HIV-1 persistence in lymph node T follicular helper cells (TFH) is mitigated by functional virus-specific T cell responses during hyperacute-treated HIV-1 infection

Omolara Baiyegunhi

Africa Health Research Institute <https://orcid.org/0000-0001-8361-5534>

Jaclyn Mann

University of KwaZulu-Natal

Trevor Khaba

HIV Pathogenesis Programme

Thandeka Nkosi

Africa Health Research Institute

Anele Mbatha

HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

Funsho Ogunshola

Africa Health Research Institute

Caroline Chasara

Africa Health Research Institute,

Nasreen Ismail

University of KwaZulu-Natal

Thandekile Ngubane

HIV Pathogenesis Programme

Ismail Jajbhay

Prince Mshiyeni Memorial Hospital,

Johan Pansegrouw

Prince Mshiyeni Memorial Hospital,

Krista Dong

Ragon Institute of MGH, MIT and Harvard

Bruce Walker

Harvard University <https://orcid.org/0000-0001-6122-9245>

Thumbi Ndung'u

Zaza Ndhlovu (✉ zndhlovu@mgh.harvard.edu)

Africa Health Research Institute <https://orcid.org/0000-0002-2708-3315>

Article

Keywords: HIV persistence, T cell responses, HIV-1 infection

Posted Date: November 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1061787/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Communications on July 12th, 2022. See the published version at <https://doi.org/10.1038/s41467-022-31692-8>.

Abstract

HIV persistence in tissue sites despite ART is a major barrier to HIV cure. Detailed studies of HIV infected cells and immune responses in native lymph node (LN) tissue environment is critical for gaining insight into immune mechanisms impacting HIV persistence and clearance in tissue sanctuary sites. We compared HIV persistence and HIV-specific T cell responses in LN biopsies obtained from 14 individuals who initiated therapy in Fiebig stages I/II, 5 persons treated (Tx) in Fiebig stages III-V and 17 late Tx individuals who initiated ART in Fiebig VI and beyond. Using multicolor immunofluorescence staining and in situ hybridization, HIV RNA and/or protein was detected in 12 of 14 Fiebig I/II Tx persons who were on suppressive therapy for 1 to 55 months, while all late Tx persons had persistent antigens. CXCR3+T follicular helper T cells harbored the greatest amounts of gag mRNA transcripts. Notably, HIV-specific CD8+ T cells responses associated with lower HIV antigen burden in LNs, suggesting that these responses may contribute to HIV suppression in LNs during therapy. These results reveal HIV persistence despite the initiation of ART in hyperacute infection and highlight the contribution of virus-specific responses to HIV suppression in tissue sanctuaries during suppressive ART.

Introduction

Antiretroviral therapy (ART) does not eradicate HIV infection due in large part to early establishment and persistence of integrated proviruses in quiescent circulating and tissue reservoirs¹⁻³, which are resistant to drug or immune-mediated clearance. Additional mechanisms of persistence that involve ongoing replication have been suggested⁴, including replication in germinal centers (GCs) within secondary lymphoid tissues, which have been described as sanctuary sites due to low CD8⁺ T cell infiltration⁵ and suboptimal penetration of antiretroviral drugs⁶. Ongoing virus replication, and to a lesser extent viral gene expression in the face of ART are contested concepts, with studies of individuals initiated on ART during chronic infection yielding conflicting results⁶⁻¹². T follicular helper (Tfh) cells have been identified as a major source of persistent virus¹³, but the precise subset of these cells enriched for HIV transcription on therapy within sanctuary sites is unknown. HIV persistence on ART is underscored by the nearly inevitable rebound of plasma viremia when treatment is interrupted, even after years of suppression, and is the major barrier to HIV cure¹⁴.

It has previously been shown that very early initiation of ART can lead to prolonged remission when treatment is interrupted^{15,16}, but this is an infrequent occurrence. In most individuals, virus rebound occurs within weeks to months even in individuals initiated on ART during Fiebig stage I (hyperacute) HIV infection^{14,17}, despite rapid suppression of viremia and dramatically lower numbers of latently infected cells in peripheral blood¹⁸⁻²¹. Intriguingly, virus rebound kinetics following treatment interruption are heterogenous, sometimes taking a year or more^{14,22}. The underlying immunological and virologic mechanisms responsible for the diverse viral rebound kinetics remain unknown. For instance, prolonged therapy in SIV infected macaques initiating therapy within 6 days of infection, prior to detectable plasma viremia, led to apparent elimination of infection after 600 days of suppressive ART in some animals²³.

However, such clearance of infection has not been observed in acute HIV infection, even in persons in whom therapy was initiated before detection of plasma viremia²⁰. We previously showed that early treatment initiation enhances T cell functions in peripheral blood and limits viral diversity²⁴, but it is not clear if functional responses occur in tissues and whether such responses play a significant role in HIV suppression during ART. Limited access to tissue samples from persons initiating therapy before peak viremia has impeded a better understanding of the impact of early therapy on the lymphoid reservoir.

Here we analyzed 64 excisional lymph node (LN) biopsies and paired peripheral blood (PB) samples obtained from a well pedigreed cohort of individuals, where some individuals initiated ART during hyperacute HIV infection, to investigate the impact of blunting peak viremia on the microanatomical location, cellular source and role of T cell responses on HIV persistence in LNs. Study participants were drawn from a unique hyperacute HIV infection cohort termed FRESH (Females Rising through Education, Support and Health). FRESH is a prospective study of uninfected 18-23-year-old women at high risk of HIV infection established at the epicenter of the HIV epidemic in South Africa, where yearly incidence rates approach 10%. Despite vigorous prevention efforts, twice weekly monitoring for viral RNA has identified and treated (Tx) persons at the onset of plasma viremia, allowing for immediate institution of ART in many cases resulting in peak plasma viral loads that are sometimes <1,000 RNA copies/ml and the preservation of CD4⁺ T cell numbers¹⁸. Our results show that despite ART-induced blunting of peak viremia¹⁸ and augmentation of functional HIV-specific T cell responses²⁴, HIV Gag p24 protein and viral RNA can persist in the LNs of Fiebig I/II Tx donors even after 4.5 years of fully suppressive ART, and these viral antigens are enriched in LN CXCR3⁺Tfh cells. We also show that superior functioning T cell responses were associated with lower HIV antigen persistence in the LNs.

Results

Hyperacute HIV infection as a model to interrogate antigen persistence in lymph nodes

To determine the impact of immediate initiation of ART in hyperacute HIV infection (before peak viremia) on HIV clearance from sanctuary sites, we studied 14 women aged 18-26 who initiated ART during hyperacute HIV infection (Fiebig I/II Tx) and achieved full suppression of plasma viremia within a median of 15 days (range, 6 to 33). LNs were obtained by excisional biopsy after treatment for a median of 370 days [range, 19 to 1647]. All remained fully suppressed except for one donor who had a transient viral load blip prior to LN excision. Five additional individuals identified in Fiebig stages III-V of infection and started on ART one day after diagnosis were also included. Three additional control groups were included: 13 HIV negative (HIVneg) donors; 17 individuals who initiated treatment in Fiebig VI and beyond (late Tx); and 15 untreated individuals whose duration on infection is unknown (unTx). Detailed characteristics of the cohorts are in Tables 1 and S1. 95% of the study participants were females.

