The receptor repertoire and functional profile of follicular T cells in HIV-infected lymph nodes

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Follicular helper CD4+ T cells (TFH) play an integral role in promoting B cell differentiation and affinity maturation. Whereas TFH cell frequencies are increased in lymph nodes (LNs) from individuals infected with HIV, humoral immunity remains impaired during chronic HIV infection. Whether HIV inhibits TFH responses in LNs remains unclear. Advances in this area have been limited by the difficulty of accessing human lymphoid tissues. Here, we combined high-dimensional mass cytometry with T cell receptor repertoire sequencing to interrogate the composition of TFH cells in primary human LNs. We found evidence for intact antigen-driven clonal expansion of TFH cells and selective utilization of specific complementarity-determining region 3 (CDR3) motifs during chronic HIV infection, but the resulting TFH cells acquired an activation-related TFH cell signature characterized by interleukin-21 (IL-21) dominance. These IL-21+ TFH cells contained an oligoclonal HIV-reactive population that preferentially accumulated in patients with severe HIV infection and was associated with aberrant B cell distribution in the same LN. These data indicate that TFH cells remain capable of responding to HIV antigens during chronic HIV infection but become functionally skewed and oligoclonally restricted under persistent antigen stimulation.

INTRODUCTION

Follicular helper T cells (TFH) provide key signals necessary for B cell recruitment and selection to generate protective antibody responses (1, 2). During untreated chronic HIV infection, TFH cells become highly expanded in the lymph nodes (LNs) (3, 4). Despite this, HIV+ patients generate diminished protective antibody responses against immune challenges. For example, HIV-infected individuals produce lower titers of antibodies and less durable responses to seasonal influenza vaccines (5, 6). The prevailing model suggests that TFH cells from HIV patients are ineffective at providing B cell help based on in vitro assays that showed less robust antibody production by B cells cocultured with TFH cells from HIV+ patients (7–9). A proposed mechanism for this involves up-regulation of programmed cell death–ligand 1 (PD-L1) by B cells, which interacts with programmed cell death–1 (PD-1) on TFH cells to inhibit T cell receptor (TCR)–dependent activation of TFH cells (7). However, the extent to which TFH cells express impaired antigen responsiveness in vivo remains unclear. Because TFH cells need to appropriately sense antigen signals to discriminate between B cells, defective response to antigen not only impairs provision of T cell help to individual B cells but also imperils the process of B cell selection on a global level.

Here, we interrogated the functional phenotype and TCR repertoire composition of primary TFH cells isolated directly from LNs from HIV+ individuals. We used the presence or absence of antigen-dependent TCR signatures to address the responsiveness of TFH cells to antigen engagement and applied high-dimensional mass cytometry to elucidate how HIV infection alters the functional phenotype of TFH cells in the lymphoid compartment. Our data revealed clonal expansion and convergent selection for Gag-reactive TCRs in TFH cells in the germinal centers (GCs) of HIV-infected LNs, indicating that TFH cells remain capable of responding to HIV antigens during chronic HIV infection. However, TFH cells in LNs from HIV+ individuals acquire an activated phenotype dominated by interleukin-21 (IL-21) production, which was less polyfunctional and correlated with aberrant changes in B cell development. By combining antigen-specific analyses with single-cell TCR sequencing, we further demonstrated that IL-21+ TFH cells contained an HIV-reactive population expressing a restricted TCR repertoire and GC phenotype. Thus, TCR-directed response to HIV alters TFH cell diversity and composition in the lymphoid compartment.

RESULTS

HIV-infected LNs contain clonally expanded GC TFH cells

LNs from untreated HIV+ patients contain a high frequency of TFH cells, but the mechanism that drives expansion of TFH cells remains unclear. The enrichment of HIV antigens (10, 11) and the highly proinflammatory milieu (12, 13) in the LNs could lead to antigen-driven and/or bystander T cell expansion. To address whether proliferation of TFH cells is antigen-dependent, we tested whether HIV induces selective proliferation of certain T cell clones. We focused on GC TFH cells because the frequency of these cells becomes greatly increased during chronic HIV infection (3, 4). To identify GC TFH
cells, we selected memory CD4+ T cells that express T<sub>FH</sub> cell markers CXCR5 and PD-1. CD57 is a glycan carbohydrate epitope expressed by T<sub>FH</sub> cells in the GC, and we used this marker to further demarcate the GC subset (14–17). Naïve CD4+ T cells were identified by CD45RO<sup>-</sup>CXCR5<sup>-</sup>CD57<sup>-</sup>CCR7<sup>+</sup> expression, and memory CD4+ T cells were identified by CD45RO<sup>+</sup>CXCR5<sup>-</sup>staining and excluded for PD-1 and inducible T cell costimulator (ICOS)–positive cells (Fig. 1A). We sorted 1464 to 15,000 naïve, memory, and GC T<sub>FH</sub> cells from freshly thawed LN samples and analyzed the TCR sequences of these subsets using a molecular identifier (MID)–based approach to increase the accuracy of repertoire sequencing (table S1) (18, 19). Because the variability of TCR sequences is encoded in the complementarity-determining region 3 (CDR3) region, we used the number of transcripts detected for a particular CDR3 sequence to define TCR clone size.

