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

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Abstract

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Author contributions: L.F.S. and N.J. designed the study. B.S.W. performed bulk TCR sequencing and data analysis, C.H. analyzed TCR sequencing data, D.D.A. performed CyTOF and IL-21 capture experiments. B.A. performed *in vitro* peptide stimulation and single-cell TCR sequencing. P.D.R., 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.B. contributed to the lymph nodes from healthy individuals. J. H. and L. P. prepared tetrameric scaffold protein. L.F.S., N.J., and B.S.W. wrote and edited the paper

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

One Sentence Summary:

Follicular T cells undergo clonal expansion and express an altered functional phenotype during chronic HIV infection.

Introduction

Follicular helper T cells (T_{FH}) provide key signals necessary for B cell recruitment and selection to generate protective antibody responses (1, 2). During untreated chronic HIV infection, T_{FH} cells become highly expanded in the lymph nodes (LN) (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 T_{FH} 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 co-cultured with T_{FH} cells from HIV⁺ patients (7–9). A proposed mechanism for this involves upregulation of PD-L1 by B cells, which interacts with PD-1 on T_{FH} cells to inhibit TCR-dependent activation of T_{FH} cells (7). However, the extent to which T_{FH} cells express impaired antigen responsiveness *in vivo* remains unclear. As T_{FH} cells need to appropriately sense antigen signals in order to discriminate between B cells, defective response to antigen not only impairs provision of T cell help to individual B cells, but would also imperil the process of B cell selection on a global level.

Here, we interrogated the functional phenotype and TCR repertoire composition of primary T_{FH} cells isolated directly from LNs from HIV⁺ individuals. We utilized the presence or absence of antigen-dependent TCR signatures to address the responsiveness of T_{FH} cells to antigen engagement and applied high dimensional mass cytometry to elucidate how HIV infection alters the functional phenotype of T_{FH} cells in the lymphoid compartment. Our data revealed clonal expansion and convergent selection for Gag-reactive TCRs in T_{FH} cells in the germinal centers (GC) of HIV-infected LNs, indicating that T_{FH} cells remain capable of responding to HIV antigens during chronic HIV infection. However, T_{FH} cells in LNs

from HIV⁺ individuals acquire an activated phenotype dominated by IL-21 production, which were 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⁺ T_{FH} cells contained an HIV-reactive population expressing a restricted TCR repertoire and GC phenotype. Thus, TCR-directed response to HIV alters T_{FH} cell diversity and composition in the lymphoid compartment.

Results

HIV infected LNs contain clonally expanded GC T_{FH} cells

LNs from untreated HIV⁺ patients contain a high frequency of T_{FH} cells, but the mechanism that drives expansion of T_{FH} cells remains unclear. The enrichment of HIV antigens (10, 11) and the highly pro-inflammatory milieu (12, 13) in the LNs could lead to antigen-driven and/or bystander T cell expansion. To address whether proliferation of T_{FH} cells is antigen-dependent, we tested whether HIV induces selective proliferation of certain T cell clones. We focused on GC T_{FH} cells because the frequency of these cells becomes greatly increased during chronic HIV infection (3, 4). To identify GC T_{FH} cells, we selected memory CD4⁺ T cells that express T_{FH} cell markers CXCR5 and PD-1. CD57 is a glycan carbohydrate epitope expressed by T_{FH} 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⁻CXCR5⁻CD57⁻CCR7⁺ expression, and memory CD4⁺ T cells were CD45RO⁺CXCR5⁺PD-1⁺ICOS⁻ (Fig. 1A). We sorted 1,464 to 15,000 naïve, memory, and GC T_{FH} 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. On average 11,839 TCR transcripts were detected for each sample (Table S2). Unique TCR frequencies range from 1 in 37,129 (0.003%) for the rarest clones to 250 in 2,498 (~10%) for the most expanded clone. To compare the degree of relative clonal expansion, we categorized TCR frequency into 6 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_{FH} cells had a much higher fraction of TCRs occupied by abundant clones (>0.1%) compared to naïve and memory CD4⁺ T cells (Fig. 1B, 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_{FH} cell frequency is due to selective proliferation of certain T cell clones, GC T_{FH} cells had a lower NSE score compared to naïve and memory cells (Fig. 1C). Taken together, our data demonstrated a notable expansion of clone size in GC T_{FH} cell populations.