Long-term persistence of HIV Gag p24 antigen in germinal centers (GCs) of individuals initiating antiretroviral therapy during hyperacute HIV-infection

To investigate HIV persistence in LNs of individuals initiating ART in Fiebig stages I/II, we measured HIV Gag p24 antigen in excisional LN biopsies by multicolor immunofluorescence staining of formalin-fixed paraffin-embedded LNs and imaging of tissue sections (Fig. S1). The transcription factor BCL-6 was used to identify active GCs²⁵ (Fig. S1) and images were quantified for Gag p24 content using the algorithm for area measurements in TissueQuest (TissueGnostics)²⁶. Fig. 1A shows a representative image of HIV Gag p24 LN staining for a participant who was diagnosed in Fiebig stage I, initiated ART within 48 hours and achieved persistent plasma viremia suppression within 33 days. The LN sample shown was obtained after 479 days of uninterrupted ART treatment with undetectable viremia, and depicts HIV Gag p24 antigen within a GC, which was present in 5 of the 7 GCs examined in this LN (Fig. S1E).

Gag p24 staining and imaging were conducted on a total of 14 LNs from fully suppressed Fiebig I/II Tx donors obtained at a median of 370 days (range, 19 to 1647 days) post-ART initiation. 12 of 14 (86%) of these Fiebig I/II Tx donors had detectable HIV Gag p24 in at least one GC, and overall 42 of 55 GCs evaluated (76%) were positive for Gag p24. In two Fiebig I/II Tx donors there was no detectable HIV Gag p24 despite examining more than more than 4 GCs (Fig. 1B). In those with detectable HIV Gag p24, quantitative image analysis revealed no correlation between the amount of HIV Gag p24 present in the LN tissue section and treatment duration prior to LN excision (Fig. 1C) or peak plasma viral load (data not shown). Notably, regardless of treatment duration, Fiebig I/II Tx donors had significantly less detectable HIV Gag p24 compared to Fiebig III-V Tx ($P<0.0001$), late Tx ($P<0.0001$) and unTx ($P<0.0001$) donors, though there was considerable overlap (Figs. 1D-E, S1F-G). This result was also consistent in a subset of donors Tx beyond 1 year (Fig. S1H). Quantitative image analysis of all treated LNs revealed greater area percent of Gag p24 staining in GCs compared to extrafollicular areas of the tissue ($P=0.04$, Fig. 1F). Together, these data demonstrate that early ART initiation in Fiebig stage I/II limits the magnitude of HIV Gag p24 antigen in LNs, but that Gag p24 can persist predominantly in follicular areas even after 4.5 years of fully suppressive treatment.

Lymph nodes of Fiebig I/II treated individuals harbor HIV-1 RNA

To determine if viral RNA transcription was occurring, which is required to produce infectious virions, we used an *in-situ* hybridization (ISH) assay called RNAscope²⁷ to probe for HIV-1 *gag-pol* RNA within LN sections. 12 Fiebig I/II Tx, 4 Fiebig III-V Tx, 2 late Tx, 4 unTx and 3 HIVneg LN samples were analyzed based on sample availability. Viral RNA was detected as punctate dots in LNs from all HIV infected persons and there were no signals in the HIVneg controls (Figs. 2A, 2B and S2). Productively infected viral RNA⁺ cells were identified as a dense spherical signal, whereas follicular dendritic cell (FDC)-bound virus particles were defined by a diffuse lattice-like pattern consistent with previous reports²⁸. Combined RNAscope[®] ISH *gag-pol* staining with IF staining for CD4⁺ T cells confirmed viral RNA (green) within CD4⁺ T cells (red, Fig. 2C). RNAscope staining was quantified using Fiji²⁹. 10 of 12 Fiebig I/II Tx donors had detectable but significantly lower amounts of HIV RNA compared to late Tx ($P=0.04$) and unTx ($P=0.001$) donors (Fig. 2D). However, there was no difference in RNA density between Fiebig I/II Tx and Fiebig III-V Tx donors. Notably, there was a positive correlation between Gag p24 density measured by IF and *gag-pol*

RNA measured by *in situ* hybridization in Fiebig I/II Tx donors ($P=0.008$; $r=0.8$, Fig. 2E). The results are consistent with the persistence of viral RNA despite very early ART initiation in hyperacute infection and durable plasma virus suppression.

Discordant HIV-1 RNA loads in plasma and lymph nodes

To better define active virus transcription within LN mononuclear (LNMCs) cells and to determine the viral loads in the LNs of aviremic individuals initiated on treatment either very early or later in infection. We measured cell-associated viral loads in LNMCs using a commercial viral load assay Cobas® Ampliprep HIV-1 test. We found a hierarchy of LNMC viral loads with the values lowest in patients that initiated therapy in Fiebig stages I/II (Fig. 2F). Interestingly, neither the peak plasma viral load (Fig. 2G), treatment duration before LN excision (Fig. 2H), nor the time to suppression (Fig. 2I) impacted viral RNA persistence in the LN. Overall, quantifiable amounts HIV RNA persists in the LNs of most Fiebig I/II Tx individuals and the magnitude of LN viral loads was not dependent on the duration of treatment.

Expansion of GCTfh cells in early ART-treated individuals

Identifying the cellular phenotypes of persistent HIV-1 protein and transcripts during therapy will be critical for future anti-HIV interventions. While follicular T helper (Tfh) cells are a key component of the adaptive immune response to HIV-1 infection and provide cognate help to B cells^{30,31} and CD8⁺ T cells^{32,33}, these cells also serve as a major HIV reservoir^{13,34}. Moreover, HIV antigen can be trapped in the follicular dendritic lymphoreticular network within LNs and persist for years³⁵, thus we interrogated persistence within these cell subsets.

Given that Tfh are major targets of HIV infection, we first sought to determine the extent to which early ART mitigates HIV induced Tfh expansion. We defined GCTfh as CD4⁺CD45RA⁻CXCR5^{hi}PD-1^{hi} and nonGCTfh cells as CD4⁺CD45RA⁻CXCR5⁺PD-1⁺ in LNMCs (Fig. 3A) consistent with previous Tfh studies³⁶. HIV negatives (n=9) had very low frequencies of GCTfh cells (median 1,3%, IQR; 0,6% to 1,5%) of antigen experienced (CD45RA⁻) CD4⁺ T cells whereas nonGCTfh cells were 11% (IQR; 8,5% to 13%, Fig. 3B). HIV infection resulted in significant expansion of GCTfh (Fig. 3C). Timing of treatment initiation impacted the extent of GCTfh expansion. Immediate therapy was associated with significant diminution of GCTfh expansion (Fiebig I/II Tx vs healthy controls $P=0.07$), whereas a slight delay in treatment initiation (Fiebig III or later) was associated with significant GCTfh expansion comparable to untreated HIV infection (Fiebig III-V Tx vs healthy controls $P=0.03$) (Fig. 3C). Notably, HIV induced Tfh expansion was restricted to GCTfh, as no significant expansion of nonGCTfh cells were observed between the groups (Fig. 3D). To verify these observations, we quantified the area densities of GCTfh *in situ* using FFPE LNs. Consistent with flow cytometry data GCTfh cell densities were significantly greater in delayed therapy and untreated infection compared to Fiebig I/II Tx and HIV negative controls (Fig. 3E). Together, these data show that early treatment initiated in Fiebig I/II mitigates HIV-induced GCTfh expansion. Reduced HIV targets in GCs might partly explain reduced HIV persistence in LN of individuals who initiate therapy very early.