We first quantified the degree of relative clonal expansion, we categorized TCR frequency into three groups, ranging from rare (<0.1%) to >2%, according to the clone size relative to the total TCR transcripts detected in that sample. As expected, the TCR repertoire of naïve CD4+ T cells was composed mostly of rare clones. In contrast, the TCR repertoire of GC T<sub>FH</sub> cells had a much higher fraction of TCRs occupied by abundant clones (>0.1%) compared with naïve and memory CD4+ T cells (Fig. 1B and fig. S1). The degree of TCR clonal expansion was quantified by normalized Shannon entropy (NSE) (20, 21). Consistent with the hypothesis that the increase in GC T<sub>FH</sub> cell frequency is due to selective proliferation of certain T cells, GC T<sub>FH</sub> cells had a lower NSE score compared with naïve and memory cells (Fig. 1C). Together, our data demonstrated a notable expansion of clone size in GC T<sub>FH</sub> cell populations.

**TCRs from GC T<sub>FH</sub> cells exhibit signatures of antigen-driven clonal convergence.**

Next, to test whether clonal expansion in GC T<sub>FH</sub> cells from HIV-infected LNs was antigen-driven, we analyzed the TCR sequences for evidence of convergence to the same amino acid sequence from distinct nucleotide sequences. Unlike B cells, which can undergo somatic hypermutation, the TCR sequence of a naïve T cell is determined during maturation in the thymus and remains fixed throughout the life spans of the T cell and its progeny. Thus, with the exception of clones that express two TCRα or TCRβ sequences, distinct TCR nucleotide sequences necessarily arise from distinct naïve T cells. However, multiple nucleotide sequences of different TCRs may encode the same amino acid sequence. These degenerate TCR sequences are typically rare, and the presence of these sequences suggests antigen selection pressure that favors certain TCR motifs that recognize particular antigen(s). Thus, having highly abundant CDR3 amino acid sequences that are encoded by multiple distinct nucleotide sequences indicates preferential expansion of T cells with that specificity (20). On the other hand, we would not expect multiple nucleotide sequences to converge on the amino acid level in the absence of strong antigen-driven selection. Following this logic, we translated the TCR nucleotide sequences into amino acid sequences and tallied the number of different nucleotide sequences that encode each CDR3 amino acid sequence. These CDR3 amino acid sequences can be broken into four quadrants—Q1, Q2, Q3, and Q4—based on the level of degeneracy and frequency in the repertoire (Fig. 2A and fig. S2). Q1 contains highly expanded amino acid CDR3 sequences that are encoded by two or more nucleotide sequences (Fig. 2B). These degenerate, abundant clones likely arose from strong antigen-driven selection and proliferation. Q2 contains low-frequency amino acid CDR3 sequences that are also encoded by two or more nucleotide sequences. Degenerate clones can stochastically arise in the repertoire, but these are typically rare as reflected by the low frequency of nonclonally expanded sequences in Q2. Q3 contains amino acid CDR3 sequences that show neither clonal expansion nor amino acid convergence and make up most of the repertoire. Q4 contains expanded amino acid CDR3 sequences derived from a single nucleotide sequence and are therefore nondegenerate. This TCR degeneracy analysis revealed a significantly higher degree of antigen-driven clonal convergence in GC T<sub>FH</sub> cells compared with naïve and memory T cells (Fig. 2C). Together with the NSE decrease in GC T<sub>FH</sub> cells, these data provide further evidence that antigen-driven clonal expansion is preserved in GC T<sub>FH</sub> cells.