TCRs from GC T_{FH} cells exhibit signatures of antigen-driven clonal convergence

Next, to test whether clonal expansion in GC T_{FH} 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 lifespans of the T cell and its progeny. Thus, with the exception of clones that express 2 TCR α or β 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 4 quadrants 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 2 or more nucleotide sequences. 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 2 or more nucleotide sequences. Degenerate clones can stochastically arise in the repertoire, but these are typically rare as reflected by the low frequency of non-clonally expanded sequences in Q2. Q3 contains amino acid CDR3 sequences that show neither clonal expansion nor amino acid convergence and make up the majority of the repertoire. Q4 contains expanded amino acid CDR3 sequences derived from a single nucleotide sequence and are therefore non-degenerate. This TCR degeneracy analysis revealed a significant degree of antigen-driven clonal convergence in GC T_{FH} cells compared to naïve and memory T cells (Fig. 2B-C). Together with the NSE decrease in GC T_{FH} cells, these data provide further evidence that antigen-driven clonal expansion is preserved in GC T_{FH} cells.

HIV promotes selective expansion of HIV-reactive T_{FH} cells.

To determine if clonally expanded and/or convergently selected TCRs include HIV-specific sequences, approximately 2 – 3 million thawed LN cells were cultured with an HIV-1 consensus B Gag peptide pool for 3–4 weeks, then restimulated with the same peptide pool for 4 hours to identify antigen-specific T cells by CD40L and CD69 upregulation (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⁺CD69⁺ 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 to determine which sequences were Gag- or HA-specific. Common sequences shared between naïve, memory, or GC T_{FH} cells were shown as connecting lines on circos plots (Fig. 3A).

We found several Gag-specific TCR sequences in the GC T_{FH} (0 to 7 clones) population. Though we did not have enough data points to reach significance, the overlapping between Gag-specific TCR sequences was minimal in memory T cells (0 or 1 clones), and no Gagspecific sequences were found in the naïve T cell population (Fig. 3B). A similar trend of enrichment of antigen-specific clones in the GC T_{FH} phenotype was also observed for

HA-specific TCR sequences (Fig. S4). This is unsurprising, as these individuals have likely been exposed to influenza infection and/or vaccinated against HA in the past. However, analysis of combined TCR sequencing data from all individuals clearly showed that these Gag-specific GC T_{FH} cells, but not the HA-specific clones, were highly expanded compared to the bulk GC T_{FH} cells of unknown specificity (Fig. 3C). Translating these antigen-specific TCR sequences into amino acid sequences showed that the Gag-specific TCR sequences within the GC T_{FH} population, but not the HA-specific sequences, have a significantly higher degree of coding degeneracy (Fig. 3D). Thus, the Gag-specific GC T_{FH} cells were preferentially expanded and degenerate. Collectively, these data indicate that Gag-specific T_{FH} cells respond to antigen stimulation and become selectively expanded in the LNs.

T_{FH} cells acquire distinct phenotypic characteristics in HIV-infected LNs

To investigate HIV-driven changes on the phenotypic level, we designed a mass cytometry panel to examine phenotypic and functional features of T_{FH} cells (Table S4). Cryopreserved cervical LNs from 25 Mexican HIV⁺ patients were obtained and analyzed together with cervical, mesenteric, and iliac LNs from 7 local healthy controls (Table S5). To interrogate the functional potential of T_{FH} cells, three to five 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 mass cytometer CyTOF 2. Bead-based standards were used to correct for machine performance and batch differences (22) (Fig. S5). To broadly define CD4⁺ T cells with T_{FH} cell features, we performed manual gating to select CD4⁺ T cells positive for CXCR5 and CD45RO staining. GC T_{FH} cells were further identified by PD-1 and CD57 expression and imported into Cytobank (Fig. 4A, 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 clearly showed a different pattern between GC T_{FH} cells from HC and HIV⁺ patient-derived samples (Fig. 4B). Hypothesizing that HIV can broadly impact T_{FH} cells beyond the GC subset, we also analyzed other CD4⁺ T cells positive for CXCR5 expression. Similar to the observation with GC T_{FH} cells, CXCR5⁺ memory CD4⁺ 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_{FH} phenotypes were the dominant HIV-related features, we performed DensVM to automatically identify t-SNE clusters in an unbiased fashion (23) (Fig. 4C, Fig. S7). We then generated a heat map to visualize the staining intensity for individual markers and the relative contribution of HC or HIV-derived cells to each clusters (Fig. 4D). This analysis revealed a high abundance of activated CD38⁺ T_{FH} cells in LN cells from HIV⁺ individuals (clusters 1 and 2). CD38⁺ T_{FH} cells also co-localized with cells that expressed the highest levels of Ki67, PD-1, ICOS, BCL-6, and IL-21 on the t-SNE maps (Fig. S8). Manual gating for CD38 on total CXCR5⁺ memory CD4⁺ T cell and GC T_{FH} cells confirmed that T_{FH} cells from HIV⁺ patients were indeed more highly activated compared to cells from HCs (Fig. 4E). A significantly higher proportion of CD38⁺CXCR5⁺ memory CD4⁺ T cells stained for the GC marker CD57, and

