Identification and characterization of HIV-specific resident memory CD8+ T cells in human lymphoid tissue

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Current paradigms of CD8+ T cell–mediated protection in HIV infection center almost exclusively on studies of peripheral blood, which is thought to provide a window into immune activity at the predominant sites of viral replication in lymphoid tissues (LTs). Through extensive comparison of blood, thoracic duct lymph (TDL), and LTs in different species, we show that many LT memory CD8+ T cells bear phenotypic, transcriptional, and epigenetic signatures of resident memory T cells (T RM). Unlike their circulating counterparts in blood or TDL, most of the total and follicular HIV-specific CD8+ T cells in LTs also resemble T RM. Moreover, high frequencies of HIV-specific CD8+ T RM with skewed clonotypic profiles relative to matched blood samples are present in LTs of individuals who spontaneously control HIV replication in the absence of antiretroviral therapy (elite controllers). Single-cell RNA sequencing analysis confirmed that HIV-specific T RM are enriched for effector-related immune genes and signatures compared with HIV-specific non-T RM in elite controllers. Together, these data indicate that previous studies in blood have largely failed to capture the major component of HIV-specific CD8+ T cell responses resident within LTs.

INTRODUCTION

It is well established that CD8+ T cells are required for effective immune control of HIV. The contemporary literature is also replete with studies that describe the qualitative characteristics of successful HIV-specific CD8+ T cell responses. For example, allotype restriction, antigen sensitivity and specificity, clonotype distribution, cytolytic activity, polyfunctionality, proliferative reserve, and response magnitude have all been associated with differential rates of disease progression (1). However, most of these correlates are based on data acquired from peripheral blood samples, despite the fact that HIV replicates primarily in lymphoid tissues (LTs).

HIV seeds, replicates, and persists in LTs (2). It is therefore important to develop an anatomically consistent view of immunosurveillance, especially in light of the fact that follicular helper T cells (T FH) facilitate viral replication and harbor much of the viral reservoir (3–5). The efficacy of live-attenuated simian immunodeficiency virus (SIV) vaccines can be predicted from the magnitude of virus-specific T cell responses in LTs (6). In addition, CD8+ T cell depletion is associated with a rapid redistribution of productive SIV infection to non-T FH in elite controller monkeys, suggesting active viral suppression by CD8+ T cells and/or natural killer cells, in LTs (7). However, very little is known about the antiviral properties of HIV/SIV-specific CD8+ T cells that reside in LTs.

Through the early work of Gowans (8), we now know that lymphocytes recirculate between tissues and blood through lymph via the thoracic and lymphatic ducts. More recent studies of human blood have categorized memory CD8+ T cells broadly into two circulating subsets based on their tissue-homing properties. Central memory T cells (T CMI) express lymphoid-homing receptors (CCR7 and CD62L) and recirculate between blood, LTs, and lymph, whereas effector memory T cells (T EM) lack CCR7 and CD62L and recirculate between blood, non-LTs (NLTs), and lymph to survey visceral organs.
and body surfaces (9). Recently, a new subset known as resident memory T cells (T_RMs), which reside within NLTs, has been identified (10–13). Most T_RMs constitutively express CD69 (14), which down-regulates the sphingosine-1-phosphate receptor 1 (S1PR1) (15), thereby preventing tissue egress. NLTs are seeded with T_RMs soon after immune priming (11), establishing a frontline tissue defense that can eliminate pathogens largely without the involvement of circulating T cells (16). T_RMs have distinct functional, phenotypic, and transcriptomic profiles attuned to their compartmentalized state (17).

T_RMs have been identified in murine LTs through parabiosis experiments (18), where they are relatively sparse compared with circulating T cells (19). CD8^+ T_RMs have also been observed in human LTs (20–22). The notion that T_RMs may exist in LTs could transform our understanding of CD8^+ T cell–mediated control of HIV infection. In line with this possibility, the frequency of HIV–specific CD8^+ T cells is generally higher in lymph nodes (LNs) compared with matched peripheral blood samples (23, 24), despite the fact that most circulating HIV–specific CD8^+ T cells lack classical lymphoid-homing characteristics (25, 26). Moreover, CD8^+ T cells accumulate in HIV–infected LTs (27) and respond poorly to S1PR1–mediated signals (28). However, it remains unclear whether HIV–specific CD8^+ T cells with a T_RM phenotype exist in HIV–infected LTs. To resolve this issue, we conducted a detailed assessment of CD8^+ T cells in the peripheral blood and LTs of healthy and HIV^+ individuals. Our data show that T_RMs most often dominate the HIV–specific CD8^+ T cell response in LTs and identify a link between increased magnitudes of HIV–specific CD8^+ T_RMs and effective immune control of HIV.

RESULTS

CD69^+CCR7^-CD8^+ T cells are expanded in HIV–infected LNs

CD69 has often been considered as a marker of early T cell activation (29). We examined CD69 expression on LN CD8^+ T cells from HIV^+ and HIV^− individuals (table S1). Irrespective of adherence to antiretroviral therapy (ART), higher frequencies of LN CD69^+ CD8^+ T cells and CCR7^- (T_EM) cells were present in HIV^+ compared with HIV^− individuals (Fig. 1A and fig. S1A). In HIV^+ individuals, higher frequencies of CD8^+ T cells expressed CD69 in LNs compared with blood (Fig. 1B). A strong association was also detected between the frequency of memory CD8^+ T cells and the frequency of CD69^+ CD8^+ T cells in LNs (Fig. 1C and fig. S1B). Moreover, LN CD69^+ CD8^+ T cells from HIV^+ individuals were biased toward a T_EM phenotype (Fig. 1D and fig. S1B).

