### Title

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# Dynamics of SIV-specific CXCR5+ CD8 T cells during chronic SIV infection

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**A significant challenge to HIV eradication is the elimination of viral reservoirs in germinal center (GC) T follicular helper (Tfh) cells. However, GCs are considered to be immune privileged for antiviral CD8 T cells. Here, we show a population of simian immunodeficiency virus (SIV)-specific CD8 T cells express CXCR5 (C-X-C chemokine receptor type 5, a chemokine receptor required for homing to GCs) and expand in lymph nodes (LNs) following pathogenic SIV infection in a cohort of vaccinated macaques. This expansion was greater in animals that exhibited superior control of SIV. The CXCR5+ SIV-specific CD8 T cells demonstrated enhanced polyfunctionality, restricted expansion of antigen-pulsed Tfh cells in vitro, and possessed a unique gene expression pattern related to Tfh and Th2 cells. The increase in CXCR5+ CD8 T cells was associated with the presence of higher frequencies of SIV-specific CD8 T cells in the GC. Following TCR-driven stimulation in vitro, CXCR5+ but not CXCR5– CD8 T cells generated both CXCR5+ as well as CXCR5– cells. However, the addition of TGF-β to CXCR5– CD8 T cells induced a population of CXCR5+ CD8 T cells, suggesting that this cytokine may be important in modulating these CXCR5+ CD8 T cells in vivo. Thus, CXCR5+ CD8 T cells represent a unique subset of antiviral CD8 T cells that expand in LNs during chronic SIV infection and may play a significant role in the control of pathogenic SIV infection.**

SIV | follicular CD8 T cells | CXCR5+CD8+ T cells | lymphoid follicles | HIV

Numerous studies conducted to date have demonstrated the critical nature of antiviral CD8 T cells in the control of human and simian immunodeficiency virus (HIV/SIV) replication (1–3). Studies also showed a direct relationship between higher frequency and function of HIV-specific CD8 T cells and enhanced viral control (4–6). In particular, early induction of HIV-specific CD8 T cells resulted in a concomitant decline in plasma viremia (7, 8), suggesting that antiviral CD8 T-cell responses elicited early after HIV/SIV infection can significantly modulate viral control outcome. Consistent with this, contemporary vaccine strategies designed to elicit high frequencies of antiviral CD8 T cells have contained pathogenic SHIV (9, 10) and SIV challenges (11, 12) in macaques. Despite a pronounced antiviral CD8 T-cell response elicited early after HIV infection and the subsequent decline in set-point viremia, the majority of HIV-infected individuals do not control HIV replication in the absence of ART and inevitably progress to disease.

It is now well appreciated that lymphoid sites, in particular B-cell follicles and T follicular helper (Tfh) cells, serve as important sites of productive HIV/SIV infection (13–15). The density of infection that is localized to secondary lymphoid sites and germinal centers (GCs), even under continuous ART, underscores the need to better understand T-cell dynamics at lymphoid sites and specific immune factors that may limit effective clearance of virally infected CD4 T cells. Studies in unvaccinated SIV-infected rhesus macaques (RMs) and HIV-infected humans indicated that antiviral CD8 T cells have a limited capacity to migrate to B-cell follicles and GCs of the lymphoid tissue during chronic infection (16–18), and the exclusion of CD8 T cells from GC sites has been posited as an important mechanism of immune evasion by HIV/SIV. However, recent studies have reported the emergence of CD8 T cells expressing the C-X-C chemokine receptor type 5 (CXCR5) that is required for homing to B-cell follicles (19, 20) during chronic LCMV and HIV infections (21–23). A remaining critical question to be addressed is whether CD8 T cells can gain access to GCs of B-cell follicles during chronic HIV/SIV infection and, if so, whether these cells can impact levels of viral replication in vivo.