the expression of a CD38⁺ activated phenotype additionally correlated with the capacity to produce IL-21 upon T cell stimulation (Fig. 4F-G). While LN location (cervical vs others) may contribute to some differences between cells from HIV⁺ patients and HCs, changes in T_{FH} cell phenotypes were also related to the severity of HIV infection in cells obtained from the same site. Within the CD57⁺ GC subset from HIV infected cervical LN samples, high IL-21 expression correlated with a higher level of peripheral CD4⁺ T cell depletion by low CD4⁺ T cell count (Fig. 4H). Other clinical features, including viral load or anti-viral treatment at the time of lymph node 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

As 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. 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) (24, 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 TCR beta-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, Fig. 5B). Clonally expanded T cells were detected in three out 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 approximately 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.

The majority of IL-21⁺ Gag-reactive T cells expressed a classic T_{FH} cell phenotype, as indicated by high CXCR5, ICOS, and PD-1 expression (Fig. 5C-D). These cells were also enriched for CD57 compared to bulk memory T cells in the same LN (Fig. 5C-D). While 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 upregulation were also enriched for CD57 expression (Fig. 5E-F). Taken 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 CD25⁺OX40⁺ phenotype following peptide stimulation. IL-21-producing cells were measured as a subset of CD25⁺OX40⁺ T_{FH} cells. Our data showed that significantly more Gag-reactive T_{FH} cells produced IL-21 compared to the HA-reactive T cells (Fig. 5G, average Gag: 14.7% vs HA: 5.7%). As a percentage of GC T_{FH} cells, Gag peptides also induced a larger IL-21 response compared to HA (Fig. 5H, average Gag: 0.83% vs HA: 0.52%). Taken 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 infected is associated with less polyfunctional T_{FH} cells

While IL-21 is critical for T_{FH} 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_{FH} cells that included effector type T_{FH} cells capable of producing different combinations of IFN- γ , IL-2, TNF- α , or IL-17A (29). To better delineate the impact of HIV infection on the functional diversity of T_{FH} cells, we performed manual gating to measure the frequency of T_{FH} 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_{FH} cell markers and utilized unstimulated cells to establish the baseline signal for each effector molecule in the absence of T cell activation (Fig. S13). Our data showed positive staining for IFN- γ , TNF- α , IL-2, IL-4, and granzyme A in T_{FH} cells, but T_{FH} cells from HIV⁺ patients secreted significantly less IFN- γ and granzyme A compared to HCs (Fig. 6A). While not statistically significant, GC T_{FH} 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 8 new HIV⁺ patients, 5 additional HCs, and two HCs analyzed previously by CyTOF. GC T_{FH} cells defined using the same gating strategy by 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 changes in T_{FH} cell subsets that favor IL-21-producing populations in the setting of chronic antigen stimulation.

Because IL-21 and other effector molecules have overlapping but also distinct patterns of distribution on the t-SNE map (Fig. S8B), next we asked how HIV impacts the effector profile of IL-21⁺ T_{FH} 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, granzyme A). IL21⁺ 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 T_{FH} cells from HIV⁺ patients compared to controls (Fig. S15). Instead, more dedicated single IL-21-producing T_{FH} 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, PBMCs were obtained from a subset of HC and HIV⁺ donors from whom we had paired LN samples. Circulating T_{FH} cells (cT_{FH}) were identified as IL-21-producing CD4⁺ T cells according to a recent study by Streeck and colleagues (24). We showed that, similar to cells in the LN, cT_{FH} cells were more functionally focused on IL-21 expression in HIV⁺ patients, and the abundance of IL-21

single positive cells was tightly associated between lymphoid and circulatory compartments (Fig. 6B-C). Collectively, these data revealed an IL-21-dominant phenotype as a prevailing feature broadly shared by different types of T_{FH} cells from HIV⁺ patients.

Untreated HIV is associated with defective B cell differentiation. Past studies clearly demonstrated that LNs from HIV⁺ patients contain fewer memory B cells and more plasma cells (3, 4), but how aberrant B cell subset distribution relates to changes in T_{FH} cells remained unclear. Hypothesizing that IL-21-dominant T_{FH} cells promote abnormal B cell development, we examined the relationship between the frequency of single IL-21⁺ T_{FH} cells and B cell phenotype. Memory B cells and plasma cells were identified using the same gating strategy as Perreau et al. (Fig. 6D and Fig. S16) (3). We found that having a low frequency of isotypeswitched memory B cells was associated with more single IL-21-producing cells (Fig. 6E, 6G). A reciprocal relationship was observed for plasma cells, which increased proportionally with single IL-21-producing cells (Fig. 6F, 6H). These data indicate that the accumulation of functionally focused IL-21⁺ T_{FH} cells is associated with lymphoid B cell pathology during HIV infection.