To gain more insight on cellular targets of HIV infection in LNs, next, we investigated if there was a particular subcellular GCTfh that was selectively expanded. We quantified previously described^{37,38} GCTfh subsets namely; GCTfh1 defined as CXCR3⁺CCR6⁻, GCTfh2 as CXCR3⁻CCR6⁻, GCTfh1-17 as CXCR3⁺CCR6⁺ and GCTfh17 defined as CXCR3⁻CCR6⁺ (Fig. 3F) among our study groups (Fig. 3G) and determined their relationship with HIV Gag p24 densities. While subsets had varying frequencies (Fig. 3G), within the early Tx donors, higher frequency of GCTfh1 was associated with greater degrees of Gag p24 positivity (Fig. 3H), whereas GCTfh17 displayed a reverse trend (Fig. 3I). Overall, these results show that while early treatment mitigates GCTfh responses, subset distribution of Tfh cells might impact virus persistence in early treated LNs.

Lastly, given the notion that HIV-specific CD4⁺ T cells might be more susceptible to infection and contribute to viral persistence³⁹, we used class II tetramers to characterize HIV-specific Tfh responses in LN tissues. DRB1*11:01 and DRB1*13:01 class II tetramers previously described^{38,40} were used to identify HIV-specific CD4⁺ T cells (Fig. 3J). Summary data from the analysis of 3 Fiebig I/II Tx, 2 late Tx and 4 unTx donors within our cohort expressing the class II DRB1*11:01 and DRB1*13:01 alleles, revealed that tetramer-specific GCTfh and nonGCTfh cells were detected in our chronic unTx and Fiebig I/II Tx participants at similar frequencies (Fig. 3K). Phenotypic analysis revealed both GC and nonGC HIV-specific Tfh. These results demonstrate that HIV-specific Tfh responses are induced during early treated HIV infection. However, we did not have sufficient quantities of tetramer⁺ cells to determine if antigen specific CD4⁺T cells are preferentially infected.

CXCR3⁺ Tfh cells harbour a greater burden of persistent HIV RNA in lymph nodes obtained from treated individuals with sustained plasma viral suppression.

Since image analysis for Gag p24 indicated that most of the HIV antigen was confined within discrete regions of GCs, and flow data showed differential correlation between levels of Gag p24 and GCTfh subsets, we next stained for other markers shown to be highly expressed on human Tfh³⁸. We also used FDC markers to identify residual Gag p24 that has been reported to persist on FDCs³⁵. Immunofluorescence imaging of serial sections stained with different combinations of antibodies and detected with Opal fluorophores revealed that Gag p24 co-localized with several phenotypic markers (Figs. 4A and S3), including PD1 (Fig. 4A, panel i), CD4 (panel ii), CXCR3 (panel iii-iv), CCR6 (panel iv) and FDC (panel v). To more definitively identify the Tfh subset that harbored the most HIV infection burden, we quantified HIV RNA in LNMCs isolated from LN tissue of 3 Fiebig I/II Tx, 2 Fiebig III-V Tx and 3 late Tx donors and FACS-sorted into the 4 different Tfh subsets (Fig. 4B). HIV mRNA was detectable using digital droplet PCR in all the subsets (Fig. 4C). Importantly, when we analyzed the cells based on expression of chemokine receptors, CXCR3 and CCR6, we found that CXCR3⁺ Tfh subsets harbored significantly greater amounts of HIV RNA than other subsets ($P=0.005$, Fig. 4C).

To further interrogate preferential infection of CXCR3⁺ Tfh cells, we used a broadly neutralizing antibody (bNAb) called 3BNC117 to stain HIV infected cells while simultaneously staining for CXCR3. 3BNC117

targets the CD4 binding site on the surface of HIV-1 Envelope (Env) glycoprotein⁴¹. LNMC and paired PBMC samples obtained from 7 Fiebig I/II treated donors were analyzed without prior manipulation. Representative flow plots for one donor and aggregate data for 7 donors showed detection of HIV-1 Env (3BNC117) positive LNMCS at significantly greater frequency compared to paired PBMC samples ($P=0.03$, Fig. 4D). To confirm detection of low frequency HIV-1 positive cells *ex vivo*, we intracellularly stained aliquots of the same samples with anti-Gag p24 antibody. Similarly, Gag p24⁺CD4⁺ T cells were readily detectable in LNMCS compared to PBMCs ($P=0.01$, Fig. 4E). We phenotyped infected cells by dual staining of 3BNC117 and CXCR3 and observed a trend towards more Env⁺CD4⁺ T cells co-expressing CXCR3 ($P=0.06$, Fig. 4F) than those not expressing CXCR3. Together, these data suggest that CD4⁺CXCR3⁺ expressing Tfh cells may be preferentially infected *in vivo* compared to other subsets.

Impact of HIV-specific CD4⁺ and CD8⁺ T cell responses on HIV persistence in the lymph node during ART

We previously showed that immediate ART initiation augments HIV-specific T cell function in peripheral blood²⁴. To investigate the effects of early ART on LN responses, we begun by investigating if there were compartmental differences in the frequency of HIV-specific responses between LN and PB. We used intracellular cytokine staining (ICS) to measure the proportions of HIV-specific CD4⁺ and CD8⁺ T cells in LNs and paired blood samples using 15 fully suppressed Fiebig I/II Tx donors on uninterrupted therapy for greater than a year. Representative flow plots for one donor and aggregate data show significantly higher frequency of Gag-specific CD8⁺ T cells ($P=0.05$) compared to PB responses (Fig. 5A). HIV-specific CD4⁺ T cell frequencies also trended towards greater frequencies in LN relative to PB ($P=0.06$; Fig. 5B). Next, we investigated whether HIV-specific CD8⁺ T cell responses limit HIV persistence in the LN, and found a negative correlation between the frequency of HIV-specific CD8⁺ T cell responses and HIV Gag p24 density ($r=-0.7$, $P=0.02$; Fig. 5C). No correlation was observed between peripheral responses and the amount of persistent Gagp24 antigen in the LN, indicating the peripheral responses do not accurately depict HIV persistence in LNs. Notably, there was no correlation between LN or peripheral CD4⁺ T cell responses and persistent HIV Gag p24 in the LN (Fig. 5D).

Considering that proliferative CD8⁺ T cell responses are often associated with protection^{42,43}, we next measured virus-specific responses by carboxyfluorescein succinimidyl ester (CFSE) dilution. Representative flow plots for a donor with low and high Gagp24 density are shown (Fig. 5E). Aggregate data show proliferative Gag-specific CD8⁺ T cell responses negatively correlated with HIV Gag p24 burden ($r=-0.7$, $P=0.04$; Fig. 5F). A similar result was obtained for Gag-specific CD4⁺ T cell responses ($r=-0.7$, $P=0.04$; Fig. 5G). Together, these data show an association between maintenance of functional cellular responses and reduced HIV viral antigens in LNs.

Given most of the residual virus was concentrated within GCs, we next assessed the capacity of HIV-specific T cell responses to traffic into the GCs by enumerating the frequencies of CXCR5⁺ HIV-specific responses in LN, which denote capacity to migrate into GCs. CXCR5⁺ HIV-specific CD8⁺ T cells were significantly lower compared to CXCR5⁺ CD8⁻ T cells ($P=0.001$), suggesting reduced capacity to migrate

into GCs, which may partly explain the observed greater HIV antigen burden in GCs relative to extrafollicular areas (Fig 5H). Moreover, SEB stimulation showed significantly lower proportion of IFN- γ ⁺ CXCR5⁺CD8⁺ T cells relative to CXCR5⁻CD8⁺ T cells (Fig 5I), indicating an inherent deficiency of CXCR5⁺ CD8⁺ T cells to secrete cytokines. Additionally, there was no correlation between plasma CXCL-13, (part of the CXCR5-CXCL-19 axis crucial for recruitment of immune cells into GCs) and density of HIV antigens in LNs (Fig. S4). Together, these data show that reduced functional HIV-specific CD8⁺ T cell responses within GCs might contribute to HIV persistence in this tissue microenvironment.