To determine whether LN CD8^+ T cells express CD69 as a consequence of activation, we obtained samples from HIV^+ individuals in the acute/early stages of infection (Fiebig IV to VI), a time when early cycling (Ki-67^+) CD8^+ T cells are prevalent in blood (30). Substantially higher frequencies of LN CD8^+ T cells expressed Ki-67 in acutely infected HIV^+ compared with HIV^− individuals (Fig. 1E). These LN Ki-67^+ CD8^+ T cells also showed evidence of HIV specificity (fig. S1C), as demonstrated previously in blood (30). However, only a minority of LN CD69^+ CD8^+ T cells expressed Ki-67 (Fig. 1F and fig. S1D). In addition, we conducted mass cytometry to assess the expression of multiple activation markers on LN CD8^+ T cells in HIV^+ individuals with chronic disease (Fig. 1G). Similar to our conventional flow cytometry data, mass cytometry also demonstrated higher CD69 levels in LNs compared with blood (fig. S1E). We next identified 14 CD8^+ T cell clusters algorithmically in t-distributed stochastic neighbor embedding (tSNE) space, where one cluster (#4) showed high coexpression of several activation markers (CD38, Ki-67, and ICOS) but not CD69 (Fig. 1H). In contrast, CD69 expression intensity was high on unique clusters (#7 and #8) with elevated levels of CD38, but also dissociated with CD38 expression in other dominant CD69 clusters (#2 and #3) (Fig. 1H). Furthermore, some naïve–like clusters (#10 and #14) demonstrated low CD69 intensity (fig. S1F), but these clusters also expressed low to high intensity of BCL-6, CD95, and CXCR5 (#10) as well as CXCR3 and CCR6 (#14), suggestive of memory stem T cell (T_SCM)–like features (31). Quantitative assessments demonstrated that several activation markers could be expressed on CD69^+ CD8^+ T cells (fig. S1G); however, hierarchical clustering confirmed that CD69 expression was more closely related to PD-1, CD95, and CXCR3 expression than CD38, Ki-67, and ICOS expression (Fig. 1I). Collectively, these data indicate that high levels of CD69 expression on memory CD8^+ T cells in HIV–infected LTs cannot be explained by an increased early/cycling (Ki-67^+) immune activation profile.

CD69^+CD8^+ T cells show enriched traits of residency in LTs

Although CD69 is classically associated with activation, it also marks noncirculating T_RMs in NLTs (32). Recent studies in humans have also identified high levels of CD69^+ CD8^+ T cells in multiple LTs (20–22). However, it remains unclear whether these findings reflect early T cell activation or proliferation in inflamed tissues and organs isolated from brain–dead individuals, as previously described in murine models (33). We therefore collected spleen, iliac LNs (IliLNs), mesenteric LNs (MesLNs), and tonsils from HIV^− individuals without known major inflammatory disease. Using conventional (fluorescence) flow cytometry (fig. S2A), we found that most LT memory CD8^+ T cells expressed CD69, irrespective of tissue origin (Fig. 2A). Most LT T_EM cells also expressed CD69 (Fig. 2B), suggestive of a T_RM phenotype (32). Similar to our previous analysis on HIV–infected individuals, we also found here some expression of CD69 on naïve-like (CCR7^+CD45RA^+) cells (fig. S2B). However, CD69 expression levels on naïve-like cells were not significantly different between blood and LTs, and these cells had lower CD69 median fluorescence intensity (MFI) than on paired memory cells (fig. S2B). Moreover, CD69^+CCR7^-CD45RA^+ cells were biased toward higher CD95 MFI levels, again indicative of a T_SCM phenotype (fig. S2C). CD69^+ CD8^+ T cells expressed lower levels of multiple activation markers compared with in vitro stimulated T cells (fig. S2D). CD69^+ CD8^+ T cells did not entirely overlap with the set of activation markers using tSNE analysis (fig. S2E), and Boolean gating analysis confirmed that the minority of CD69^+ CD8^+ T cells (median, 33.7%) expressed any of the assessed activation markers, suggesting that CD69 expression on these cells is not driven exclusively by activation.