Discussion

How HIV impacts lymphoid T_{FH} cells have 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⁺ cohort. The data described here represent a comprehensive phenotype and TCR analysis of T_{FH} cells in the LNs, including that of HIVreactive T cells. We also analyzed LN samples from HIV negative HCs, but due to ethical and practical limitations, HC-derived LNs were obtained from different body sites and should be interpreted with this potential caveat. Our data based on TCR repertoire sequencing analyses provided clear evidence for antigen-driven expansion of T_{FH} cells and selection for certain preferred CDR3 sequences during chronic HIV infection. We further demonstrated these GC T_{FH} cells acquire a distinct functional phenotype and become dominated by an IL-21⁺ functional subset.

The biological relevance for having different subsets of T_{FH} cells is just now beginning to be understood. Using IL-21 and IL-4 reporter genes in mice to trace T_{FH} cells that produce, IL-21, IL-4 or both, Weinstein et al. demonstrated temporal differences in the kinetics of IL-21 and IL-4 production and showed that IL-21⁺, IL-21⁺IL-4⁺, and IL-4⁺ T_{FH} cells each provide specialized follicular helper function and support distinct aspects of B cell function and development (30). Here, we also detected various functional T_{FH} cell subsets in healthy human LNs and showed that T cells identified using the classic T_{FH} lineage markers have the potential to secrete many distinct types of effector molecules. Our data is consistent with the diversity of functional phenotypes previously observed in tonsils (29), and highlights the heterogeneity of T_{FH} cells at a healthy baseline.

We used HIV infection to ask how prolonged antigen stimulation alters the composition of T_{FH} cells in the LN. *In vitro* studies have suggested that T_{FH} 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_{FH} 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). While our data do not rule out a contribution from bystander T cell expansion, our data are most consistent with the model where T_{FH} 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_{FH} cells. Notably, 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_{FH} cells. We measured the extent of clonal restriction by single cell TCR sequencing. We found different clonal frequencies in Gag-reactive IL-21⁺ T cells between different HIV⁺ patients, with expanded clones occupying a significant proportion of Gag-reactive response in some individuals. While we did not evaluate Env-reactivity directly, reduced TCR diversity will likely also impact T cells that recognize other HIV antigens. How T_{FH} cell repertoire relates to the selection of protective and/or neutralizing antibody responses remains poorly understood. The density of peptide-MHC complexes presented by competing B cells clearly 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_{FH} cells may be necessary to capture the breadth of viral variants, and an oligoclonal T_{FH} 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 if individuals with more diverse HIV-specific T_{FH} 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_{FH} 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_{FH} cells and shifts T_{FH} 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_{FH} cell phenotype in infected LNs. In addition, as the majority of GC T_{FH} cells also acquire an altered phenotype, there are likely other secondary effects of HIV infection that extend beyond HIV-specific T cells. Notably, IL-21⁺ T_{FH} cells in HIV infected LNs produce less of other types of cytokines. This IL-21-focused T_{FH} cell subset could represent a more differentiated T_{FH} cell population in response to persistent antigen stimulation. However, as having more IL-21⁺ T_{FH} cells in the LN was associated with severe HIV infection and aberrant B cell distribution, the accumulation of IL-21⁺ T_{FH} 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 that showed IL-21 is required to maintain BCL-6 expression in B

cells and promotes plasma cells differentiation (30, 36, 37). Changes in B cell subsets could also result from a relative decrease in other T_{FH} cell subsets, which would agree with recent studies that showed specialization of T_{FH} cell subsets in regulating distinct aspects of B cell differentiation (34). Finally, whether having a highly specialized population of clonally restricted T_{FH} cells could divert T cell help to unprotective B cells remains to be determined.

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

Material and Methods

Study Design

The goal of the study was to define T_{FH} cell diversity in primary human LNs. All samples were de-identified and obtained with IRB approval from the University of Pennsylvania. Subject 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 metal-conjugated antibody panel (Table S4), following a 5 hour stimulation with PMA and ionomycin in the presence 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 Nolan lab normalizer. Data analyses were performed using Cytobank and “cytokit” package in R.

TCR β sequencing and analyses

TCR sequences from single cells were obtained by a series of three nested PCR reactions as previously described (26, 27). TCR junctional region analysis was performed using IMGT/VQuest. For bulk cell analyses, TCR library generation and raw sequence processing were performed using MIDs with primers listed in Table S1. (18, 19).