Discussion

Comprehensive understanding of ART-mediated HIV suppression in tissue sanctuary sites is critical to the design, optimization, and evaluation of curative strategies. Moreover, a therapeutic vaccine for HIV-1 infection would need to induce robust anti-HIV immune responses in ART suppressed individuals to mediate post-treatment viral control. Here, we used a very well characterized cohort of persons with hyperacute HIV infection to conduct a comprehensive analysis of HIV persistence in LNs following ART initiation in Fiebig stage I/II and to elucidate Tfh cell responses which are critical for robust B cell and CD8⁺ T cell functions.

Most donors exhibited persistent HIV antigens in LN despite prompt blunting of initial peak viremia and sustained plasma viral suppression for as long as 55 months, suggesting that early therapy initiation may not fully eradicate persistent virus in lymphoid tissue sites. Immediate therapy reduced GCTfh expansion which is typically associated with dysregulation of B cell responses due to excessive GC reactions in untreated HIV infection^{36,44}. Moreover, mitigated GCTfh responses decreased the number of cellular targets of HIV infection. Importantly, the association between functional immune responses and reduced viral burden in LNs indicates that T cell responses contribute towards elimination of infected cells during therapy. Combined, these data highlight the need to prioritize elimination of active HIV persistence in LNs as a critical step to achieving a cure or prolonged HIV remission off therapy.

Our unique ability to obtain excisional lymph node biopsies in the FRESH cohort allowed for characterization of sites of virus persistence within the LN architecture in persons in whom peak viremia is blunted. The topological analysis of persistent HIV antigens within intact LN tissues identified greater HIV protein antigen burden within B cell follicles. Notably, onset and duration on therapy did not significantly affect the amount of detectable Gag p24 protein, consistent with the notion of rapid HIV reservoir establishment followed by very slow decay rate⁴⁵. Importantly, our data highlight LN GCs as major sites of HIV persistence, with the potential to be a major source of rebound viremia upon treatment interruption.

Using a highly specific *in-situ* hybridization assay, RNAscope^{46,47}, which, in addition to HIV antigen detection provided further evidence in support of persistent HIV transcription in LNs in the face of ART in 86% of very early treated donors. We identified densely spherical signals by RNAscope staining in some

early treated individuals suggestive of productively infected viral RNA⁺ cells, consistent with a previous report in which active HIV RNA transcription was detected in the LNs of patients who initiated therapy in the chronic phase of illness¹³. Moreover, our data reveal heterogeneity in the amount of persistent HIV transcripts in very early treated aviremic individuals despite similar levels of peak viremia and rapid plasma viral suppression kinetics following ART initiation. Viral antigen persistence and ongoing transcription could indicate that HIV continues to cause immune damage in anatomical sites despite full suppression in peripheral blood, but it may also suggest that even in early treated individuals, priming, stimulation and harnessing of HIV-specific immunity for curative strategies will not be insurmountable because functional HIV-specific immunity is preserved.

Identifying cellular reservoirs of HIV in tissues has been a major area of research, (reviewed in ⁴⁸). These studies describe GCTfh subset compositions anatomically and phenotypically during HIV infection and their contributions to persistent virus. The observed positive correlation between the proportions of GCTfh1 cells (which are CXCR3⁺CCR6⁻GCTfh) and detection of greater amounts of HIV RNA relative to CXCR3⁺ Tfh cells, indicates that CXCR3 may yet be another phenotypic marker of Tfh cells that have greater HIV transcription activity on ART. These data are consistent with a study that reported greater amounts of SIV DNA in CXCR3⁺ GCTfh compared to CXCR3⁻ GCTfh in macaques⁴⁹. It is reasonable to attribute increased HIV burden in the CXCR3⁺ Tfh subset to CXCR3 being used as an alternative co-receptor for HIV entry, which has previously been reported⁵⁰. However, confirmatory work is needed. In any case, we have identified a marker for HIV infected cells during ART that could be targeted for elimination as part of an HIV eradication strategy. Whether or not the CXCR3⁺ Tfh population identified in this study represents the same population as the PD-1⁺ subset that was recently implicated in HIV persistence is intriguing and warrants further investigation^{13,51}.

Low CD8⁺ T cell density in GCs is thought to be a major reason for persistently high HIV antigen burden in this anatomical niche^{52,53}. Interestingly, we detected significantly greater proliferative responses in individuals with little to no detectable HIV antigens compared to those with greater LN HIV antigen burden. These findings are consistent with our recent study showing that very early ART is associated with functionally superior cellular responses²⁴. Together, these data suggest that HIV-specific T cell responses contribute to HIV suppression in LN during therapy⁵⁴. However, longitudinal studies using serial biopsies from the same donor are needed to confirm these findings.

A notable limitation of the study is that we could only obtain one LN sample per study participant, thus we could not conduct intra-individual longitudinal HIV decay kinetics, or longitudinally define the immune responses in tissues associated with control. This limitation was partly overcome by sampling LNs from many participants over a very wide time range. Serial LN studies have been reported in the absence of complications ⁵⁵. Alternatively, future studies could attempt serial fine needle aspirates, though cell yields may be limiting for some of the studies described here and the architectural structure would be disrupted. An additional limitation is the lack of gender diversity. Although we studied only women, this is the group

at disproportionate risk of infection and women are underrepresented in most studies to date⁵⁶. These issues can and should be addressed in future studies focused by our findings.

In conclusion, our results demonstrate HIV persistence in LNs despite prompt and durable ART-mediated plasma viral suppression. HIV structural proteins and HIV RNA persist in LNs of in 12 of 14 individuals, albeit at lower levels compared to treatment in chronic infection. Given sample limitations, one cannot rule out persistent infection in the other two individuals. Among those with detectable infection, GCs serve as the primary anatomical site of HIV persistence and the major cellular source is CXCR3 expressing Tfh cells. Together, our results emphasize the importance of very early initiation of ART in Fiebig stage I/II to reduce the amounts of persistent virus in the LN and highlight the need for interventions to completely eradicate residual viremia in immune privileged and anatomically compartmentalized tissue sites.

Methods

Study population, samples, and performance site

Study participants were drawn from the HIV Pathogenesis Programme (HPP) lymph node study (LNS) cohort, Durban, South Africa. Recruitment into the HPP LNS cohort were from the FRESH cohort described in ¹⁶ (n=41) and another primary HIV infection cohort in Durban, South Africa where participant's time of infection is less defined (n=35). Axillary, cervical or inguinal LNs were surgically excised at Prince Mshiyeni Hospital in Umlazi, and 120 ml paired peripheral blood was also obtained from each participant. Viral load measurements were performed by HIV-1 RNA testing using the NucliSens EasyQ v2.0 assay (BioMérieux Clinical Diagnostics, Marcy-l'Étoile, France), through a certified commercial laboratory. CD4⁺ T cell counts were enumerated by Tru-Count technology and analyzed on a FACSCalibur flow cytometer [Becton Dickinson (BD) New Jersey, USA]. Sample processing and laboratory studies were performed at the Africa Health research Institute in Durban, South Africa.

Study approval

All study participants provided written informed consent prior to inclusion in the study. Ethical approval for the study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (protocol number BF298/14) and the Institutional Review Board of Massachusetts General Hospital (protocol number 2015-P001018).

Lymph node and blood sample processing

Excised LNs were sectioned into two, one section was fixed in 10% formal-saline (Sigma-Aldrich, St. Louis, Missouri, USA) for immunofluorescence microscopy (IF) studies, while the second section was macerated to release LN mononuclear cells (LNMCs) according to the method of Schacker, et al. ⁵⁷. The cells were passed through a mesh screen and harvested by centrifugation [625 x *g*, 6 min, room temperature (RT)].