**HIV promotes selective expansion of HIV-reactive T<sub>FH</sub> cells.**

To determine whether clonally expanded and/or convergently selected TCRs include HIV-specific sequences, about 2 to 3 million thawed LN cells were cultured with an HIV-1 consensus B Gag peptide pool for 3 to 4 weeks and then restimulated with the same peptide pool for 4 hours to identify antigen-specific T cells by CD40L and CD69 up-regulation (fig. S3). LN cells were also stimulated with an overlapping set of hemagglutinin (HA) peptides from influenza virus (A/California/7/2009) as a non-HIV control. TCRs from CD40L<sup>+</sup>CD69<sup>+</sup> Gag- or HA-reactive T cells were used to generate a reference TCR panel (table S3). These antigen-specific TCR sequences were mapped onto our bulk T cell sequencing data from freshly thawed LN cells.
features of T<sub>FH</sub> cells (table S4). Cryopreserved cervical LN from 25 Mexican HIV<sup>+</sup> patients were obtained and analyzed together with cervical, mesenteric, and iliac LNs from seven local healthy controls (HCs) (table S5). To interrogate the functional potential of T<sub>FH</sub> cells, 3 to 5 million cryopreserved LN cells were thawed and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A and monensin for 5 hours. Cells were then stained with metal-conjugated antibodies and acquired on the CyTOF 2 mass cytometer. Bead-based standards were used to correct for machine performance and batch differences (fig. S5) (22). To broadly define CD4<sup>+</sup> T cells with T<sub>FH</sub> cell features, we performed manual gating to select CD4<sup>+</sup> T cells positive for CXCR5 and CD45RO staining. GC T<sub>FH</sub> cells were further identified by PD-1 and CD57 expression and imported into Cytobank (Fig. 4A and fig. S6). T-distributed stochastic neighbor embedding (t-SNE) was performed using the viSNE implementation to create two-dimensional plots that placed cells with similar phenotypic characteristics in close proximity.

We used contour maps to facilitate visualization of cellular distribution, which to determine which sequences were Gag- or HA-specific. Common sequences shared between naïve, memory, or GC T<sub>FH</sub> cells were shown as connecting lines on circos plots (Fig. 3A).

We found several Gag-specific TCR sequences in the GC T<sub>FH</sub> (zero to seven clones) population. Although we did not have enough data points to reach significance, the overlap between Gag-specific TCR sequences was minimal in memory T cells (zero or one clone), and no Gag-specific sequences were found in the naïve T cell population (Fig. 3B). A similar trend of enrichment of antigen-specific clones in the GC T<sub>FH</sub> cells was observed for HA-specific TCR sequences but not the HA-specific sequences, have a significantly higher degree of coding degeneracy (Fig. 3D). Thus, the Gag-specific GC T<sub>FH</sub> cells, CXCR5<sup>+</sup> memory CD4<sup>+</sup> T cells from patient samples occupied regions on the t-SNE map that overlapped with cells from HCs and additional areas that were unique (Fig. 4B). To delineate which T<sub>FH</sub> phenotypes were the dominant HIV-related features, we performed density-based local maxima cluster with support vector machine (DensVM) to automatically identify t-SNE clusters in an unbiased fashion (Fig. 4C and fig. S7) (23). We then generated a heatmap to visualize the staining intensity for individual markers and the relative contribution of HC- or HIV-derived cells to each cluster (Fig. 4D).

This analysis revealed a high abundance of activated CD38<sup>+</sup> T<sub>FH</sub> cells in LN from HIV<sup>+</sup> individuals (clusters 1 and 2). CD38<sup>+</sup> T<sub>FH</sub> cells also colocalized with cells that expressed the highest levels of Ki67 and T<sub>FH</sub> markers PD-1, ICOS, B cell lymphoma 6 (BCL-6), and IL-21 on the t-SNE maps (fig. S8). Manual gating for CD38 on total CXCR5<sup>+</sup> memory CD4<sup>+</sup> T cells and memory CD4<sup>+</sup> T cells confirmed that T<sub>FH</sub> cells from HIV<sup>+</sup> patients were more highly activated compared with cells from HCs (Fig. 4E). A significantly higher proportion of CD38<sup>+</sup>CXCR5<sup>+</sup> memory CD4<sup>+</sup> T cells stained for the GC marker CD57 and the expression of CD38<sup>+</sup>-activated phenotype additionally correlated with the capacity to produce IL-21 upon T cell stimulation (Fig. 4, F and G). Whereas LN location (cervical versus others) may contribute to some differences between cells from HIV<sup>+</sup> patients and HCs, changes in T<sub>FH</sub> cell phenotypes were also related to the severity of HIV infection in cells from the same site. For HIV-infected cervical LN samples,