Recently, others and we reported an aberrant accumulation of virus-infected Tfh cells in the lymph nodes (LNs) and rectal mucosa of SIV-infected RMs with high viral load (VL) (14, 15, 24–27), which was not evident in vaccinated SIV-infected RMs with low VL during a pathogenic SIVmac251 infection (15). In the current study, we sought to understand the role of antiviral CD8 T cells in limiting the virus-infected Tfh cells. In particular, we studied the nature of CXCR5 expression on SIV-specific CD8 T cells in blood and LNs. The chemokine receptor CXCR5 is required for homing to B-cell follicles/GCs (19, 20), and a prior human study showed the presence of CXCR5+ SIV-specific CD8 T cells in tonsils (28). We also sought to understand phenotypic and functional differences in the CD8 T cells based on CXCR5 expression. We observed a strong induction of CXCR5 on SIV-specific CD8 T cells in the blood and LNs of animals that exhibited superior viral control. These CXCR5+ CD8 T cells showed a unique gene expression profile, were able to limit the expansion of antigen-pulsed Tfh cells in vitro,

## Significance

**Simian immunodeficiency virus (SIV)-specific follicular CD8 T cells represent a unique subset of antiviral CD8 T cells that rapidly expand during pathogenic SIV infection, localize within B-cell follicles, and contribute to control of chronic SIV replication. The potential for these cells to infiltrate sites of ongoing viral replication and viral persistence and the ability to induce these cells by vaccination provide a tremendous opportunity to develop and optimize therapeutic strategies to target and reduce the HIV reservoirs in lymphoid tissues.**

Author contributions: G.H.M., R.A., I.R.W., P.J.S., V.V., and R.R.A. designed research; G.H.M., D.R., H.M.A., G.T., M.M., S.H., and V.V. performed research; S.I. contributed new reagents/analytic tools; G.H.M., D.R., H.M.A., G.T., M.M., A.C., S.E.B., I.R.W., P.J.S., V.V., and R.R.A. analyzed data; and G.H.M., R.A., V.V., and R.R.A. wrote the paper.

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Conflict of interest statement: R.R.A. is a coinventor of DNA/MVA vaccine technology, and Emory University licensed this technology to Geovax Inc.

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Data deposition: Microarray results have been deposited in the Gene Expression Omnibus database, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=epmdikuulfmbhmj&acc=GSE74751 (accession no. GSE74751).

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and were associated with a lower viral burden within the Tfh subset. These findings demonstrate that CXCR5+ CD8 T cells represent a unique subset of vaccine-induced antiviral CD8 T cells with the potential to home to B-cell follicles and limit HIV replication in vivo.

## Results

**Study Overview.** Despite comprehensive analyses on the role of CXCR5 on CD4 T cells during chronic SIV/HIV infection (14, 15, 24–26), much less is known about the role of CXCR5 on CD8 T cells. Moreover, previous studies have not characterized antigen-specific CXCR5+ CD8 T cells and their role in HIV/SIV pathogenesis and viral control. We therefore studied the CXCR5 expression on SIV-specific CD8 T cells in the LNs during chronic SIVmac251 infection in a group of 20 DNA/MVA (Modified Vaccinia Ankara)-vaccinated SIV-infected RMs with a spectrum of viral control (Fig. 1 and Fig. S1). We considered animals with set point (week 24) VL at or below $10^4$ copies per mL of plasma as low-VL animals, as described previously. There were 12 animals with low VL and 8 animals with high VL. This was a retrospective study, and thus, we used samples from LNs and blood where available.

**SIV-Specific CXCR5+ CD8 T Cells Expand in LNs Following Pathogenic SIV Infection and at Higher Levels in Animals That Exhibited Superior Viral Control.** To understand the contribution of SIV-specific CXCR5+ CD8 T cells to viral control during chronic SIV infection, we determined the magnitude of Gag CM9 tetramer+ CD8 T cells (Tet+) and their coexpression of CXCR5 in the LNs during the acute (week 2) and chronic (week 24) phases of SIV infection in a group of Mamu A*01+-vaccinated RMs that exhibited a varying degree of viral control (Fig. 1 and Fig. S1*A*). During the acute phase, the CXCR5+ Tet+ cells were readily detectable in the LNs; however, the magnitude of these cells was quite variable and ranged from 0.01% (limit of detection) to 6% (Fig. 1 *A* and *B*). At this time, the magnitude of CXCR5+ Tet+ cells (geometric mean of 0.32%) was about 14 times lower compared with CXCR5– Tet+ CD8 T cells (geometric mean of 4.4%) (Fig. 1*B*). During the chronic phase, the magnitude of CXCR5– Tet+ cells decreased by 2.5-fold (geometric mean of 1.7%) compared with acute phase. However, we observed a twofold increase in the magnitude of CXCR5+ Tet+ cells (geometric mean of 0.75%) that was associated with a fourfold increase in the proportion of Tet+ cells expressing CXCR5 during the chronic phase (24%) compared with the acute phase (6%) (Fig. 1*C*). Similar to LNs, about 5% of the Tet+ cells in the blood expressed CXCR5 during the acute phase, and this expression increased to 10% during the chronic phase (Fig. 1*C*). In addition, we observed a direct correlation between the magnitude of CXCR5+ Tet+ cells in the LNs and the proportion of Tet+ cells expressing CXCR5 in the blood during chronic phase (Fig. S1*B*). These results clearly demonstrated that the SIV-specific CXCR5+ CD8 T cells expand in LNs and blood following pathogenic SIV infection in vaccinated macaques.