In more detailed analyses (fig. S3A), we found that LT memory CD8^+ T cells expressed higher levels of integrins (CD103 and CD49a) and chemokine receptors (CXCR5, CXCR3, and CCR5) compared with blood memory CD8^+ T cells (Fig. 2C and fig. S3B). In contrast, blood memory CD8^+ T cells expressed higher levels of circulation markers (CD52) (34), effector (T-bet, Eomes, perforin, and granzyme B) proteins, as well as the fractalkine receptor CX3CR1 (Fig. 2C and fig. S3B). tSNE analysis further showed that LT memory CD8^+ T cells formed distinct clusters compared with blood memory CD8^+ T cells (Fig. 2D). Different clusters were observed in spleen and tonsils compared with LNs. We also examined LT CD103^+, CXCR5^+, CD49a^+, CCR5^+, and CXCR6^+ CD8^+ T cells and found that these populations were more likely to express CD69 (Fig. 2E and fig. S4). Likewise, LT CD69^+ CD8^+ T cells expressed higher levels of CD103, CXCR5, CD49a, CCR5,
Fig. 1. CD69 is not coupled to an early immune activation signature in HIV-infected LNs. (A) Frequency of CD69 expression on total LN CD8+ T cells from HIV+ and HIV− individuals. Red indicates ART−, and orange indicates ART+ in the HIV+ scatter plot group. (B) Frequency of CD69 expression on total blood (top) and LN (bottom) CD8+ T cells. Matched samples from one individual are shown in the representative flow cytometry plots. Lines connect matched samples across all individuals in the graph. PBMC, peripheral blood mononuclear cell. (C) Correlation between the frequency of memory (non-CCR7hi non-CD45RO−) CD8+ T cells and CD69 expression on total blood (top) and LN (bottom) CD8+ T cells. Blue, HIV− individuals; red, HIV+ ART− individuals; orange, HIV+ ART+ individuals. (D) Frequency of CCR7 − CD69+ memory CD8+ T cells in HIV− (blue) and HIV+ (red, ART−; orange, ART+) individuals. (E) Coexpression pattern of Ki-67 and CD69 in/on total LN CD8+ T cells from an HIV− individual (left) and an HIV+ ART− individual (right) with acute infection (Fiebig V). (F) Frequency of Ki-67−/+ CD69+ CD8+ T cells in LNs from HIV+ individuals with acute infection (n = 7). (G) Expression intensity of CD69, ICOS, Ki-67, and CD38 in multidimensional viSNE space. viSNE plots were derived using 27,576 cells (n = 17 individuals), and the markers are shown in (I). (H) Same viSNE with subpopulations colored using the PhenoGraph implementation of Cytofkit (top). Distribution of CD69 expression over all 14 subpopulations derived using PhenoGraph (bottom). (I) Hierarchical clustering of expression intensity (z score) for all assessed markers within the different subpopulations derived using PhenoGraph. Median and interquartile range (IQR) are shown for all scatter/bar plots. **P < 0.01, ***P < 0.001. Conventional (fluorescence) flow cytometry was used for all stains in (A) to (F), and mass cytometry (CyTOF) was used in (G) to (I).
Fig. 2. CD69+ CD8+ T cells show TEM characteristics in LTs. (A) Frequency of CD69 expression on memory CD8+ T cells (blue) in blood, spleen, IliLNs, MesLNs, and tonsils from HIV− individuals. Asterisks denote statistical comparisons versus blood. (B) Frequency of CD69−/+ cells among CCR7+ (TCM) and CCR7− (TEM) CD8+ T cells. Bar plots are based on all individuals in (A). (C) Heat map showing expression frequency (blue, 0%; red, 100%) for the indicated markers among blood, spleen, IliLN, MesLN, and tonsil memory CD8+ T cells. (D) tSNE clustering on blood (red), spleen (orange), IliLN (blue), MesLN (green), and tonsil (black) memory CD8+ T cells. Values for the tSNE analysis are derived from all markers in (C). (E) CD69 expression on CD103+, CXCR5+, CD49a+, CCR5+, CXCR6+, and CD52+ memory CD8+ T cells from MesLNs. (F) Average expression frequency (blue, 0%; red, 100%) for the indicated markers among blood, spleen, IliLN, MesLN, and tonsil CD69− and CD69+ memory CD8+ T cells. Colors denote average frequencies, derived from all individuals in (C). Median and IQR are shown for all scatter/bar plots. *P < 0.05, **P < 0.01, ***P < 0.001. Conventional flow cytometry was used for all stains in this figure.
CXCR6, PD-1, and granzyme B compared with CD69−CD8+ T cells, which expressed higher levels of CD52 (Fig. 2F).

**LT CD69+CD8+ T cells have transcriptional, epigenetic, and functional T RM signatures**

To determine whether human CD69+CD8+ T cells have a transcriptional signature resembling TRMs, we sorted MesLN memory CD69+ and CD69−CD8+ T cells (fig. S5A) and performed RNA sequencing (RNA-seq) analysis. We identified a core signature of genes that were either up-regulated or down-regulated in LN memory CD69+ and CD69−CD8+ T cells (fold change > 2; P < 0.05), where particular genes that are down-regulated in NLT TRMs, such as S1pr1, Klf3, and Sell (35), were also poorly expressed in LN memory CD69+CD8+ T cells (Fig. 3A and table S2). Znf683 (Hobit), previously associated with murine TRM formation (36), was down-regulated in LN memory CD69+CD8+ T cells. Gene set enrichment analysis revealed that genes linked with human lung CD69+CD103+CD8+ T cells (37) were also enriched in LN CD69+CD8+ T cells (fig. S5B). Ingestiny pathway analysis further demonstrated that the major differences between CD69+ and CD69−CD8+ T cells included cell-mediated immune responses and cell trafficking (Fig. 3B). In addition, many genes typically associated with TRMs and TCMs/TEMs (17, 38) were up-regulated (Igtae, Rgs1, Rgs2, Xcl1, Tbx21, and Ifng) or down-regulated (Fam65b, Klf2, Rasgrp2, S1pr1, Sell, Ccr7, Lef1, Tcf7, Cxcr3, and Cxcr4) in CD69+ CD8+ T cells (Fig. 3C). Many of these genes followed the patterns observed in splenic CD69+ CD8+ T cells and TCMs (CD69−CD103+CD8+) isolated from P14 T cell receptor (TCR)–transgenic mice (Fig. 3C).

We further conducted assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) to map potential differences in open chromatin regions (OCRs) between LN memory CD69+ and CD69−CD8+ T cells. OCRs adjacent to genes known to be involved in the establishment of residency and immune responses showed marked differences between these two subsets of LN memory CD8+ T cells (Fig. 3D and table S3). For example, several OCRs were present next to Ifng, Xcl1, and Xcl2 primarily in CD69+CD8+ T cells (Fig. 3E), suggesting a readiness to up-regulate these effector functions (17, 37). Prdm1 (encoding Blimp-1), recently associated with TRM formation (36), was also enriched for OCRs in CD69+CD8+ T cells (fig. S5C). In contrast, CD69−CD8+ T cells were more likely to have multiple OCRs adjacent to genes associated with trafficking, including Klf2 and S1pr1 (Fig. 3F), and the Znf683 locus (fig. S5C). We further inferred transcription factor occupancy in regulatory elements by motif analysis and found that RUNX, AP-1, and ETS families belonged to the most enriched motifs in CD69+CD8+ T cells, whereas CD69−CD8+ T cells showed enriched motifs belonging to the PAX, TCF, and KLF families, demonstrating that unique transcription factor families are likely cooperatively responsible for regulating resident and recirculating T cell states (Fig. 3G).