Peripheral blood mononuclear cells (PBMCs) were isolated from patient's blood samples by density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in liquid nitrogen⁵⁸.

Viral RNA quantification in lymph node mononuclear cells (LNMCs)

Cryopreserved LNMCs (10 million cells) were lysed, and viral RNA was quantified using the Cobas[®] AmpliPrep HIV-1 test (Roche, Mannheim, Germany) at an accredited clinical laboratory using standardized protocols.

Immunofluorescence (IF) microscopy

IF microscopy staining was performed on 4 µM sections of formalin fixed paraffin embedded (FFPE) LNs using the Opal 4-color fluorescent IHC kit (PerkinElmer, Waltham, MA, USA). Briefly, following antigen retrieval, two blocking steps (2 x 10 min, RT) were performed using the Dako peroxidase-blocking reagent (Agilent Technologies, Glostrup, Denmark) and Bloxall block (Vector Laboratories, Burlingame, CA, USA). The slides were washed with 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 5 min, sequentially probed with the primary antibody (30 min, RT), and Opal polymer HRP [20 min, RT (PerkinElmer)] and detected using the Opal polymer 520 (10 min, RT). This protocol was repeated for the second and third antibodies with Opal polymers 570 and 690 respectively, followed by counterstaining with spectral DAPI (PerkinElmer) to make a total of 4 different fluorochromes. Primary antibodies used in these combinations include anti-human BCL-6 [(clone PG-B6p) Dako/Agilent Technologies], CCR6 [(R6H1) ThermoFisher Scientific, Waltham MA, USA], CD4 [(clone 4B12) Dako/Agilent Technologies], CXCR3 [(clone 6H1L8) Thermo Fisher Scientific], follicular dendritic cells [(clone CNA.42), Dako/Agilent Technologies] p24 [(clone Kal-1), Dako/Agilent Technologies], and PD-1 [clone NAT105] Abcam, Cambridge, MA, USA]. Slides were mounted with Dako fluorescence mounting medium (Agilent Technologies) and imaged with the Axio Observer, 20x objective lenses, a Hamamatsu C13440-20C camera and TissueFAXS imaging software (TissueGnostics, Vienna, Austria).

RNAscope[®] *in situ* hybridization (ISH)

RNAscope[®] ISH was conducted using the RNAscope[®] 2.5 HD assay kit [Advanced Cell Diagnostics (ACD), Newark, CA, USA, Cat No: 322300] and the RNAscope[®] multiplex fluorescent kit v2.0 (ACD, Cat No: 323100) as per manufacturer's instructions. Briefly, pre-treated samples were hybridized with the clade C HIV-1 *gag-pol* probe (Cat No: 317691) at 40°C for 16 hours. Next, the samples were incubated with signal amplification probes and horseradish peroxidase conjugated secondary antibodies. The signal was detected with either diaminobenzidine for the RNAscope[®] 2.5 HD assay (ACD) or with Opal fluorophores (PerkinElmer) for the multiplex fluorescent assay. Slides were imaged with Axio Observer and TissueFAXS imaging software (TissueGnostics).

Quantitative Image analysis

Quantitative image analysis of Gag p24 in IF images of whole tissue section scans was conducted with TissueQuest software (TissueGnostics). Two independent experiments of total area measurements and nuclear segmentation analyses were performed on each whole tissue scan. The numerical data generated from the analyses are displayed in scattergrams. Greyscale images were analysed and each channel was processed separately by the software using DAPI as a master marker. In cases where images were stained with another nuclear marker such as BCL-6, then the FITC channel was used as a virtual channel for nuclei identification. Negative control slides were used to set the threshold values in the scattergrams and to distinguish specific staining signals from non-specific or background fluorescence signals. Although HIV Gag p24 staining was generally intense, there was no notable spillover of the signal to other channels (Fig. S1A-B). Also, p24 co-staining was only observed with FDCs and CD4 markers but not CD8 cells (Fig. S1C-D).

Analysis of *gag-pol* RNA signals was done using Fiji, an open-source software based on ImageJ which is optimized for biological image analysis²⁹. Briefly, images were segmented using the color segmentation plugin with the algorithm for Hidden Markov Model. Thresholding was applied to the segmented image and the total area of brown RNA signals was measured and recorded. Five images were analysed per sample and averaged. Pixel measurements were converted to mm using the scale bar.

Flow cytometry analysis

Freshly isolated or frozen LNPCs and PBMCs were characterized using flow cytometry analysis with standardized protocols⁵⁹. Cells were stained with LIVE/DEAD Fixable Blue dead cell stain kit (Thermo Fisher Scientific), CD3 Brilliant Violet (BV) 711 (BioLegend, San Diego, CA, USA), CD8 BV786 (BD Biosciences, San Jose, CA), CD4 BV650 (BD Biosciences) CXCR5 Alexa Fluor (AF) 488 (BD Biosciences), PD-1 BV421 (BioLegend), CCR6 Phycoerythrin [(PE) BioLegend], CXCR3 BV605 (BioLegend) and CD45RA PE-Cyanine (Cy)-7 (BioLegend), for 30 mins at RT.

For intracellular cytokine staining, PBMCs or LNPCs were either left unstimulated or stimulated with HIV clade C overlapping peptide (OLP) pools spanning Gag, Nef, or Env proteins or Staphylococcal enterotoxin B (SEB, 0.5 µg/ml) in the presence of GolgiStop and GolgiPlug protein transport inhibitors (BD Biosciences) for 16 hours at 37°C, prior to surface staining with the panel of antibodies comprising LIVE/DEAD fixable Aqua dead cell stain (Thermo Fisher Scientific), CD3 BV711, CD4 BV650 and CD8 BV786. After fixation and permeabilization with the BD Cytofix/Cytoperm kit (BD Biosciences), cells were again stained using TNF-α A700 (BD Biosciences) and IFN-γ PE-Cy7 (BioLegend) antibodies.

T cell proliferation was measured by labelling LNPCs with carboxyfluorescein succinimidyl ester (CFSE), stimulating cells with HIV clade C OLP pools for 7 days and staining with CD3 BV711, CD4 BV650 and CD8 BV786. Stained cells were acquired using an LSRFortessa (BD Biosciences) with FACSDiva™ software or sorted using the FACS Aria Fusion (BD Biosciences). Data were analysed using the FlowJo version 10.0.8 (Flowjo, LLC, Ashland, Oregon).

HIV infected cells were identified by surface staining with biotinylated 3BNC117 antibody followed by streptavidin PE (ThermoFisher Scientific) and/or intracellular staining with HIV Gag p24 RD1 [(clone KC57), Beckman Coulter, Indianapolis, USA] after fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences).

HLA Class II tetramer studies

HIV-specific Tfh responses were defined using fluorochrome conjugated HLA class II tetramers. Briefly, cells were stained for 1 hour at 37°C with APC and PE conjugated HLA Class II tetramer complexes, washed in 2% FCS-PBS and then stained with these antibodies: LIVE/DEAD Fixable Blue dead cell stain kit (ThermoFisher Scientific), CD3 BV711 (Biolegend), CD4 BV650 (BD Biosciences), CD8 BV786 (BD Biosciences), CXCR5 AF488 (BD Biosciences), CXCR3 BV605 (Biolegend), PD-1 BV421 (Biolegend) and CD45RA AF700 (Biolegend); for 20 mins at RT. Cells were washed and acquired on the LSRFortessa (BD Biosciences).