We next investigated the relationship between the magnitude of total, CXCR5+, and CXCR5– Tet+ cells in the LNs and plasma viremia during the chronic phase (Fig. 1*D*). Impressively, the frequency of CXCR5+ Tet+ CD8 T cells in the LNs showed a strong inverse association with plasma viremia, and this association was not evident with CXCR5– Tet+ CD8 T cells. The magnitude of total Tet+ cells also showed an inverse association with plasma viremia; however, this association was much weaker than the association observed with CXCR5+ Tet+ cells. This association between CXCR5+ CD8 T cells and viral control was not influenced by a specific vaccine group (Fig. S1*C*). In a limited number of animals, we also observed a strong inverse association between the magnitude of CXCR5+ Tet+ CD8 T cells and multiply spliced SIV TatRev RNA levels in GC–Tfh in LNs (Fig. 1*E*). A similar inverse association was also observed with cell-associated viral DNA within the Tfh subset ($P = 0.003$, $r = -0.85$; Fig. S1*D*). We also observed a similar association with total Tet+ CD8 T cells but did not with CXCR5– Tet+ CD8 T cells. Collectively, these data demonstrated that the magnitude of CXCR5+ Tet+ CD8 T cells within the LNs potentially contribute to both control of plasma viremia and viral replication within the GC–Tfh during pathogenic SIV infection. These data highlight an important role for a SIV-specific CXCR5+ follicular CD8 T-cell subset in control of viral replication during chronic SIV infection.

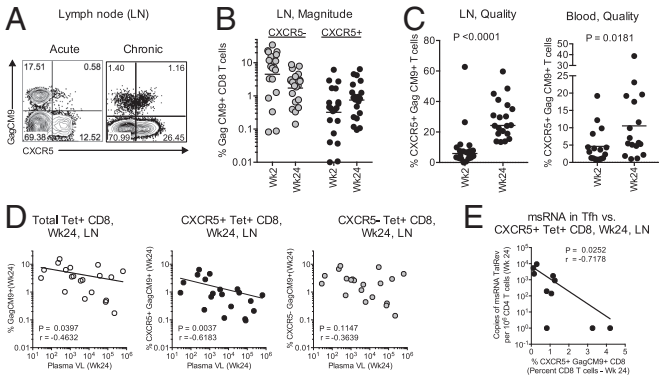


**Fig. 1.** Rapid expansion of CXCR5+ SIV-specific CD8 T cells is associated with enhanced control of chronic SIV infection. (*A*) Representative FACS plots showing CXCR5 expression on Gag CM9 tetramer+ CD8 T cells (Tet+) following SIVmac251 infection in LNs. (*B*) Magnitude of CXCR5– and CXCR5+ Tet+ CD8 T cells in the LNs during acute (Wk2) and chronic (Wk24) phases of SIV infection ($n = 20$). (*C*) The proportion of Gag CM9 tetramer+ cells expressing CXCR5 in LNs ($n = 20$) and blood ($n = 17$). (*D*) Correlation between the magnitude of total, CXCR5+, or CXCR5– tetramer+ CD8 T cells and set-point plasma VLs in the LNs ($n = 20$). (*E*) Correlation between the frequency of CXCR5+ Tet+ CD8 T cells and the msRNA of TatRev in GC–Tfh cells in the LNs at week 24 postinfection ($n = 10$). Horizontal lines show the median.