Because IL-21 plays a key role in B cell selection and differentiation, we further dissected the sequence repertoire, antigen specificity, and functional potential of IL-21–producing T cells. We adapted the method by Schultz et al. (24) and optimized cell capture using a streptavidin-based scaffold protein to couple anti–IL-21 antibodies onto the surface of memory T cells (fig. S10) (25). LN cells were incubated with IL-21 capturing complex and Gag peptides for 18 hours and then stained with an IL-21 detection antibody. Individually sorted single IL-21+ T cells were processed for TCRβ chain amplification and sequencing (Fig. 5A) (26, 27). In total, we analyzed TCR sequences from 185 Gag-reactive T cells in four untreated HIV+ patients (table S6 and Fig. 5B). Clonally expanded T cells were detected in three of the four individuals. In samples where clonal expansion was observed, 3.6, 12.5, or 24.5% of Gag-reactive T cells express the exact same TCR sequence as that of another cell from the same person, with a single TCR clonotype occupying about a tenth of sequence repertoire from IL-21+ T cells in two individuals (8.5 and 12.5%; Fig. 5B). These data provided a quantitation of clonotypic diversity of the T cell response to Gag peptides in HIV-infected LNs and demonstrated a highly restricted repertoire of IL-21–producing T cells in some HIV+ patients.

Most of the IL-21+ Gag-reactive T cells expressed a classic T FH cell phenotype, as indicated by high CXCR5, ICOS, and PD-1 expression (Fig. 5, C and D). These cells were also enriched for CD57 compared with bulk memory T cells in the same LN (Fig. 5, C and D). Although we did not detect a significant correlation between CD57 signal intensity and IL-21 frequency on CyTOF analyses (fig. S11), we additionally identified Gag-reactive T cells in a subset of donors using a cytokine-independent approach (28) and showed that Gag-reactive T cells identified by CD25 and OX40 up-regulation were also enriched for CD57 expression (Fig. 5, E and F). Together, these data indicate that T cell response to HIV antigens alters T FH cell phenotypes.

Because IL-21+ GC T FH cells were most abundant in patients with more severe HIV infection, we hypothesized that HIV-specific T cells preferentially contributed to the increased IL-21 functional phenotype in the infected lymphoid environment. To test this idea, we compared IL-21 expression between Gag- and HA-reactive T cells from the same donor (fig. S12). LN-derived T cells specific for each antigen were identified by the CD25OX40 phenotype after peptide stimulation. IL-21–producing cells were measured as a subset of CD25OX40 T FH cells. Our data showed that significantly more Gag-reactive T FH cells produced IL-21 compared with HA-reactive T cells [average: 14.7% (Gag) versus 5.7% (HA); Fig. 5G]. As a percentage of GC T FH cells, Gag peptides also induced a larger IL-21 response compared with

Fig. 3. GC T FH cells exhibit HIV antigen–driven clonal expansion and selection. (A) Gag-specific TCR clones overlap with HIV+ LN CD4+ T cell populations. Each thin slice of the arc represents a unique TCR sequence, ordered by the clone size (darker green for larger clones, inner circle). Gray curves indicate Gag-specific TCR nucleotide sequences found in naïve (gray, outer circle), memory (blue, outer circle), and GC T FH (orange, outer circle) populations. No Gag-overlapping clones were detected for one individual, H8 (not shown). (B) Number of Gag-specific TCR clones observed in naïve, memory, and GC T FH populations. Gray lines link the same patient. Bars indicate means (P values by two-tailed paired t test). (C) Mean clone size of Gag-specific T cells, HA-specific T cells, and bulk clones of unknown specificity from the GC T FH population. (D) Number of distinct nucleotide (nt) sequences per CDR3 amino acid (aa) sequence for Gag-specific T cells, HA-specific T cells, or bulk GC T FH cells. Data from all four individuals were aggregated for (C) and (D). Error bars indicate SEM. N.S., not significant. ***P < 0.001 by two-tailed t test.

high IL-21 expression in GC T FH cells correlated with peripheral CD4+ T cell depletion by low CD4+ T cell count (Fig. 4H). Other clinical features, including viral load or antiviral treatment at the time of LN excision, were not significantly associated with CD38 or IL-21 positivity in GC T FH cells (fig. S9). Collectively, high-dimensional T FH cell analyses identified an activation-related T FH cell signature in infected LNs characterized by an IL-21–dominant functional phenotype.

Gag-reactive T cells preferentially express GC markers and produce IL-21 during chronic HIV infection

Because IL-21 plays a key role in B cell selection and differentiation, we further dissected the sequence repertoire, antigen specificity, and functional potential of IL-21–producing T cells. We adapted the method...
HA [average: 0.83% (Gag) versus 0.52% (HA); Fig. 5H]. Together, these data indicate that Gag-reactive T cells preferentially acquire a GC phenotype and produce IL-21 during chronic antigen stimulation in infected LNs.