Next, we sorted human LT memory CD69+ and CD69−CD8+ T cells and found that CD69+ was stably expressed on the surface of TRMs for up to 9 days (Fig. 3G). Classical TRMs express low levels of CCR7 and CD62L (32). We found no evidence that supplementation with CCR7 ligands (CCL19 or CCL21) influenced CD69 down-regulation on LT memory CD69+CD8+ T cells (fig. S5D). However, CD69 was down-regulated significantly on CCR7+CD69+CD8+ T cells after 9 days in culture (fig. S5E), and markers associated with residency and effector functions were predominantly expressed by CCR7−CD69−CD8+ T cells ex vivo (fig. S5F). Together, these data show that LT CD69+CD8+ T cells show enriched phenotypic, transcriptional, epigenetic, and functional traits of tissue residency.

**Lack of CD69+CD8+ T cells in thoracic duct lymph**

We next applied multiplexed confocal imaging and histocytometry to quantify and position where cells with a TRM phenotype were localized in human LNs (fig. S6A) (39). CD20 was used to distinguish B cell follicles and did not overlap to a great extent with the anatomical location of CD8+ cells as expected (Fig. 4A and fig. S6B). Imaging and quantitative analysis demonstrated that most CD8+CD69+CD103− and CD69+CD103− cells were present in extrafollicular areas (Fig. 4B and fig. S6B). Further analysis revealed that CD69+CD103− cells expressed PD-1 to a higher degree than CD69+ cells. The CD69+CD103−PD-1+ CD8+ population was localized to an equal degree in B cell follicles and extrafollicular areas (fig. S6C).

To determine whether CD8+ T cells maintain CD69 expression after egress from LNs, we collected efferent lymph from HIV− individuals via cannulation of the human thoracic duct (Fig. 4C). Thoracic duct lymph (TDL) contained very low frequencies of CD69+ CD8+ T cells compared with those detected in LTs (Fig. 4D). Both TDL and LT contained a significant proportion of single CD69+CD103+CCR7+ CD8+ T cells, indicating that CD103 expression alone is insufficient to maintain residency in LTs (Fig. 4D and fig. S6D). The overall frequency of CD103+ CD8+ T cells was higher in LTs compared with TDL though, due to the large population of CD69+CD103−CCR7− CD8+ T cells present in LTs. In addition, we collected TDL, blood, and L Ts from healthy rhesus macaques (Fig. 4E). Again, very few blood and TDL CD8+ T cells expressed CD69 compared with donor-matched LT CD8+ T cells (Fig. 4C). Collectively, these data suggest that CD69+CD8+ T cells likely do not leave L Ts.

**HIV-specific CD8+ T cells show a TRM phenotype in LNs**

The observation that memory CCR7+CD69+CD8+ T cells may have TRM characteristics. In contrast to matched blood and LN bulk memory CD8+ T cells, most LN HIV-specific CD8+ T cells expressed CD69 (Fig. 5A and fig. S7A). To confirm that HIV-specific CD69+ CD8+ T cells do not leave L Ts, we detected only very low levels of CD69 on bulk and HIV-specific CD8+ T cells in TDL (Fig. 5A). CD69 expression on HIV-specific CD8+ T cells was unaffected by ART status (Fig. 5A), indicating that antigen load is not necessarily a determinant of CD69 expression, in contrast to classical immune activation markers in blood (40, 41).

In line with our previous findings, most HIV-specific CD69+CD8+ T cells (median, 84.1%) did not express CCR7 (Fig. 5B), similar to bulk memory CD8+ T cells (fig. S7A). Moreover, HIV-specific CD8+ T cells in LNs demonstrated a predominant CD69+CD103− or CD69+CD103− profile, in contrast to HIV-specific CD8+ T cells in blood and TDL (Fig. 5C). We further examined cytomegalovirus (CMV)–specific CD8+ T cells for markers of residency, because CMV does not replicate primarily in L Ts (42, 43). In individuals with detectable CMV- and HIV-specific CD8+ T cell responses, we consistently found that HIV-specific CD8+ T cells were present in blood and LNs, whereas CMV-specific CD8+ T cells were always more prevalent in blood compared with LNs (Fig. 5D) (44). HIV-specific CD8+ T cells also expressed higher levels of CD69 and CD103 than CMV-specific CD8+ T cells in LNs (Fig. 5E). In addition, HIV-specific CD69+CD8+ T cells were more likely to express cytolytic proteins (perforin and/or...
Fig. 3. CD69⁺ CD8⁺ T cells show functional and transcriptional T RM signatures in LTs. (A) RNA-seq heat map showing differentially expressed genes (fold change > 2; \( P < 0.05 \)) between human MesLN memory CD69⁺ and CD69⁻ CD8⁺ T cells. (B) Ingenuity pathway analysis based on all differentially expressed genes (\( P < 0.05 \)) between CD69⁺ and CD69⁻ CD8⁺ T cells in the RNA-seq data set. The top pathways involved in the physiological system development and function arm are shown in the table. (C) Differential expression patterns of T RM- and T CM/EM-related genes between human LN memory CD69⁺ and CD69⁻ CD8⁺ T cells (top) and between splenic CD69⁺ and CD69⁻ CD8⁺ T cells from P14 TCR transgenic mice (bottom). (D) ATAC-seq volcano plot showing OCRs adjacent to specific genes. Green marks ATAC-seq peaks that are differentially enriched (fold change > 2; \( P < 0.05 \)) in human hepatic LN memory CD69⁺ and CD69⁻ CD8⁺ T cells. (E) ATAC-seq tracks from human LN memory CD69⁺ (red) and CD69⁻ (blue) CD8⁺ T cells. (F) Enriched ATAC-seq de novo motifs for CD69⁺ (left) and CD69⁻ (right) CD8⁺ T cells. (G) Human memory CD69⁺ and CD69⁻ CD8⁺ T cells from spleen, tonsil, and hepatic LNs (\( n = 2 \) each) were assessed over time for CD69 expression in the presence or absence of recombinant human interleukin-7 (IL-7). Lines represent means ± SD. Conventional flow cytometry was used for all stains in this figure.
granzyme B) than HIV-specific CD69⁺ CD8⁺ T cells (Fig. 5F). The distribution of cytolytic proteins was similar on CD69⁺ bulk memory and HIV-specific CD8⁺ T cells (fig. S7A), indicating that many characteristics of CD69⁺ bulk and HIV-specific CD8⁺ T cells are indistinguishable.