Statistical Methods

Assessment of normality was performed using D'Agostino-Pearson test. Pearson or Spearman 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, using a p-value of <0.05 as a cutoff to determine statistical significance. Multiple-way comparisons were corrected using Holm-Sidak method. Statistical analyses were performed using GraphPad Prism. Please see the Supplementary Materials for detailed methods on T cell staining for phenotypic characterization and TCR sequencing experiments and data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Vinuesa CG, Linterman MA, Yu D, MacLennan IC, Follicular Helper T Cells. *Annu Rev Immunol* 34, 335–368 (2016). [PubMed: 26907215]
2. Crotty S, T follicular helper cell differentiation, function, and roles in disease. *Immunity* 41, 529–542 (2014). [PubMed: 25367570]
3. Matthieu P, Anne-Laure S, Elisa De C, Jean-Marc C, Rafael C, Elias KH, Laurence De L, Cecilia G, Giuseppe P, Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* 210, 143156 (2013).
4. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, Flanders MD, Cutler S, Yudanin N, Muller MI, Davis I, Farber D, Hartjen P, Haag F, Alter G, Schulze zur Wiesch J, Streeck H, Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* 122, 3271–3280 (2012). [PubMed: 22922259]
5. Nancy FC-C, Erik I, Gabriel D, Patrick JB, Lynn EE, Jason M, Anuradha G, Dennis F, Christopher D, Tahaniyat L, Timothy W, Carolyn B, Grace M, Eugene VM, Timothy B, Durability of antibody responses after receipt of the monovalent 2009 pandemic influenza A (H1N1) vaccine among HIV-infected and HIV-uninfected adults. *Vaccine* 29, 3183–3191 (2011). [PubMed: 21371580]
6. Tebas P, Frank I, Lewis M, Quinn J, Zifchak L, Thomas A, Kenney T, Kappes R, Wagner W, Maffei K, Sullivan K, Center for AR, Clinical P Trials Unit of the University of, Poor immunogenicity of the H1N1 2009 vaccine in well controlled HIV-infected individuals. *AIDS* 24, 2187–2192 (2010). [PubMed: 20616698]
7. Rafael AC, Joseph CM, Anne-Laure S, Matthieu P, Julien van G, Talibah M, Elizabeth C, Amie M, Gordon JF, Guillermo A-T, Jeffrey MJ, Ari DB, Shane C, Jacob DE, Giuseppe P, Michael ML, Elias KH, Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* 19, 494–499 (2013). [PubMed: 23475201]
8. Boswell KL, Paris R, Boritz E, Ambrozak D, Yamamoto T, Darko S, Wloka K, Wheatley A, Narpala S, McDermott A, Roederer M, Haubrich R, Connors M, Ake J, Douek DC, Kim J, Petrovas C, Koup RA, Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog* 10, e1003853 (2014). [PubMed: 24497824]
9. Cubas R, van Grevenynghe J, Wills S, Kardava L, Santich BH, Buckner CM, Muir R, Tardif V, Nichols C, Procopio F, He Z, Metcalf T, Ghneim K, Locci M, Ancuta P, Routy JP, Trautmann L, Li Y, McDermott AB, Koup RA, Petrovas C, Migueles SA, Connors M, Tomaras GD, Moir S, Crotty S, Haddad EK, Reversible Reprogramming of Circulating Memory T Follicular Helper Cell Function during Chronic HIV Infection. *J Immunol* 195, 5625–5636 (2015). [PubMed: 26546609]
10. Kohler SL, Pham MN, Folkvord JM, Arends T, Miller SM, Miles B, Meditz AL, McCarter M, Levy DN, Connick E, Germinal Center T Follicular Helper Cells Are Highly Permissive to HIV-1 and Alter Their Phenotype during Virus Replication. *J Immunol* 196, 2711–2722 (2016). [PubMed: 26873986]
11. Hufert FT, van Lunzen J, Janossy G, Bertram S, Schmitz J, Haller O, Racz P, von Laer D, Germinal centre CD4+ T cells are an important site of HIV replication in vivo. *AIDS* 11, 849–857 (1997). [PubMed: 9189209]
12. Biancotto A, Grivel JC, Iglehart SJ, Vanpouille C, Lisco A, Sieg SF, Debernardo R, Garate K, Rodriguez B, Margolis LB, Lederman MM, Abnormal activation and cytokine spectra in lymph