**HIV infection is associated with less polyfunctional T<sub>FH</sub> cells**

Although IL-21 is critical for T<sub>FH</sub> cell function, we also detected expression of other cytokines on CyTOF (Fig. 4D). These data are consistent with past analyses of pediatric tonsil cells, which revealed an abundance of cellular diversity within T<sub>FH</sub> cells that included effector-type T<sub>FH</sub> cells capable of producing different combinations of interferon-γ (IFN-γ), IL-2, tumor necrosis factor–α (TNF-α), or IL-17A (29). To better delineate the impact of HIV infection on the functional diversity of T<sub>FH</sub> cells, we performed manual gating to measure the frequency of T<sub>FH</sub> cells that produced IL-21, IFN-γ, TNF-α, IL-2, IL-4, and granzyme A. We restricted this part of the analyses to the GC subset using strictly defined T<sub>FH</sub> cell markers and used unstimulated cells to establish the baseline signal for each effector molecule in the absence
GC TFH cells from HIV+ patients also secreted less TNF-α compared with HCs (Fig. 6A). Although not statistically significant, CD57+PD-1+ frequency of Gag-reactive T cells overlaid onto bulk CD4+ T cells (gray). (H) Frequency of each indicated phenotypic subset within IL-21+ Gag-reactive T cells or IL-21− bulk memory T cells determined using IL-21 capture or by CD25 and OX40 up-regulation. (G) Frequency of CD25+OX40+CXCR5+PD-1+ TFH cells that coexpressed IL-21 after Gag or HA peptide stimulation from six HIV+ individuals. (I) IL-21 expression within GC TFH subset in LN cells from 11 HIV+ patients stimulated by Gag or HA peptides. (J) Phenotype of Gag-reactive IL-21+ T cells (red) overlaid onto bulk CD4+ T cells (gray). (K) Frequency of single IL-21–producing cells (red) within IL-21+ TFH and IL-21− bulk memory T cells identified using IL-21 capture or by CD25 and OX40 up-regulation. (L) Phenotype of memory B cells and plasma cells were identified using the same gating strategy as Perreau et al. (3) (Fig. 6D and Fig. S16). We found that having a low frequency of isotype-switched memory B cells was associated with more single IL-21–producing cells (Fig. 6, E and G). A reciprocal relationship was observed for plasma cells, which was positively correlated with single IL-21–producing cells (Fig. 6, F and H). These data indicate that the accumulation of functionallyfocused IL-21+ TFH cells is associated with lymphoid B cell pathology during HIV infection.

Fig. 5. Gag-reactive TFH cells express IL-21 and acquire GC phenotype. (A) Representative plots showing IL-21 stained using to identify Gag-reactive T cells. LN cells were stimulated for 18 hours with vehicle alone using dimethyl sulfoxide (DMSO) (left) or Gag peptides (right). (B) TCR sequencing of Gag-reactive IL-21+ T cells. Each pie chart represents TCR sequences from one individual. Light gray color represents unique TCRs. Filled colors represent the fraction of cells expressing a TCR identical to that of another cell in each individual. The number of TCR sequences analyzed is indicated at the center of pie chart. (C) Phenotype of Gag-reactive IL-21+ T cells (red) overlaid onto bulk CD4+ T cells (gray). (D) Frequency of each indicated phenotypic subset within IL-21+ Gag-reactive T cells or IL-21+ bulk memory T cells from HIV+ individuals (n = 11). **P < 0.005 and ***P < 0.0005 by two-tailed t test. (E) Representative plots showing identification of Gag-reactive T cells by CD25 and OX40 expression. (F) CD57+PD-1+ frequency of IL-21+ Gag-reactive T cells from HIV+ individuals (n = 6) identified using IL-21 capture or by CD25 and OX40 up-regulation. (G) Frequency of CD25+OX40+CXCR5+PD-1+ TFH cells that coexpressed IL-21 after Gag or HA peptide stimulation from six HIV+ individuals. (H) IL-21 expression within GC TFH subset in LN cells from 11 HIV+ patients stimulated by Gag or HA peptides. The lines connect data from the same donor. For (F) and (H), *P < 0.05 and **P < 0.005 by paired two-tailed Wilcoxon signed-rank test.

of T cell activation (Fig. S13). Our data showed positive staining for IFN-γ, TNF-α, IL-2, IL-4, and granzyme A in TFH cells, but TFH cells from HIV+ patients secreted significantly less IFN-γ and granzyme A compared with HCs (Fig. 6A). Although not statistically significant, GC TFH cells from HIV+ patients also secreted less TNF-α and had lower IL-2 production. To validate these observations, we used polychromatic flow cytometry to analyze a second set of samples that included eight new HIV+ patients, five additional HCs, and two HCs analyzed previously by CyTOF. GC TFH cells defined using the same gating strategy as CXCR5, CD45RO, PD-1, and CD57 expression on CD4+ T cells also showed an HIV-associated decrease in IFN-γ and granzyme A production (Fig. S14). Collectively, our data revealed B cell subset distribution relates to changes in TFH cell subsets that favor IL-21–producing populations in the setting of chronic antigen stimulation.