**SIV-Specific CD8 T Cells Localize to GCs in SIV-Infected Low-VL RMs.** We next investigated the presence and distribution of Tet+ CD8 T cells in the B-cell follicles and extrafollicular regions of LNs, spleen, and rectum of vaccinated and SIV-infected RM with low VL. We carried out in situ Gag CM9 tetramer staining using samples obtained from four vaccinated animals with low VL using antibodies to CD3 and CD20 (the marker used to delineate GC when identifying Tet+ CD8 T cells in situ) (16–18) and Gag CM9 Tetramer. We observed the presence of CD3+ Tet+ cells within both the extrafollicular as well as intrafollicular regions of the sampled lymphoid tissue (Fig. 2*A* and Fig. S2). The density of Tet+ cells in the follicular and extrafollicular regions was either comparable or marginally higher in the latter region (Fig. 2*B*). We observed a strong correlation between the frequency of CXCR5+ Tet+ CD8 T cells in the LNs defined by flow cytometry and the density of Tet+ CD8 T cells in GCs determined by in situ tetramer analysis (Fig. 2*C*). Unfortunately, we could not perform the in situ tetramer analysis on samples obtained from high-VL RMs due to the lack of fresh tissue, which is a requirement for this assay. However, a previous study had demonstrated a relatively lower ratio of Tet+ cells in the follicular region compared with extrafollicular region in RMs with high VL (17). However, using frozen sections, we performed in situ immunofluorescence staining on LN sections of three low-VL and three high-VL RMs to study the localization of total CD8 T cells and found the presence of CD8 T cells in the follicles primarily in the low-VL RMs but not high-VL RMs (Fig. S3*A*). To confirm that the GC resident CD8 T cells are positive for CXCR5, we stained sections for CD8, CXCR5, and IgD and found that some of the CD8 T cells localized in GC are positive for CXCR5 (Fig. S3*B*). Taken together, these data demonstrate that antiviral CXCR5+ CD8 T cells in vaccinated RMs with low VL localize to GC regions of lymphoid follicles and this may provide them with an immunological advantage to target and eliminate virus-infected Tfh cells.

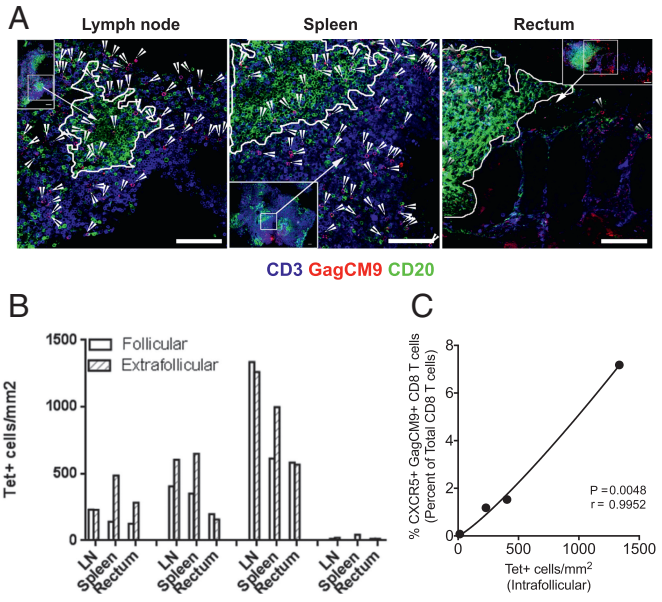


**Fig. 2.** CXCR5+ CD8 T cells are localized in the GCs of vaccinated low-VL RMs. (*A*) Representative in situ tetramer staining of Gag CM9 (red), CD20 (green), and CD3 (blue) of LN, spleen, and rectal tissue sections from a SIV low-VL RM showing the presence of Gag CM9 tetramer+ cells in the GC. The confocal images were collected with a 20× objective and each scale bar indicates 100 μm. Arrowheads indicate tetramer+ cells. (*B*) Density of follicular and extrafollicular tetramer+ cells in LN, spleen, and rectum ($n = 4$) of four low-VL RMs. (*C*) Correlation between the frequency of CXCR5+ Gag CM9+ CD8 T cells (% of CD8) measured by flow cytometry and absolute number of follicular Gag CM9+ CD8 T cells measured by in situ tetramer staining ($n = 4$).