- nodes of people chronically infected with HIV-1. *Blood* 109, 4272–4279 (2007). [PubMed: 17289812]
13. Younes SA, Freeman ML, Mudd JC, Shive CL, Reynaldi A, Panigrahi S, Estes JD, Deleage C, Lucero C, Anderson J, Schacker TW, Davenport MP, McCune JM, Hunt PW, Lee SA, Serrano-Villar S, Debernardo RL, Jacobson JM, Canaday DH, Sekaly RP, Rodriguez B, Sieg SF, Lederman MM, IL-15 promotes activation and expansion of CD8+ T cells in HIV-1 infection. *J Clin Invest* 126, 27452756 (2016).
 14. Pizzolo G, Semenzato G, Chilosi M, Morittu L, Ambrosetti A, Warner N, Bofill M, Janossy G, Distribution and heterogeneity of cells detected by HNK-1 monoclonal antibody in blood and tissues in normal, reactive and neoplastic conditions. *Clin Exp Immunol* 57, 195–206 (1984). [PubMed: 6744669]
 15. Bowen MB, Butch AW, Parvin CA, Levine A, Nahm MH, Germinal center T cells are distinct helper-inducer T cells. *Hum Immunol* 31, 67–75 (1991). [PubMed: 1715336]
 16. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC, Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 193, 1373–1381 (2001). [PubMed: 11413192]
 17. Kim JR, Lim HW, Kang SG, Hillsamer P, Kim CH, Human CD57+ germinal center-T cells are the major helpers for GC-B cells and induce class switch recombination. *BMC Immunol* 6, 3 (2005). [PubMed: 15694005]
 18. Wendel BS, He C, Qu M, Wu D, Hernandez SM, Ma KY, Liu EW, Xiao J, Crompton PD, Pierce SK, Ren P, Chen K, Jiang N, Accurate immune repertoire sequencing reveals malaria infection driven antibody lineage diversification in young children. *Nature communications* 8, 531 (2017).
 19. Ke-Yue Ma CH, Wendel Ben S., Williams Chad M., Xiao Jun, Yang Hui, and Jiang Ning, Immune Repertoire Sequencing using Molecular Identifiers Enables Accurate Clonality Discovery and Clone Size Quantification. *Frontiers in immunology* In press, (2018).
 20. Jia Q, Zhou J, Chen G, Shi Y, Yu H, Guan P, Lin R, Jiang N, Yu P, Li QJ, Wan Y, Diversity index of mucosal resident T lymphocyte repertoire predicts clinical prognosis in gastric cancer. *Oncoimmunology* 4, e1001230 (2015). [PubMed: 26137399]
 21. Lin J, Divergence measures based on the Shannon entropy. *IEEE Transactions on Information theory* 37, 145–151 (1991).
 22. Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, Pe'er D, Nolan GP, Bendall SC, Normalization of mass cytometry data with bead standards. *Cytometry A* 83, 483–494 (2013). [PubMed: 23512433]
 23. Shekhar K, Brodin P, Davis MM, Chakraborty AK, Automatic Classification of Cellular Expression by Nonlinear Stochastic Embedding (ACCENSE). *Proc Natl Acad Sci U S A* 111, 202–207 (2014). [PubMed: 24344260]
 24. Schultz BT, Teigler JE, Pissani F, Oster AF, Kranias G, Alter G, Marovich M, Eller MA, Dittmer U, Robb ML, Kim JH, Michael NL, Bolton D, Streeck H, Circulating HIV-Specific Interleukin-21(+)CD4(+) T Cells Represent Peripheral Tfh Cells with Antigen-Dependent Helper Functions. *Immunity* 44, 167–178 (2016). [PubMed: 26795249]
 25. Huang J, Zeng X, Sigal N, Lund PJ, Su LF, Huang H, Chien YH, Davis MM, Detection, phenotyping, and quantification of antigen-specific T cells using a peptide-MHC dodecamer. *Proc Natl Acad Sci U S A* 113, E1890–1897 (2016). [PubMed: 26979955]
 26. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM, Virus-Specific CD4(+) MemoryPhenotype T Cells Are Abundant in Unexposed Adults. *Immunity* 38, 373–383 (2013). [PubMed: 23395677]
 27. Han A, Glanville J, Hansmann L, Davis MM, Linking T-cell receptor sequence to functional phenotype at the single-cell level. *Nat Biotechnol* 32, 684–692 (2014). [PubMed: 24952902]
 28. Dan JM, Lindestam Arlehamn CS, Weiskopf D, da Silva Antunes R, Havenar-Daughton C, Reiss SM, Brigger M, Bothwell M, Sette A, Crotty S, A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4+ T Cells in Blood. *J Immunol* 197, 983–993 (2016). [PubMed: 27342848]
 29. Wong MT, Chen J, Narayanan S, Lin W, Anicete R, Kiaang HT, De Lafaille MA, Poidinger M, Newell EW, Mapping the Diversity of Follicular Helper T Cells in Human Blood and Tonsils