Virus-specific CXCR5⁺ CD8⁺ T cells have recently been shown to play a key role in immune protection against chronic viral infections (45–48). However, it is unclear whether human CXCR5⁺ CD8⁺ T cells exhibit TRM properties akin to TFHs (49). In HIV⁺ individuals, we observed higher frequencies of CXCR5⁺ CD8⁺ T cells in LNs compared

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Fig. 4. Anatomical distribution of CD69⁺ CD8⁺ T cells in LNs and TDL. (A) Whole imaged confocal microscopy of MesLN from human (left) and zoomed-in area (middle). Histocytometry gating strategy to identify localization of CD8⁺ T and B cells in the LNs (right). (B) Identification of B cell follicles (red) and extrafollicular areas (green) in the whole imaged area using histocytometry (left). Middle graph shows absolute numbers of CD69⁺ CD103⁻ and CD69⁺ CD103⁺ within all imaged extrafollicular (EF) and B cell follicular (F) areas for three imaged LNs. Example of histocytometry imaging showing the distribution of CD69⁺ CD69⁺ cells within the zoomed-in LN area. (C) Localization in the human body of the thoracic duct. (D) Flow plots of CD69 expression on human TDL and MesLN memory CD8⁺ T cells from two unmatched individuals. Frequency of CD69⁺ CD8⁺ T cells in TDL (n = 7 individuals) and LTs from all individuals (n = 25) in Fig. 2A. (E) Frequency of CD69 expression on blood, inguinal LN (IngLN), MesLN, tonsil, spleen, and TDL memory (non-CD28⁺ non-CD95⁻) CD8⁺ T cells from sample-matched rhesus macaques. Each color indicates fluid/tissue from one specific rhesus macaque. Asterisks denote statistical comparisons versus TDL. *P < 0.05, **P < 0.01, ***P < 0.001. Conventional flow cytometry was used for all stains in this figure.
Fig. 5. Majority of HIV-specific CD8\(^+\) T cells show a T\(_{RM}\) phenotype in LNs.

(A) Flow cytometry plots of memory CD8\(^+\) T cells showing CD69 expression on matched HIV-tetramer\(^+\) cells in blood and LNs (left) and matched HIV-tetramer\(^+\) cells in blood and TDL (middle). Frequency of CD69 expression on HIV-tetramer\(^+\) cells in blood (n = 13 individuals), ART\(^-\) LNs (n = 4 individuals), ART\(^+\) LNs (n = 4 individuals), and TDL (n = 7 individuals) (right).

(B) Distribution of LN HIV-tetramer\(^+\) (spec) CD69\(^+\) CD8\(^+\) T cells among the naive (CD45RO\(^-\) CCR7\(^+\)), TCM (CD45RO\(^-\) CCR7\(^+\)), TEB (CD45RO\(^+\) CCR7\(^+\)), and effector (T\(_{EF}\); CD45RO\(^+\) CCR7\(^-\)) compartments.

(C) CD69 and CD103 expression profiles for blood (blue), LN (red, ART\(^-\)); orange, ART\(^+\)), and TDL (yellow) HIV-specific CD8\(^+\) T cells.

(D) Representative flow cytometry plots showing memory CMV-tetramer\(^+\) (left) and HIV-tetramer\(^+\) (right) CD8\(^+\) T cell frequencies in blood (top) and LNs (bottom) from the same individual.

(E) Frequency of CD69 and CD103 expression on LN CMV-NV9–specific and HIV-FK10–specific CD8\(^+\) T cells in the same individuals with detectable tetramer\(^+\) responses to both viruses. Each color represents a matched individual and a matched tetramer\(^+\) response.

(F) Representative flow cytometry plots showing CD69 expression on perforin\(^+\) granzyme B\(^+\) HIV-specific CD8\(^+\) T cells in LNs (left).

(G) Flow plots showing CXCR5 and CD69 coexpression on memory CD8\(^+\) T cells in blood, LN (red, ART\(^-\) LNs; orange, ART\(^+\)), and TDL.

(H) Frequency of CD69 expression on blood, LN, and TDL CXCR5\(^+\) memory CD8\(^+\) T cells in HIV\(^+\) individuals.

(I) Frequency of CCR7 expression on LN CD69\(^+\) CXCR5\(^+\) CD8\(^+\) T cells in HIV\(^+\) individuals. Bar plots are based on all individuals in (A).

(J) Representative flow cytometry plots showing CXCR5 and CD69 coexpression on blood, LN, and TDL HIV-tetramer\(^+\) CD8\(^+\) T cells (matched for blood and LNs but not for TDL).

(K) Frequency of CD69 expression on blood (n = 18 responses), LN (n = 21 responses), and TDL (n = 10 responses) HIV-specific CXCR5\(^+\) CD8\(^+\) T cells.