**SIV-Specific CXCR5+ CD8 T Cells Express Cytolytic Molecules and Limit Expansion of SIV Antigen-Pulsed Tfh in Vitro.** To understand the functional quality of CXCR5+ and CXCR5– SIV-specific CD8 T cells, we characterized their ability to coproduce cytokines IFNγ, TNF-α, and IL-2 in response to stimulation with SIV Gag CM9 peptide (Fig. 3). The frequency of CD8 T cells coproducing all three cytokines (TP) was greater ($P = 0.01$) in the CXCR5+ subset compared with the CXCR5– subset (Fig. 3*A* and Fig. S4*A*). All of these TPs also showed degranulation as evidenced by CD107a expression on the cell surface. These results demonstrated that CXCR5+ cells retain a polyfunctional advantage to CXCR5– CD8 T cells for triple cytokine production, a measure that has been shown to be associated with enhanced control of human chronic viral infections (6, 29).

We next characterized the expression of molecules associated with cytolytic function in CXCR5+ and CXCR5– SIV-specific CD8 T cells from the LNs post-SIV infection (Fig. 3*B* and Fig. S4*B*). These analyses revealed that a significant fraction of CXCR5+ Tet+ cells express cytolytic molecules granzyme B and perforin; however, granzyme B was marginally lower in CXCR5+ cells compared with CXCR5– CD8 T cells. These results suggested that CXCR5+ CD8 T cells may have the potential to eliminate SIV-infected GC–Tfh cells in vivo. To address this, we sorted Tfh cells from the LNs of six Mamu A*01+ SIV-infected low-VL RMs and stimulated them with anti-CD3 and anti-CD28 antibodies for 5 d in the absence and presence of sorted autologous CXCR5+ or CXCR5– CD8 T cells (Fig. 3*C*). Tfh cells were additionally pulsed with P11C peptide so they could serve as ideal target cells for the responding CXCR5+ and CXCR5– Tet+ cells present in the coculture. At the end of 5 d, we determined the number of Tfh cells that remained after stimulation with or without CXCR5+ and CXCR5– CD8 T cells. As can be seen in Fig. 3*C*, the number of Tfh cells increased significantly following anti-CD3/CD28 stimulation in the absence of CD8 T cells. However, inclusion of either CXCR5+ or CXCR5– CD8 T cells in the culture reduced the number of Tfh cells significantly in five out of six animals, demonstrating that both CXCR5+ and CXCR5– cells are capable of limiting the expansion of SIV antigen-pulsed Tfh in vitro. This decrease was not evident when we used unpulsed Tfhs as target cells in four out of five animals tested (Fig. S4*C*). However, for one animal (#1), we observed a decrease in the number of both pulsed Tfhs and unpulsed Tfhs, but the decrease was much greater for pulsed Tfhs. It is not unexpected to see this decrease in expansion of unpulsed Tfhs in the presence of CD8 T cells, as we are using Tfhs from SIV-infected animals and it is likely that a fraction of these Tfhs are infected with SIV and thus can serve as targets.

To understand the mechanism involved in limiting the expansion of TCR-stimulated Tfh cells, we studied the expression of the cytolytic molecules granzyme B and perforin on responding SIV-specific CD8 T cells and observed a significant increase in the expression of these effector molecules on both CXCR5+ and CXCR5– CD8 T cells (Fig. 3*D* and Fig. S4*D*). The increase in coexpression of granzyme B and perforin was higher in the presence of peptide-pulsed target Tfh cells. In addition, to further investigate the mechanism behind the diminished TCR-driven expansion of Tfh cells in vitro, we assessed the transfer of granzyme B to the target Tfh cells when cocultured with the CXCR5+ and CXCR5– CD8 T-cell subsets. We observed a higher frequency of granzyme B+ Tfhs when CXCR5+ or CXCR5– CD8 T cells were included in the culture (Fig. S3*C* and Fig. S4*E*), suggesting the cytolytic function of CXCR5+ and CXCR5– CD8 T cells in the culture. Collectively these results demonstrate that both CXCR5+ and CXCR5– CD8 T cells can limit the expansion of antigen-pulsed Tfhs in vitro potentially through cytolytic molecules. However, it is important to note that, in vivo, only CXCR5+ CD8 T cells but not CXCR5– CD8 T cells will likely gain access to the Tfh population due to the expression of CXCR5 and that CXCR5+ CD8 T cells are more polyfunctional than CXCR5– CD8 T cells.