Digital droplet PCR

Total RNA was extracted from FACS-sorted LNMC Tfh subsets using Qiagen RNeasy kit (Qiagen) after lysing cells with QIAzol lysis reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions, and used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was used as a template for HIV *gag* mRNA quantification by TaqMan digital droplet PCR assay using custom probes (Assay ID: APCE4R6, Thermo Fisher Scientific) in a two-step digital droplet PCR reaction. PCR thermal cycling was conducted following optimized cycling conditions: an initial denaturation at 95°C for 10 min, 40 cycles of 30 seconds at 94°C, 1 min at 60°C, followed by a final incubation at 98°C for 10 min and holding at 4°C until reading time. After PCR amplification, droplets were measured in the QX200 ddPCR Droplet Reader (Bio-Rad), and target gene copy number was analyzed using QuantaSoft analysis software (Bio-Rad) and recorded as mRNA copies/20µL. Absolute *gag* mRNA counts were normalized to the expression of the housekeeping gene b2M.

Statistical analyses

All statistical analyses were conducted with GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA) and *P* values were considered significant if less than 0.05. Specifically, the Mann-Whitney U and Kruskal-Wallis H tests were used for group comparisons. Additional post hoc analyses were performed using the Dunn's multiple comparisons test. Correlations between variables were defined by the Spearman's rank correlation test.

Declarations

Conflict of interest statement

The authors have no conflicting financial interests.

Online supplemental material

Fig. S1 shows that there is no co-localization of Gag p24 signals with non-infectious cell subsets like CD8⁺ T cells and validates the image quantitative analyses using a different approach of nuclear segmentation. Fig. S2 displays representative images of HIV RNA detected in lymph node sections by *in situ* hybridization. Fig. S3 demonstrates the co-localization of Gag p24 antigens with cells expressing PD1⁺, CD4⁺, CXCR3⁺ and CCR6⁺ surface markers. Fig. S4 shows that the low antigen persistence within lymph nodes is not associated with plasma CXCL-13 levels.

AUTHOR CONTRIBUTIONS

ZMN, BDW, KD and TN initiated the study-cohorts. ZMN conceived the study. ZMN and OOB, designed the experiments. IJ and JP performed the lymph node biopsies. OOB performed digital droplet PCR, flow cytometry, viral RNA measurements and imaging experiments assisted by TK, ThaN and FO under the supervision of ZMN. OOB and ZMN analyzed the data. OOB, ZMN and JM wrote the manuscript. TN and BDW edited the manuscript.

ACKNOWLEDGEMENTS

We would like to thank our study participants, the laboratory and clinic staff at FRESH and the HIV Pathogenesis Programme (HPP), Durban, South Africa and Drs Shiv Pillai and Takashi Maehara of the Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA, USA, for training on immunofluorescence microscopy. We acknowledge Dr Faatima Laher of HPP and Dr Jackson Marakalala of the Africa Health Research Institute, Durban, South Africa for technical assistance with digital droplet PCR and CXCL-13 ELISA studies, respectively.

We would like to acknowledge the following funding sources; HHMI International research scholar award (Grant #55008743 to ZMN); the US National Institutes of Health (NIAID) (R01AI145305 to ZMN, R37 AI67073 to BDW); a Dan and Marjorie Sullivan Research scholar 97 Award (Grant # 224910); a Doctoral Innovation Scholarship from the South African National Research Foundation (NRF) (2014-2016 to OOB); the International AIDS Vaccine initiative (IAVI, grant number UKZNRSA1001), and the South African Research Chairs Initiative. Additional funding was from the Mark and Lisa Schwartz Foundation, the Bill and Melinda Gates Foundation, the International AIDS Vaccine Initiative (IAVI, grant number UKZNRSA1001), the Ursula Brunner Fund at the Ragon Institute and the Victor Daitz Foundation. This work was also partially supported by Gilead Sciences Incorporated and the Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative (grant # DEL-15-006). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD 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 author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

References

1. Murray, A.J., Kwon, K.J., Farber, D.L. & Siliciano, R.F. The Latent Reservoir for HIV-1: How Immunologic Memory and Clonal Expansion Contribute to HIV-1 Persistence. *J Immunol* **197**, 407-417 (2016).
2. Puertas, M.C., *et al.* Lack of concordance between residual viremia and viral variants driving de novo infection of CD4(+) T cells on ART. *Retrovirology* **13**, 51 (2016).
3. Chun, T.W. Tracking replication-competent HIV reservoirs in infected individuals. *Curr Opin HIV AIDS* **8**, 111-116 (2013).
4. Martinez-Picado, J. & Deeks, S.G. Persistent HIV-1 replication during antiretroviral therapy. *Curr Opin HIV AIDS* **11**, 417-423 (2016).
5. Connick, E., *et al.* CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol* **178**, 6975-6983 (2007).
6. Fletcher, C.V., *et al.* Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. *Proceedings of the National Academy of Sciences* **111**, 2307-2312 (2014).
7. Lorenzo-Redondo, R., *et al.* Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature* **530**, 51-56 (2016).
8. McManus, W.R., *et al.* HIV-1 in lymph nodes is maintained by cellular proliferation during antiretroviral therapy. *The Journal of Clinical Investigation* **129**, 4629-4642 (2019).
9. Musick, A., *et al.* HIV Infected T Cells Can Proliferate in vivo Without Inducing Expression of the Integrated Provirus. *Front Microbiol* **10**, 2204 (2019).
10. Rasmussen, T.A., *et al.* The effect of antiretroviral intensification with dolutegravir on residual virus replication in HIV-infected individuals: a randomised, placebo-controlled, double-blind trial. *Lancet HIV* **5**, e221-e230 (2018).
11. Hatano, H., *et al.* Increase in 2-long terminal repeat circles and decrease in D-dimer after raltegravir intensification in patients with treated HIV infection: a randomized, placebo-controlled trial. *J Infect Dis* **208**, 1436-1442 (2013).
12. Real, F., *et al.* Platelets from HIV-infected individuals on antiretroviral drug therapy with poor CD4(+) T cell recovery can harbor replication-competent HIV despite viral suppression. *Sci Transl Med* **12**, eaat6263 (2020).

13. Banga, R., *et al.* PD-1(+) and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals. *Nature medicine* **22**, 754-761 (2016).
14. Colby, D.J., *et al.* Rapid HIV RNA rebound after antiretroviral treatment interruption in persons durably suppressed in Fiebig I acute HIV infection. *Nature medicine* **24**, 923-926 (2018).
15. Saez-Cirion, A., *et al.* Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS Pathog* **9**, e1003211 (2013).
16. Namazi, G., *et al.* The Control of HIV After Antiretroviral Medication Pause (CHAMP) Study: Posttreatment Controllers Identified From 14 Clinical Studies. *The Journal of Infectious Diseases* **218**, 1954-1963 (2018).
17. Deeks, S.G., *et al.* International AIDS Society global scientific strategy: towards an HIV cure 2016. *Nature medicine* **22**, 839-850 (2016).
18. Dong, K.L., *et al.* Detection and treatment of Fiebig stage I HIV-1 infection in young at-risk women in South Africa: a prospective cohort study. *Lancet HIV* **5**, e35-e44 (2018).
19. Buzon, M.J., *et al.* Long-term antiretroviral treatment initiated at primary HIV-1 infection affects the size, composition, and decay kinetics of the reservoir of HIV-1-infected CD4 T cells. *J Virol* **88**, 10056-10065 (2014).
20. Henrich, T.J., *et al.* HIV-1 persistence following extremely early initiation of antiretroviral therapy (ART) during acute HIV-1 infection: An observational study. *PLOS Medicine* **14**, e1002417 (2017).
21. Hatano, H., *et al.* Prospective Antiretroviral Treatment of Asymptomatic, HIV-1 Infected Controllers. *PLoS pathogens* **9**, e1003691 (2013).
22. Frange, P., *et al.* HIV-1 virological remission lasting more than 12 years after interruption of early antiretroviral therapy in a perinatally infected teenager enrolled in the French ANRS EPF-CO10 paediatric cohort: a case report. *Lancet HIV* **3**, e49-54 (2016).
23. Okoye, A.A., *et al.* Early antiretroviral therapy limits SIV reservoir establishment to delay or prevent post-treatment viral rebound. *Nature medicine* **24**, 1430-1440 (2018).
24. Ndhlovu, Z.M., *et al.* Augmentation of HIV-specific T cell function by immediate treatment of hyperacute HIV-1 infection. *Sci Transl Med* **11**, eaau0528 (2019).
25. Goteri, G., *et al.* Comparison of germinal center markers CD10, BCL6 and human germinal center-associated lymphoma (HGAL) in follicular lymphomas. *Diagnostic Pathology* **6**, 97-97 (2011).