(L) Frequency of CCR7 expression on LN (n = 21 responses) HIV-specific CD69\(^+\) CXCR5\(^+\) CD8\(^+\) T cells.

(M) Frequency of CD69\(^+\) CXCR5\(^+\) cells among LN CMV- and HIV-specific CD8\(^+\) T cells in individuals with detectable tetramer\(^+\) responses to both viruses. Each dot represents a single tetramer\(^+\) response in all scatter plots. Each color represents a matched individual and a matched tetramer\(^+\) response. *P < 0.05, **P < 0.01, ***P < 0.001. Conventional flow cytometry was used for all stains in this figure.
with blood and TDL (Fig. 5G). Most CXCR5+ CD8+ T cells in LNs coexpressed CD69 and lacked CCR7 (Fig. 5, H and I). Similarly, we found very low levels of HIV-specific CXCR5+ CD8+ T cells in peripheral blood and TDL compared with LTs (Fig. 5J), likely because most LT HIV-specific CXCR5+ CD8+ T cells showed a residency phenotype, including high levels of CD69 (Fig. 5K) and low levels of CCR7 (Fig. 5L). In addition, we found that LT HIV-specific CD8+ T cells more commonly expressed a CD69+ CXCR5+ phenotype compared with LT CMV-specific CD8+ T cells (Fig. 5M). These data suggest that HIV-specific CXCR5+ cells are T RMs and are induced due to preferential localization of HIV in B cell follicles.

**CD69 expression increases on SIV-specific CD8+ T cells over time**

To assess the kinetics of T RM formation, we infected Mamu A*01+ rhesus macaques with SIVmac251 and collected blood and LTs over time. Peripheral blood SIV-specific CD8+ T cells showed a pronounced transient burst of Ki-67 and CD69 expression at days 10 to 14 after infection (Fig. 6 and fig. S7B). In contrast, LT SIV-specific CD8+ T cells showed an inverse association between Ki-67 and CD69 over the same time period, indicating that most early cycling cells were not retained in LTs. After resolution of the acute phase, cycling decreased and CD69 expression steadily increased on LT SIV-specific CD8+ T cells (Fig. 6), suggesting that residency is established late and that CD69 expression is most likely only downregulated after extensive cycling in response to antigen (50).

**Elite controllers demonstrate high magnitudes of LN HIV-specific T RMs**

Recent work has shown that LT CD8+ T cells suppress viral replication in SIV elite controller rhesus macaques (7). In human elite controllers, we found that most LN HIV-specific CD8+ T cells showed a T RM phenotype (Fig. 7A). Human elite controllers also showed higher LN/blood ratios of HIV-specific CD8+ T cells (13 dpi, n = 12; 90 dpi, n = 3). The longitudinal SIV-specific CD8+ T cell response is plotted at each time point as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. Conventional flow cytometry was used for all stains in this figure.

**HIV-specific T RMs demonstrate enriched effector-related immune genes and signatures compared with HIV-specific non-T RMs in LNs**

We next index-sorted HLA-B*2705–restricted and/or HLA-B*5701–restricted Gag-specific CD8+ T cells from LNs and blood of two elite controllers and conducted single-cell RNA-seq (scRNA-seq) (fig. S8A). HIV-specific CD8+ T cells from LN and blood clustered differentially in the single-cell space (fig. S8B and table S4). Immune-related pathways, including interferon signatures, were higher in blood, whereas pathways related to maintenance of cell location were higher in LNs, suggesting that both immunological and trafficking properties distinguish the LN response from blood (fig. S8C). We next compared blood versus LN CD69+ HIV-specific CD8+ T cells and found that specific cytolytic genes and pathways involved in lymphocyte degranulation were higher in blood (fig. S8D). Cytolytic differences at the transcript level were not seen between blood and LN CD69+ HIV-specific CD8+ T cells (fig. S8F).

**CD69 expression on HIV-specific clonotypes is associated with higher clonotype distribution in LN**

On the basis of the enriched effector signature of CD69+ HIV-specific T RMs in LT, we finally examined whether these cells exhibited
Fig. 7. Elite controllers exhibit high magnitudes of CD69+ HIV-specific CD8+ T cells in LNs. (A) Representative flow cytometry plots of memory CD8+ T cells showing CD69 expression on HIV-tetramer+ cells in matched blood and LN samples from an HIV elite controller. (B) Relative distribution of HIV-specific CD8+ T cells in blood and LNs from elite controllers (black) and other HIV+ individuals (red, ART−; orange, ART+). (C) Magnitude of LN HIV-tetramer+ CD69+ CD8+ T cell responses in elite controllers and other HIV+ individuals (red, ART−; orange, ART+). (D) Magnitude of LN HIV-tetramer+ CD69+ CD8+ T cell responses specific for immunodominant HLA-B*5701-restricted and non-HLA-B*5701-restricted epitopes in elite controllers (n = 2). Data points were only included, where both HLA-B*5701+ and HLA-B*5701- tetramers were tested to avoid analysis biases. Each dot represents a single tetramer+ response in all scatter plots. *P < 0.05, **P < 0.01. Conventional flow cytometry was used for all stains in this figure.

differential clonotypic segregation between blood and LT. Individual EC1526 expressed lower levels of CD69 on LN HIV-specific CD8+ T cells (Fig. S9B) and showed a mixed clonotype distribution between blood and LNs (Fig. 8B). In contrast, individual EC1788 expressed higher levels of CD69 on LN HIV-specific CD8+ T cells (Fig. S9B) and showed marked repertoire skewing between blood and LNs. Clonotypes present at high frequencies in LNs relative to blood expressed high levels of CD69 in LNs (Fig. 8C). Moreover, the frequency of CD69 expression on clonotypes in LNs was associated with the relative frequency of clonotypes in LNs (Fig. 8D). Together, these data show that HIV-specific CD69− CD8+ T cell clonotypes present in blood can also be found in LNs, whereas HIV-specific CD69+ CD8+ TRM clonotypes with effector-like functional signatures preferentially localize to LNs.