Because IL-21 and other effector molecules have not only overlapping but also distinct patterns of distribution on the t-SNE map (fig. S8B), we next asked how HIV affects the effector profile of IL-21+ TFH cells. We used Boolean combination gates to create all possible combinations of IL-21 with other effector molecules stained by CyTOF (IFN-γ, TNF-α, IL-2, IL-4, and granzyme A). IL-21+ cells were then grouped according to the number of additional effector molecules produced. Our data showed a decrease in polyfunctional IL-21+ T cells that can produce multiple effector molecules in TFH cells from HIV+ patients compared with HCs (fig. S15). Instead, more dedicated single IL-21–producing TFH cells were found in HIV+ patients (Fig. 6B). To determine whether a similar type of IL-21+ cells also circulated in the blood during chronic HIV infection, peripheral blood mononuclear cells (PBMCs) were obtained from a subset of HC and HIV+ donors from whom we had paired LN samples. Circulating TFH cells (cTFH) were identified as IL-21–producing CD4+ T cells (24). We showed that, similar to cells in the LN, cTFH cells were more functionally focused on IL-21 expression in HIV+ patients, and the abundance of IL-21 single-positive cells in the blood was highly correlated with the frequency of IL-21 single-positive TFH cells in the LNs (Fig. 6, B and C). Collectively, these data revealed an IL-21–dominant phenotype as a prevailing feature broadly shared by different types of TFH cells from HIV+ patients.

Untreated HIV is associated with defective B cell differentiation. Past studies demonstrated that LNs from HIV+ patients contain fewer memory B cells and more plasma cells (3, 4), but how aberrant changes in TFH cell subsets that favor IL-21–producing populations in the setting of chronic antigen stimulation.
DISCUSSION

How HIV affects lymphoid T<sub>FH</sub> cells has been studied under limited settings. In part, the challenge has been the inaccessibility of human lymphoid tissues and the tools available to interrogate a small number of cells. Here, we overcame these challenges using LNs obtained for clinical diagnostics from a mostly untreated HIV<sup>+</sup> cohort. The data described here represent a comprehensive phenotype and TCR repertoire sequencing analyses provided evidence for antigen-driven expansion of TFH cells in the LNs, including that of HIV-reactive T cells. We also analyzed LN samples from HIV<sup>-</sup> HCs, but because of ethical and practical limitations, HC-derived LNs were obtained from different body sites and should be interpreted with this potential cave- at. Our data are consistent with the diversity of functional phenotypes previously observed in tonsils (29) and highlight the heterogeneity of TFH cells at a healthy baseline.

We found different clonal frequencies in Gag-reactive IL-21<sup>+</sup> T cells and selection for certain preferred CDR3 sequences during chronic HIV infection. We further demonstrated that these GC TFH cells acquire a distinct functional phenotype and become dominated by an IL-21<sup>+</sup> functional subset. The biological relevance for having different subsets of T<sub>FH</sub> cells is just now beginning to be understood. Using IL-21 and IL-4 reporter genes in mice to trace T<sub>FH</sub> cells that produce IL-21<sup>-</sup>, IL-4, or both, Weinstein et al. (30) demonstrated temporal differences in the kinetics of IL-21 and IL-4 production and showed that IL-21<sup>-</sup>, IL-21<sup>+</sup>IL-4<sup>-</sup>, and IL-4<sup>+</sup> T<sub>FH</sub> cells each provide specialized follicular helper function and support distinct aspects of B cell function and development. Here, we also detected various functional T<sub>FH</sub> cell subsets in healthy human LNs and showed that T cells identified using the classic T<sub>FH</sub> lineage markers have the potential to secrete many distinct types of effector molecules. Our data are consistent with the diversity of functional phenotypes previously observed in tonsils (29) and highlight the heterogeneity of T<sub>FH</sub> cells at a healthy baseline.