26. Schmid, M., *et al.* An Emerging Approach for Parallel Quantification of Intracellular Protozoan Parasites and Host Cell Characterization Using TissueFAXS Cytometry. *PLoS One* **10**, e0139866 (2015).
27. Wang, F., *et al.* RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn* **14**, 22-29 (2012).
28. Pantaleo, G., *et al.* HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362**, 355-358 (1993).
29. Schindelin, J., *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682 (2012).
30. Crotty, S. Follicular helper CD4 T cells (TFH). *Annual review of immunology* **29**, 621-663 (2011).
31. Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* **41**, 529-542 (2014).
32. Chevalier, M.F., *et al.* HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function. *J Virol* **85**, 733-741 (2011).
33. Schultz, B.T., *et al.* Circulating HIV-Specific Interleukin-21(+)CD4(+) T Cells Represent Peripheral Tfh Cells with Antigen-Dependent Helper Functions. *Immunity* **44**, 167-178 (2016).
34. Perreau, M., *et al.* Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* **210**, 143-156 (2013).
35. Popovic, M., *et al.* Persistence of HIV-1 structural proteins and glycoproteins in lymph nodes of patients under highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14807-14812 (2005).
36. Lindqvist, M., *et al.* Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *The Journal of clinical investigation* **122**, 3271-3280 (2012).
37. Schmitt, N., Bentebibel, S.E. & Ueno, H. Phenotype and functions of memory Tfh cells in human blood. *Trends Immunol* **35**, 436-442 (2014).
38. Baiyegunhi, O., *et al.* Frequencies of circulating Th1-biased T follicular helper cells in acute HIV-1 infection correlate with the development of HIV-specific antibody responses and lower set point viral load. *J Virology* **92**, e00659-00618 (2018).
39. Hunt, P.W., *et al.* HIV-Specific CD4+ T Cells May Contribute to Viral Persistence in HIV Controllers. *Clinical Infectious Diseases* **52**, 681-687 (2011).
40. Laher, F., *et al.* HIV Controllers Exhibit Enhanced Frequencies of Major Histocompatibility Complex Class II Tetramer+ Gag-Specific CD4+ T Cells in Chronic Clade C HIV-1 Infection. *J Virol* **91**(2017).

41. Scheid, J.F., *et al.* HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. *Nature* **535**, 556-560 (2016).
42. Gaiha, G.D., *et al.* Structural topology defines protective CD8⁺ T cell epitopes in the HIV proteome. *Science* **364**, 480-484 (2019).
43. Ndhlovu, Z.M., *et al.* High-dimensional immunomonitoring models of HIV-1 –specific CD8 T-cell responses accurately identify subjects achieving spontaneous viral control. *Blood* **121**, 801-811 (2013).
44. Cubas, R.A., *et al.* Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nature Medicine* **19**(2013).
45. Whitney, J.B., *et al.* Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* **512**, 74-77 (2014).
46. Deleage, C., *et al.* Defining HIV and SIV Reservoirs in Lymphoid Tissues. *Pathog Immun* **1**, 68-106 (2016).
47. Barton, K.M. & Palmer, S.E. How to Define the Latent Reservoir: Tools of the Trade. *Current HIV/AIDS reports* **13**, 77-84 (2016).
48. Wong, J.K. & Yukl, S.A. Tissue reservoirs of HIV. *Current opinion in HIV and AIDS* **11**, 362-370 (2016).
49. Velu, V., *et al.* Induction of Th1-Biased T Follicular Helper (Tfh) Cells in Lymphoid Tissues during Chronic Simian Immunodeficiency Virus Infection Defines Functionally Distinct Germinal Center Tfh Cells. *J Immunol* **197**, 1832-1842 (2016).
50. Hatse, S., *et al.* Modest human immunodeficiency virus coreceptor function of CXCR3 is strongly enhanced by mimicking the CXCR4 ligand binding pocket in the CXCR3 receptor. *J Virol* **81**, 3632-3639 (2007).
51. Banga, R., *et al.* Blood CXCR3(+) CD4 T Cells Are Enriched in Inducible Replication Competent HIV in Aviremic Antiretroviral Therapy-Treated Individuals. *Front Immunol* **9**, 144 (2018).
52. Fukazawa, Y., *et al.* B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nature medicine* **21**, 132-139 (2015).
53. Li, S., *et al.* Low levels of SIV-specific CD8⁺ T cells in germinal centers characterizes acute SIV infection. *PLoS pathogens* **15**, e1007311-e1007311 (2019).
54. Miles, B. & Connick, E. TFH in HIV Latency and as Sources of Replication-Competent Virus. *Trends in Microbiology* **24**, 338-344 (2016).

55. Chintanaphol, M., *et al.* Brief Report: Safety and Tolerability of Inguinal Lymph Node Biopsy in Individuals With Acute HIV Infection in Thailand. *J Acquir Immune Defic Syndr* **79**, 244-248 (2018).
56. Karim, S.S.A. & Baxter, C. HIV incidence rates in adolescent girls and young women in sub-Saharan Africa. *Lancet Glob Health* **7**, e1470-e1471 (2019).
57. Schacker, T.W., *et al.* Lymphatic Tissue Fibrosis Is Associated with Reduced Numbers of Naïve CD4 T Cells in Human Immunodeficiency Virus Type 1 Infection. *Clinical and Vaccine Immunology* **13**, 556-560 (2006).
58. McCoy, J.P., Jr. Handling, storage, and preparation of human blood cells. *Curr Protoc Cytom* **Chapter 5**, Unit 5 1 (2001).
59. Ndhlovu, Z.M., *et al.* Magnitude and Kinetics of CD8+ T Cell Activation during Hyperacute HIV Infection Impact Viral Set Point. *Immunity* **43**, 591-604 (2015).

Tables

Table 1. Characteristics of study participants at the time of lymph node excision

Characteristic	Fiebig stage I/II treated	Fiebig stage III-V treated	Late treated ^A	Untreated	HIV negative
No. of participants (% female)	14 (100%)	5 (100%)	17 (94%)	15 (80%)	13 (100%)
Median (IQR)^C age of participants (years)	22 (20-24)	22 (19-24)	24 (22-27)	24 (24-29)	22 (21-23)
Median (IQR) CD4 Count (no. of cells/ml)	911 (717-1120)	942 (696-1086)	589 (454-792)	595 (401-720)	976 (792-1180)
Median (IQR) plasma viral load copies/ml	<20 (<20-<20)	<20 (<20-<20)	<20 (<20-<20)	11000 (1900-22000)	NA ^B
Median (IQR) days on treatment	370 (31-550)	120 (46-677)	571 (100-762)	NA	NA

^ADonors whose Fiebig stage of infection was either Fiebig VI or unknown are defined as late treated.

