. Piper J, Assi SA, Cauchy P, et al. Wellington-bootstrap: differential DNase-seq footprinting identifies cell-type determining transcription factors. *BMC Genomics.* 2015;16(1):1000.

42. Piper J, Elze MC, Cauchy P, Cockerill PN, Bonifer C, Ott S. Wellington: a novel method for the accurate identification of digital genomic footprints from DNase-seq data. *Nucleic Acids Res.* 2013;41(21):e201.

43. Yang J, Tian X, Yang J, et al. 5-Aza-2'-deoxycytidine, a DNA methylation inhibitor, induces cytotoxicity, cell cycle dynamics and alters expression of DNA methyltransferase 1 and 3A in mouse hippocampus-derived neuronal HT22 cells. *J Toxicol Environ Health A.* 2017;80(22):1222-1229.

44. Ayala VI, Deleage C, Trivett MT, et al. CXCR5-dependent entry of CD8 T cells into rhesus macaque B-cell follicles achieved through T-cell engineering. *J Virol.* 2017;91(11):e02507-16.

45. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature.* 2000;406(6793):309-314.

46. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell.* 1996;87(6):1037-1047.

47. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature.* 1998;391(6669):799-803.

48. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol.* 2010;11(2):114-120.

49. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* 2012;209(2):243-250.

50. Choy JS, Wei S, Lee JY, Tan S, Chu S, Lee TH. DNA methylation increases nucleosome compaction and rigidity. *J Am Chem Soc.* 2010;132(6):1782-1783.

51. Ploquin MJ, Desoutter JF, Santos PR, et al. Distinct expression profiles of TGF-beta1 signaling mediators in pathogenic SIVmac and non-pathogenic SIVagm infections. *Retrovirology.* 2006;3(1):37.

52. Poli G, Kinter AL, Justement JS, Bressler P, Kehrl JH, Fauci AS. Transforming growth factor beta suppresses human immunodeficiency virus expression and replication in infected cells of the monocyte/macrophage lineage. *J Exp Med.* 1991;173(3):589-597.

53. Wilson EB, Kidani Y, Elsasser H, et al. Emergence of distinct multiarmed immunoregulatory antigen-presenting cells during persistent viral infection. *Cell Host Microbe.* 2012;11(5):481-491.