We used HIV infection to ask how prolonged antigen stimulation alters the composition of T<sub>FH</sub> cells in the LN. In vitro studies have suggested that T<sub>FH</sub> cells could become inhibited in the context of chronic inflammation and fail to activate appropriately to TCR stimulation via induction of PD-1–mediated inhibitory signals (7). The increase in T<sub>FH</sub> cells could then be explained by an overabundance of cytokine signals in HIV-infected LNs that activated T cells in an antigen-independent fashion (12, 13). Although our data do not rule out a contribution from bystander T cell expansion, our data are most consistent with the model where T<sub>FH</sub> cell pathology, manifested as clonal expansion and reduced polyfunctionality, is primarily an antigen-driven process. By TCR repertoire sequencing, we showed that certain TCR clonotypes become expanded within GC T<sub>FH</sub> cells. A small portion of HIV-specific clones harbor distinct nucleotide sequences that converge to the same amino acid sequence—a signature of antigen-driven selection. Convergent selection of TCR sequences is expected only when there is external pressure to select for certain CDR3 binding motifs. These data provide strong evidence for an antigen-driven process and additionally suggest that B cells, in their capacity as antigen-presenting cells, also shape the composition of T<sub>FH</sub> cells. We measured the extent of clonal restriction by single-cell TCR sequencing. We found different clonal frequencies in Gag-reactive IL-21<sup>+</sup> T cells between different HIV<sup>+</sup> patients, with expanded clones occupying a significant proportion of Gag-reactive response in some individuals.
Although we did not evaluate Env-reactivity directly, reduced TCR diversity will likely also affect T cells that recognize other HIV antigens. How T<sub>FH</sub> cell repertoire relates to the selection of protective and/or neutralizing antibody responses remains poorly understood. The density of peptide-major histocompatibility complex (MHC) presented by competing B cells has been shown to be a major factor that determines the magnitude of T cell help (31–33), but the diversification of antigenic variants by viral mutation provides an additional layer of complexity in the selection of relevant B cells during chronic HIV infection (34). Previous studies have shown that early HIV Env gene diversity predicts development of antibody breadth (35). This raised the possibility that a diverse repertoire of HIV-specific T<sub>FH</sub> cells may be necessary to capture the breadth of viral variants, and an oligoclonal T<sub>FH</sub> population that focuses T cell reactivity to nonproductive but common antigenic viral sequences may neglect rare B cells that have neutralizing potential. Future studies to determine whether individuals with more diverse HIV-specific T<sub>FH</sub> cell repertoire are more successful at generating broadly neutralizing antibodies will provide additional insights.

One important open question is why an excess of expanded T<sub>FH</sub> cells in the LNs fails to provide superior B cell help during chronic HIV infection. Our data provided evidence that HIV infection alters the functional quality of T<sub>FH</sub> cells and shifts T<sub>FH</sub> cells toward an IL-21–dominant subset. In support of an antigen-dependent model of T cell activation, Gag-reactive T cells preferentially acquire GC features and secrete IL-21, indicating that HIV-reactive T cells directly contributed to altered T<sub>FH</sub> cell phenotype in infected LNs. In addition, because most of the GC T<sub>FH</sub> cells also acquire an altered phenotype, there are likely other secondary effects of HIV infection that extend beyond HIV-specific T cells. IL-21<sup>+</sup> T<sub>FH</sub> Cells in HIV-infected LNs produce less of other types of cytokines. This IL-21–focused T<sub>FH</sub> cell subset could represent a more differentiated T<sub>FH</sub> cell population in response to persistent antigen stimulation. However, because having more IL-21<sup>+</sup> T<sub>FH</sub> cells in the LN was associated with severe HIV infection and aberrant B cell distribution, the accumulation of IL-21<sup>+</sup> T<sub>FH</sub> cells is likely counterproductive in the setting of HIV infection. The notion that an excess of the IL-21–producing subset could contribute to HIV-related B cell changes is consistent with previous studies in mice showing that IL-21 is required to maintain BCL-6 expression in B cells and promotes plasma cell differentiation (30, 36, 37). Changes in B cell subsets could also result from a relative decrease in other types of T<sub>FH</sub> cells, which would agree with recent studies that showed specialization of T<sub>FH</sub> cell subsets in regulating distinct aspects of B cell differentiation (34). Lastly, whether having a highly specialized population of clonally restricted T<sub>FH</sub> cells could divert T cell help to unprotective B cells remains to be determined.

We have defined the TCR and phenotypic repertoire of T<sub>FH</sub> cells during chronic HIV stimulation. Our work highlights an antigen-driven process that alters the composition of T<sub>FH</sub> cells in the lymphoid compartment. Collectively, our data suggest that, in addition to the size of the GC reaction, the functional profile and the compositional complexity of T<sub>FH</sub> cells are additional key measurements that could affect the quality of T<sub>FH</sub> cell responses to vaccines and infections.

**MATERIALS AND METHODS**

**Study design**

The goal of the study was to define T<sub>FH</sub> cell diversity in primary human LNs. All samples were de-identified and obtained with Institutional Review Board approval from the University of Pennsylvania. Participant characteristics are shown in table S5. Please see the Supplementary Materials for details on sample collection.