- Using High-Dimensional Mass Cytometry Analysis. *Cell reports* 11, 1822–1833 (2015). [PubMed: 26074076]
30. Weinstein JS, Herman EI, Lainez B, Licona-Limon P, Esplugues E, Flavell R, Craft J, TFH cells progressively differentiate to regulate the germinal center response. *Nat Immunol* 17, 1197–1205 (2016). [PubMed: 27573866]
31. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC, Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* 143, 592–605 (2010). [PubMed: 21074050]
32. Victora GD, Nussenzweig MC, Germinal centers. *Annu Rev Immunol* 30, 429–457 (2012). [PubMed: 22224772]
33. Depoil D, Zaru R, Guiraud M, Chauveau A, Harriague J, Bismuth G, Utzny C, Muller S, Valitutti S, Immunological synapses are versatile structures enabling selective T cell polarization. *Immunity* 22, 185–194 (2005). [PubMed: 15723807]
34. Cuevas JM, Geller R, Garijo R, Lopez-Aldeguer J, Sanjuan R, Extremely High Mutation Rate of HIV-1 In Vivo. *PLoS Biol* 13, e1002251 (2015). [PubMed: 26375597]
35. Piantadosi A, Panteleeff D, Blish CA, Baeten JM, Jaoko W, McClelland RS, Overbaugh J, Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. *J Virol* 83, 10269–10274 (2009). [PubMed: 19640996]
36. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, Nutt SL, Tarlinton DM, IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* 207, 365–378 (2010). [PubMed: 20142430]
37. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE, IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol* 175, 7867–7879 (2005). [PubMed: 16339522]

Figure 1:

GC T_{FH} cells become clonally expanded. (A) Representative plots showing sorting strategy to identify naïve, memory, and GC T_{FH} cells. (B) Breakdown of the proportion of the TCR repertoire represented by clones of different sizes for sorted naïve, memory, and GC T_{FH} cells from HIV⁺ LNs. TCR clone size was normalized by the total number of TCR transcripts on nucleotide sequences. (C) Normalized Shannon entropy of the TCR repertoire of sorted naïve, memory, and GC T_{FH} cells. Grey lines link the same patient. Bars indicate means. * $P < 0.05$ by two-tailed Wilcoxon signed-rank test, $n = 8$ HIV-infected LNs.

Figure 2:

Antigen-driven clonal selection signature in GC T_{FH} cells of HIV-infected LNs. **(A)** Representative degeneracy plot from sample H2. Coding degeneracy level (number of unique TCR nucleotide (nt) sequences encoding a common CDR3 amino acid (aa) sequence) of each CDR3 aa sequence is plotted against their frequency (measured as % of total TCR transcripts) in naïve, memory, and GC T_{FH} cells. Each dot is a unique CDR3 aa sequence. Red dashed lines indicate cutoffs for degenerate (2 or more nt sequences coding for the same aa sequence, horizontal) and expanded (0.1% or more of TCR transcripts, vertical) clones. Red arrow points to example degenerate clone in **(B)**. **(B)** An example of CDR3 aa degeneracy. aa (top row) and nt (bottom row) sequences for 3 distinct nt sequences (0.41% of total TCR transcripts) that code for the same aa sequence as indicated by arrow in **(A)**: Y=3, X=0.41%. Red boxes and highlights indicate redundant codons. **(C)** Comparison of Q1 degenerate-abundant clone percentage in naïve, memory, and GC T_{FH} cells. Grey lines link the same patient. Bars indicate means. * $P < 0.05$ by two-tailed Wilcoxon signed-rank test, $n = 8$ HIV-infected LNs.

Figure 3:

GC T_{FH} cells exhibit HIV-antigen-driven clonal expansion and selection. **(A)** Gagspecific 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). Grey curves indicate Gag-specific TCR nucleotide sequences found in naïve (black, outer circle), memory (blue, outer circle), and GC T_{FH} (orange, outer circle) populations. No Gag-overlapping clones were detected for one subject, H8 (not shown). **(B)** Number of Gagspecific TCR clones observed in naïve, memory, and GC T_{FH} populations. Grey 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)** The number of distinct nucleotide sequences per CDR3 amino acid sequence, of Gag-specific T cells, HA-specific T cells, or bulk GC T_{FH} cells. Data from all 4 subjects were aggregated for C and D. Error bars indicate SEM. N.S. indicates not significant, *** *P* < 0.001 by two-tailed *t*-test.