^BNA, not applicable.

QIQR: Interquartile range.

Figures

Figure 1

HIV Gag p24 persists in germinal centers (GCs) despite early ART initiated in Fiebig stages I/II. (A) IF images of Gag p24 antigen (yellow) and BCL-6 (green) staining in lymph node (LN) sections and a scatter plot showing CD4⁺ T cell counts, plasma viral loads and the time of LN excision for a Fiebig I treated (Tx) participant. Nuclei are counterstained with DAPI (blue) and scale bar is 20 μ m. (B) Gag p24 density per GC computed from TissueQuestTM analysis of IF LN sections for 2 HIV negative and 14 Fiebig I/II Tx donors and (C) correlation analyses of average density of Gag p24 per donor and the treatment duration prior to LN excision. (D) Representative IF images of Gag p24 antigen (yellow) and BCL-6 (green) staining (scale bar is 50 μ m) and (E) aggregate data from TissueQuestTM analysis of IF LN sections for 14 Fiebig I/II Tx, 3 Fiebig III-V Tx, 8 late Tx and 13 untreated (unTx) HIV-infected individuals. Each dot represents the density of Gag p24 per GC and the total number of GCs analysed per group is displayed. (F) Comparison of the total percentage area staining for Gag p24 within GCs and outside the GCs (EF) for all donors. The median and interquartile range adjusted P values from Dunn's test (E) or a Mann U-Whitney P value (F) are shown. Spearman rho (r) values and P values are reported for correlation analyses (C). Dotted line denotes threshold of detection (B).

Figure 2

Figure 2

HIV-RNA detection in lymph nodes (LN) of Fiebig I/II treated individuals using RNAscope (A-E) and Cobas® AmpliPrep HIV-1 test (F-I). (A) RNAscope hybridization for HIV gag-pol RNA was detected using 3, 3'-diaminobenzidine (DAB, brown) or (B) fluorescent Opal polymers (green). Representative images for HIV negative, Fiebig I/II treated (Tx), late Tx and untreated (unTx) HIV-infected LN sections are shown. Single RNA transcripts are seen as punctate dots; clusters of transcripts are also observed. Red

arrowheads identify HIV RNA+ cells and green arrowheads identify virions on follicular dendritic cells. (C) Images showing multiplexed RNAscope gag-pol hybridization (green) coupled with IF staining for CD4+ cells (red). Scale bars are 50 μm (A) or 20 μm (B & C). (D) RNA signals quantified using Fiji was compared for 12 Fiebig I/II Tx, 4 Fiebig III-V Tx, 2 late Tx and 4 unTx LNs. (E) A correlation analysis of area staining of gag-pol RNA and Gag p24 density for Fiebig I/II Tx LNs. (F) Viral RNA loads are quantified in lymph node mononuclear cells (LNMCS) isolated from 7 Fiebig I/II Tx, 4 Fiebig III-V Tx, 7 late Tx and 9 unTx LNs. Viral loads below the limits of detection of the assay are assigned a value of 20. (G-I) Correlation analysis of LNMCS' viral loads with (G) peak plasma viral loads, (H) treatment duration, and (I) time to suppression for 7 Fiebig I/II Tx and 4 Fiebig III-V donors. Mann U-Whitney P and Spearman rho (r) and P values are reported. Dotted line denotes threshold of viral load detection.

Figure 3

Figure 3

Expansion of Germinal center T follicular helper (GCTfh) cells during HIV-1 infection. (A-D) (A) Representative flow cytometry plots, (B) pie chart and (C) summary plots comparing the proportions of GCTfh (CXCR5hiPD1hi), and (D) nonGCTfh (PD-1+CXCR5+) cells in HIV negative (HIVneg), Fiebig I/II treated (Tx), Fiebig III-V Tx, Late Tx and untreated HIV-infected (unTx) groups. (E) Representative images showing lymph node sections stained with BCL-6 (green) to define germinal centers and CD4 (red), or PD-

1 (red) to localize GCTfh cells and aggregate results for the area density of GCTfh cells computed using TissueQuest (TissueGnostics, Vienna) software. (F) Representative flow cytometry plot showing gating for Tfh subsets and (G) aggregate data across the groups. (H) Correlation analysis of Gag p24 density measured using image cytometry with GCTfh1 and (I) GCTfh17 subsets distribution measured using flow cytometry. (J) Gating strategy for identifying double-positive tetramer specific (Tet++) CD4+ T cells and overlay plots of Tet++ CD4+ T cells (red dots) on Tfh subsets with (K) aggregate data of Tet++ Tfh subsets across the groups. Mann U-Whitney P and Spearman rho (r) and P values are reported.

Figure 4

CXCR3⁺ Tfh cells contribute to HIV persistence in treated hyperacute HIV-1 infection. (A) The co-localization of Gag p24 antigens with cells expressing (i) PD1⁺, (ii) CD4⁺, (iii) CXCR3⁺ and (iv) CCR6⁺ surface markers and (v) follicular dendritic cells (FDC) are assessed by immunofluorescence microscopy. Representative IF images characterizing HIV Gag p24⁺ cells in the germinal centers (BCL-6⁺). Green, red, and orange signals are from Opal fluorophores 520, 570 and 690 (PerkinElmer) and nuclei are counterstained with DAPI (blue)]. (B) Representative flow cytometry plots showing gating for FACs sorted Tfh subsets. (C) HIV mRNA quantified in FACs sorted Tfh subsets using digital droplet PCR. Absolute numbers of quantified HIV transcripts are equated to absolute cell numbers determined using the expression of 2M. Amounts of HIV mRNA within CXCR3⁺ and CXCR3⁻ subsets are also compared. (D) LNMCs surface stained with 3BNC117 monoclonal antibody, flow plots and aggregate data show proportion of HIV-infected (3BNC117) CD4⁺ T cells, (E) LNMCs intracellularly stained with KC57 (Gag p24), flow plots and aggregate data show proportion of HIV-infected (KC57⁺) CD4⁺ T cells. (F) Representative flow plot and aggregate data show proportion of 3BNC117⁺ CD4⁺ T cells that either co-express or do not express CXCR3. Statistical differences are calculated using Mann U-Whitney and Kruskal-Wallis tests.

Figure 5

Figure 5

HIV specific CD8+ T cell responses limit the amount of persistent HIV antigens in lymph nodes during ART. (A-B) Intracellular cytokine staining was conducted after stimulating PBMCs and lymph node mononuclear cells (LNMCs) with HIV-Gag. (A) Representative flow cytometry plots and aggregate data of 15 donors showing IFN- γ + CD8+ T cells, and (B) IFN- γ + CD4+ T cells after stimulation with HIV-1 clade C Gag peptide pools. (C-D) Correlation analysis of average Gag p24 density; measured from image

analysis, with the frequency of (C) IFN- γ +CD8+ T cells and (D) IFN- γ +CD4+ T cells in LNMCs and PBMCs. (E) Representative flow cytometry plots of CFSE-labelled CD4+ and CD8+ T cells after 7-days of stimulation of LNMCs with HIV-1 clade C Gag peptide pools. (F) Aggregate data correlating IFN- γ +CD8+ T cell responses and Gag p24 density. (G) Aggregate data correlating IFN- γ +CD4+ T cell responses and Gag p24 density. (H) Representative flow plot and aggregate data showing frequency of HIV Gag-specific CXCR5+ CD8+ T cells. (I) SEB-specific (IFN γ +) CD8+ T cells expressing CXCR5. Mann U-Whitney P and Spearman rho (r) and P values are reported.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryfiguresandtablesOct2021.pdf](#)