DISCUSSION

T_RMs provide constant immunosurveillance and early protection against secondary challenge in NLTs. Here, we show that CD8+ T cells with a T_RM phenotype also accumulate in LTs and dominate the LT CD8+ T cell response against HIV. Our key findings were as follows: (i) CD69+ CD8+ T cells are highly expanded in HIV-infected LNs; (ii) LT CD69+ CD8+ T cells are largely not recently activated, based on Ki-67 expression, and instead bear transcriptional and epigenetic signatures of T_RMs distinct from nonresident T cells in LNs; (iii) LT CD69+ T_RMs do not recirculate to the blood via the thoracic duct; and (iv) LT CD69+ T_RMs most often dominate the HIV-specific CD8+ T cell response in LNs. Moreover, we found high magnitudes of LT HIV-specific T_RMs, in elite controllers and demonstrate that CD69 expression is closely linked to the distribution of HIV-specific clonotypes between LN and blood. Collectively, these data inform our current understanding of immunosurveillance in LNs and define that ongoing CD8+ T cell–mediated immunity against HIV in LNs is in part mediated by T_RMs.

Previous studies have identified bona fide LT CD8+ T_RMs in mice (18), but generally at low levels (19), especially compared with the frequencies of human LT CD69+ CD8+ T cells recently described by Farber and colleagues (20, 21). These human studies have generated some controversy, because CD69 expression may occur as a consequence of brain death–induced immune activation (33). Our data show that high frequencies of noncycling CD69+ CD8+ T cells are generally present in multiple human LNs and further show that LN CD69+ CD8+ T cells share many of the core transcriptomic signatures of NLT T_RMs (17, 35, 37). A recent study confirmed that human LT and NLT CD69+ T cells share several prototypical genes of our LN T_RM signature—such as lower levels of Slpr1, Krf3, and Sell—and pathways that are involved in immune defense and lymphocyte migration (51). However, the core signature from Kumar et al. (51) was extracted from both CD4+ and CD8+ T cells and a blood–contaminated lymphoid organ (spleen), whereas we solely focused on LN CD8+ T cells from live individuals. Hence, specific genes that were higher on CD69+ (e.g., Cx3cr1) or CD69− (e.g., Pdcd1, Il10, and Il2) T cells were not seen in our data set (51). Similar to this (51) and other studies in the human T_RM field, we did not find an association between residency in human LNs and the murine T_RM-associated transcription factor Hobit (37, 52). We also report the epigenetic structure of human LT CD8+ T_RMs; epigenetic differences were apparent between the proximal regulatory regions of many genes in CD8+ T_RMs versus non-T_RMs, including genes associated with cellular motility, migration, adhesion, and function. These data provide evidence that T_RMs have a unique epigenetic imprinting for genes that are involved in residency and also aid to explain that certain genes are more highly expressed in T_RMs. These results strongly support the notion that LT CD69+ cells are enriched for T_RMs.

Our data indicate that CD69 can be expressed on CCR7+ cells, including naïve-like cells, whereas CD69+ CCR7− cells usually represent bona fide T_RMs in both NLTs and LTs in mice. We cannot formally exclude the possibility that CCR7+ CD69+ could modulate CD69 to exit LTs. Likewise, it remains unclear whether
A scRNA-seq

**Fig. 8. Distribution of HIV-specific clonotypes between LN and blood.** (A) scRNA-seq was conducted on CD69+ and CD69− index-sorted HLA-B*2705–restricted and/or HLA-B*5701–restricted Gag-specific CD8+ T cells from LNs of two elite controllers. The heat map (left) illustrates the single-cell gene expression variability of differentially expressed genes (P < 0.01), and violin plots (right) illustrate immune-related genes differentially expressed between CD69+ and CD69− single HIV-specific CD8+ T cells. (B) CD38 amino acid sequence and percent frequency are shown for each HLA-B*2705–restricted and HLA-B*5701–restricted clonotype. The frequency of CD69 expression for each clonotype was obtained from single-cell index data (right). (C) CD69 expression on LN clonotypes, where higher frequencies of specific clonotypes are present in LNs versus blood (LN > blood; n = 7) or vice versa (LN < blood; n = 7). Bars show means ± SD. (D) Correlation between CD69 expression on LN clonotypes and the distribution of clonotypes between LNs and blood (frequency of a given clonotype in LNs minus the corresponding frequency in blood). Data points were only included, where at least one clonotype was present in both compartments. N/A, not applicable. *P < 0.05, **P < 0.01. Conventional flow cytometry was used for all stains in this figure.

CD103+CD69−CCR7+CD8+ T cells also originate from CD8+ T RMs in LTs that modulate expression of CD69 and CCR7 or whether these cells represent a recirculating CD8+ T cell memory population transiently observed in both LT and efferent lymph. Previous studies in KAEDE mice have identified CD103+CD69−CCR7+ migrating CD4+ T cells in both draining LNs and blood originating from the skin after photoactivation, which share the same phenotype we observed in both LT and TDL CD8+ T cells (53). One possible mechanism by which T cells can down-regulate CD69 is through extensive cycling, leading to increased KLF2 and S1PR1 expression (50). This concept aligns with our acute SIV infection data, which show that CD69+ CD8+ T cell frequencies decline markedly in LTs
when antigen-specific T cells reach a cycling plateau, and then accumulate progressively as LT SIV-specific CD8+ T cells no longer cycle. It remains unclear whether these kinetic changes reflect homeostatic expansion of the original population or the conversion of recirculating cells into TRMs. However, if all LT CD8+ T RMs were free to transiently modulate CD69 and equally recirculate, then it would be difficult to explain the observed clonotypic disequilibrium between LN and blood in individuals with prominent HIV-specific CD8+ CD69+ responses. Clonotypic disequilibrium has also been observed in LTs with high levels of CD4+ TRMs, such as in Peyer’s patches (54). Coupled with the lack of CD69+ cells in the thoracic duct and extensive expansion of CD69+CCR7- cells in HIV infection, these data suggest that LT HIV-specific CD8+ T cells, which mostly bear the CD69+CCR7- phenotype, become resident as a consequence of local antigen exposure in LTs.