Figure 4:

High-dimensional analysis of lymphoid CD4⁺ T cells identified distinct T_{FH} cell subsets in HIV⁺ patients and healthy controls. **(A)** Representative gates used to identify GC T_{FH} cells for t-SNE analysis. **(B)** t-SNE plots of GC T_{FH} T cells and CXCR5⁺CD45RO⁺CD4⁺ T cells generated using “cytokit” package in R. Data include cells from 25 HIV⁺ patients and 7 HC. **(C)** Phenotypic clusters identified from CXCR5⁺ memory CD4⁺ T cells using DensVM. **(D)** Heatmap shows the average staining signal of indicated markers within each of the clusters identified in **(C)**. Memory and naïve cells are shown at the bottom of the heatmap for comparison. Red-blue scale indicates staining intensity. Green-brown scale represents the relative frequency of HIV⁺ cells to total numbers of cells in each cluster. **(E)** The frequency of CD38 expression in CXCR5⁺ T_{FH} cells or GC T_{FH} cells from HC (n = 7) or HIV⁺ patients (n = 25). Data are mean ± SEM. ** *P* < 0.005; *** *P* < 0.0005 by two-tailed t-test. **(F)** The frequency of CD57⁺PD1⁺ GC subset within CXCR5⁺ T_{FH} cells that was positive or negative for CD38 expression (n = 25 HIV). *** *P* < 0.0005 by paired two-tailed t-test. **(G)** The relationship between IL-21 expression and CD38 frequency in CXCR5⁺ T_{FH} cells. **(H)** The relationship between peripheral CD4⁺ T cell count and IL-21⁺ GC T_{FH} cells frequency. For G and H, data are from 25 HIV⁺ patients, association was determined by Pearson correlation.

Figure 5:

Gag-reactive T_{FH} cells express IL-21 and acquire GC phenotype. **(A)** Representative plots showing IL-21 staining used to identify Gag-reactive T cells. LN cells were stimulated for 18 hours with vehicle alone (left) or Gag peptides (right). **(B)** TCRb 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)** The phenotype of Gag-reactive IL-21⁺ T cells (red) overlaid onto bulk CD4⁺ T cells (gray). **(D)** The 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, *** *P* < 0.0005 by two-tailed t-test. **(E)** Representative plots showing identification of Gagreactive T cells by CD25 and OX40 expression. **(F)** CD57⁺PD-1⁺ frequency of Gag-reactive T cells from HIV⁺ individuals (n = 6) identified using IL-21 capture or by CD25 and OX40 upregulation. **(G)** The frequency of CD25⁺OX40⁺CXCR5⁺PD-1⁺ T_{FH} cells that co-expressed IL-21 following Gag- or HA-peptide stimulation from 6 HIV⁺ individuals. **(H)** IL-21 expression within GC T_{FH} subset in LN cells from 11 HIV⁺ patients stimulated by Gag or HA peptides. The lines connect data from the same donor. For F, G, H, * *P* < 0.05, ** *P* < 0.005 by paired two-tailed Wilcoxon signed-rank test.

Figure 6:

Dominant IL-21 expression in T_{FH} cells correlate with B cell pathology in HIV-infected LNs. (A) The frequency of GC T_{FH} cells that positively stained for each indicated effector molecule as determined by CyTOF. ** $P < 0.005$, *** $P < 0.0005$ by two-tailed t-test ($n = 7$ HC, 25 HIV). (B) Bar-graph showing the frequency of single-IL-21-producing T cells as a percentage of total IL-21 $^{+}$ T cells within each indicated T_{FH} cell subset. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ by two-tailed Wilcoxon signed-rank test (PBMCs: $n = 4$ HC, 16 HIV; LN: $n = 6$ HC, 25 HIV). (C) Plot shows the correlation between the abundance of single-IL-21 $^{+}$ T cells (as a percentage of total IL-21 $^{+}$ T cells) in paired PBMC and LN from 4 HC and 15 HIV $^{+}$ individuals. Association is determined by Pearson correlation. (D) IgD $^{+}$ CD27 $^{-}$ naïve B cells were excluded (also see Fig. S16). Plots showing gating strategy to identify switched memory B cells (IgD $^{+}$ CD38 low) and plasma cells (IgD $^{+}$ CD38 high) on non-naïve B cells. (E-H) Correlation between switched memory B cell and plasma cells with the abundance of single IL-21 $^{+}$ T cells in GC T_{FH} cells (E-F) or CXCR5 $^{+}$ CD45RO $^{+}$ CD4 $^{+}$ T cells (G-H), $n = 7$ HC and 25 HIV. Association was measured by Pearson correlation (E and G) or Spearman's rank correlation (F and H), depending on data normality as determined by D'Agnostina-Pearson test.