**CyTOF staining and data analyses**

Cryopreserved cells were thawed and stained with a metal-conjugated antibody panel (table S4), after a 5-hour stimulation with PMA and ionomycin in the presence of monensin and brefeldin A. Antibody-stained cells were mixed with normalization beads and acquired on CyTOF 2. Bead standards were used to normalize CyTOF runs with the MATLAB-based normalizer (22). Data analyses were performed using Cytobank and “cytofkit” package in R. Please see the Supplementary Materials for detailed methods on cell staining and CyTOF analysis.

**Statistical methods**

Assessment of normality was performed using D’Agostino-Pearson test. Pearson or Spearman’s rank correlation was used depending on the normality of the data to measure the degree of association. The best-fitting line was calculated using least-squares fit regression. Statistical comparisons were performed using two-tailed Student’s t test or Wilcoxon signed-rank test, with a P value of <0.05 as a cutoff to determine statistical significance. Multiple comparisons were corrected using Holm-Sidak method. Statistical analyses were performed using GraphPad Prism.

**SUPPLEMENTARY MATERIALS**

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Methods

Fig. S1. GC T<sub>FH</sub> cells are clonally expanded.

Fig. S2. Antigen-driven clonal selection signature in GC T<sub>FH</sub> cells of HIV-infected LNs.

Fig. S3. Identification of Gag- or HA-reactive T cells in cultured cells.

Fig. S4. HA-specific CD4 T cell clones detected in HIV-infected LNs.

Fig. S5. Batch normalization of CyTOF data using calibration beads.

Fig. S6. Identification of GC T<sub>FH</sub> cells from LN samples.

Fig. S7. High-dimensional analysis of CXCR5<sup>+</sup>CD45RO<sup>+</sup>CD4<sup>+</sup> T cells.

Fig. S8. Signal intensity of individual markers on t-SNE plots.

Fig. S9. Correlation between IL-21 frequency and CD38 expression with viral load and antiviral treatment.

Fig. S10. IL-21 surface capture effectively identified IL-21–producing T cells.

Fig. S11. Correlation between the frequency of IL-21<sup>+</sup> GC T<sub>FH</sub> cells and signal intensity of CD57 staining.

Fig. S12. T<sub>FH</sub> cells respond to Gag or HA peptides.

Fig. S13. Identification of effector molecule–producing CD4<sup>+</sup> T cells on CyTOF.

Fig. S14. Identification of effector molecule–producing CD4<sup>+</sup> T cells by flow cytometry.

Fig. S15. Frequency of IL-21–producing subsets in T<sub>FH</sub> cells in HIV<sup>+</sup> and HC samples.

Fig. S16. Identification of B cell subsets.

Table S1. TCR<sub>β</sub> sequencing primers.

Table S2. TCR repertoire sequencing cell and transcript counts.

Table S3. Gag and HA TCR sequence reference panel.

Table S4. CyTOF antibody staining panel.

Table S5. Clinical characteristics and demographic information of LN samples.

Table S6. Gag-reactive TCR sequences from single-cell TCR sequencing of IL-21<sup>+</sup> cells.

Table S7. Tabulated raw data sets.
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capture experiments. B.A. performed in vitro peptide stimulation and single-cell TCR sequencing. P.M.D.R.-E., Y.A.-T., and G.R.-T. established the infrastructure for HIV+ patient recruitment and provided LN samples and the associated clinical information. S.M.H. assisted with TCR sequencing library preparation. K.-Y.M. adapted the TCR repertoire sequencing protocol. M.R.B. contributed to the LNs from healthy individuals. J.H. and L.P. prepared tetrameric scaffold protein. P.A.G. provided statistical support. L.F.S., N.J., and B.S.W. wrote and edited the paper. **Competing interests:** N.J. is a scientific adviser of ImmuDX LLC. The other authors declare that they have no competing financial interests. **Data and materials availability:** The data for this study have been deposited in the database of Genotypes and Phenotypes under the accession number phs001548.v1.p1.

The receptor repertoire and functional profile of follicular T cells in HIV-infected lymph nodes

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Zooming in on human lymph nodes

Follicular helper T cells (TFH) play an essential role in shaping B cell–mediated antibody responses. By obtaining lymph nodes from HIV+ individuals, Wendel et al. have used mass cytometry and TCR sequencing to directly examine the TFH response to HIV. They report that HIV infection alters the clonality of TFH cells with severe infections resulting in pronounced oligoclonal TFH responses. From a functional standpoint, they found that TFH cells in the lymph nodes of HIV+ individuals secreted interleukin-21 but were less polyfunctional as compared with TFH cells from healthy individuals and that this lack of polyfunctionality correlated with impaired isotype switching of B cells in the lymph nodes.

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