In light of the fact that CD8+ T RMs are abundant in all human LTs, it seems plausible that these cells serve as regional sentinels that scan all antigen-presenting cells originating from areas that drain to their respective LN. CD8+ T RMs may therefore acquire unique population-level specificities as a function of pathogen exposure history within different LN. There are estimated to be at least 600 distinct LN/LTs in the human body (55), without necessarily including ectopic/tertiary lymphoid structures at mucosal sites (56) that can number in the thousands in the large intestine alone (57). HIV replicates actively in all of these tissues. Accordingly, HIV-specific CD8+ T cell responses must be deployed to all LT sites at all times to maintain overall control of viremia in HIV+ individuals. It should be noted in this context that we only compared the clonal distribution of HIV-specific CD8+ T cells between peripheral blood and a single LT site. In previous studies of peripheral blood, we showed that individual CD8+ T cell clonotypes specific for the same viral antigen can have distinct functional and phenotypic properties (58). The observation that residency status directly influences the anatomical distribution of HIV-specific CD8+ T cell clonotypes therefore has far-reaching implications for our understanding of protective immune responses against HIV. First, our data indicate that HIV-specific CD8+ T cells in blood do not necessarily parallel HIV-specific CD8+ T cells in LTs. Second, there may be distinct HIV-specific CD8+ T RM populations and clonotypes between different LTs, which could impart differential immune pressure on viral replication at each site. Third, in future studies, we need to further explore the functional abilities and potential deficiencies of LT HIV-specific T RMs, because these cells exist in continuous proximity to HIV-infected CD4+ T cells and likely spearhead the cellular immune response against HIV. This view is supported by the observation that elite controllers maintain high levels of HIV-specific T RMs with enhanced effector capabilities, despite low levels of viral replication in LTs.

In summary, our data indicate that CD8+ T RMs likely play a central role in immunosurveillance against HIV. Further studies are now warranted to examine the precise mechanisms through which LT CD8+ T RMs control HIV replication, potentially informing new approaches to the development of vaccines or immunotherapeutic strategies designed to eliminate the viral reservoir in established HIV infection.

MATERIALS AND METHODS

Study design

The present study was an exploratory study aimed at understanding whether T RMs are present in HIV-infected LN and part of HIV-specific immunosurveillance mechanisms. Recruitment of human samples occurred at four sites: Instituto Nacional de Enfermedades Respiratorias in Mexico City, Case Western Reserve University, University of Pennsylvania, and University of California, San Francisco. C57BL/6j (B6) mice were housed in specific pathogen-free conditions at the University of Minnesota. Rhesus macaque samples were obtained from primates housed at the University of Pennsylvania and Yerkes National Primate Research Center. Human samples were collected in accordance with the Declaration of Helsinki, and study protocols were approved by the regional review boards. Animals were housed in accordance to regional ethical guidelines, and protocols were approved by the regional review boards. Sample sizes were based on the availability of biological samples rather than a prespecified effect size. Investigators were not blinded to group identity during the course of experimentation. For more technical details, see Supplementary Materials and Methods.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Maturation of and cycling of LN CD8+ T cells.
Fig. S2. CD69 expression on naive CD8+ T cells and immune activation profile of CD69+ and CD69+ CD8+ T cells in LTs.
Fig. S3. Phenotypic characteristics of memory CD8+ T cells in blood and LTs.
Fig. S4. Residency characteristics of CD69+ and CD69+ CD8+ T cells.
Fig. S5. RNA-seq, ATAC-seq, and CCR7 analysis of LN T<sub>RMs</sub>.
Fig. S6. Imaging analysis and CD103 expression in TDL.
Fig. S7. Bulk and HIV-specific analysis and rhesus gating strategy.
Fig. S8. Index sorting strategy and scRNA-seq analysis.
Fig. S9. scRNA-seq analysis of LN HIV-specific CD8+ T cells.

Table S1. Cohort characteristics.
Table S2. List of significant genes up-regulated/down-regulated from RNA-seq.
Table S3. List of significant ATAC-seq tracks.
Table S4. List of top 200 significant genes up-regulated/down-regulated from scRNA-seq.

REFERENCES AND NOTES


Identification and characterization of HIV-specific resident memory CD8+ T cells in human lymphoid tissue


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Taking residence to defend

In HIV+ individuals receiving antiretroviral therapy, lymphoid tissues (LTs) that CD4+ T cells home to are a key site of HIV persistence. Studying the immune response to HIV in LTs has remained a challenge. By obtaining LTs from HIV+ individuals, Buggert et al. have carried out comprehensive transcriptional and epigenetic analyses on CD8+ T cells in these LTs. They report that CD8+ T cells in LTs of HIV+ individuals have a signature associated with resident memory T cells (T RMs) and that the frequency of these HIV-responsive LT-resident CD8+ T cells was considerably increased in elite controllers. The study brings to the fore the importance of HIV-specific LT-resident T RMs in restraining HIV replication in LTs.