**SIV-Specific CXCR5+ CD8 T Cells Share Features of Tfh and Th2 Cells.** To elucidate the differences in cellular processes and functional states between the CXCR5+ and CXCR5– CD8 T-cell subsets during chronic SIV/HIV infection, we performed global gene

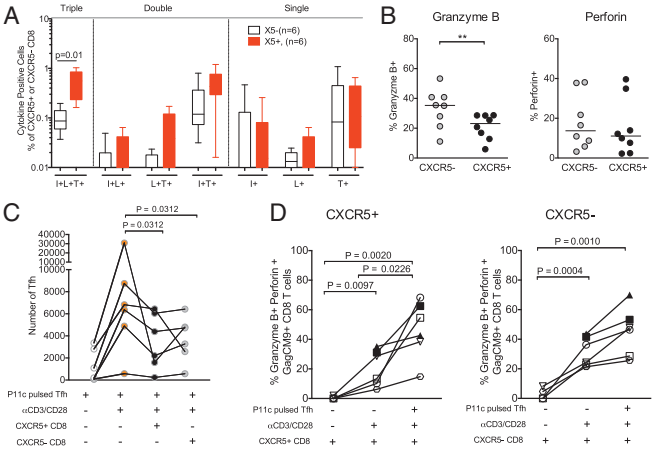


**Fig. 3.** CXCR5+ SIV-specific CD8 T cells express cytolytic molecules and limit SIV antigen-pulsed Tfh expansion in vitro. (*A*) The frequency of cytokine coexpressing cells in response to P11c (Gag CM9) peptide stimulation. I, IFN-γ; L, IL-2; T, TNF-α. Scatter plots show the median. (*B*) Expression of granzyme B and perforin on CXCR5+ and CXCR5– Gag CM9 tetramer+ CD8 T cells at week 24 postinfection in the LNs ($n = 8$). (*C*) Tfh cell-limited expansion assay showing sorted GC–Tfh cells pulsed with P11c peptide and cultured alone, driven to expand with αCD3/CD28 stimulation with or without autologous sorted CXCR5+ and CXCR5– CD8 T cells from six Mamu A01+ SIV-infected low-VL RMs. (*D*) Expression of granzyme B and perforin on sorted CXCR5+ and CXCR5– CD8 T cells following a 5-d in vitro Tfh expansion assay ($n = 6$). $^{**}P < 0.01$.

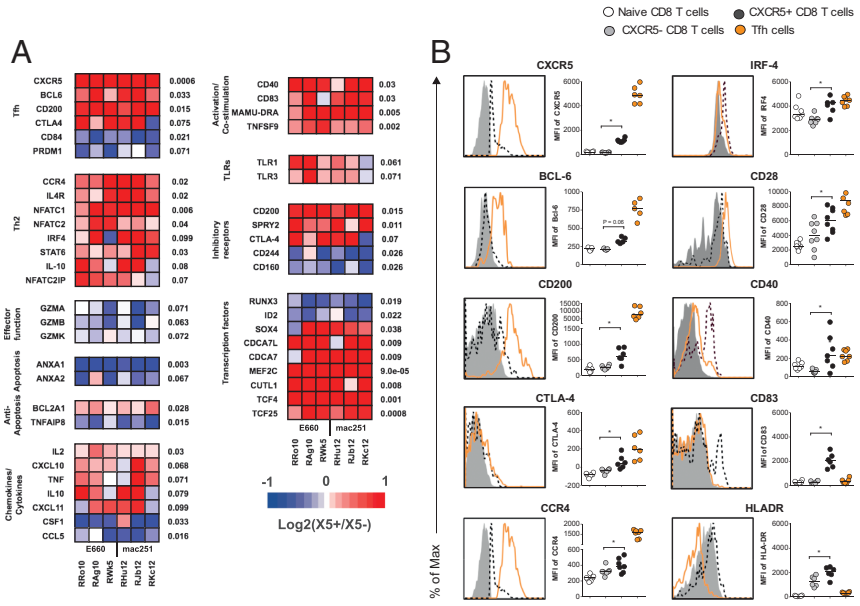


**Fig. 4.** Global gene expression analysis revealed distinct gene expression profile for CXCR5+ SIV-specific CD8 T cells. (*A*) Microarray analysis of sorted CXCR5+ and CXCR5– Gag CM9 tetramer+ CD8 T cells from the LNs of six vaccinated SIV-infected low-VL RMs. The color intensity for heat maps represents expression by CXCR5+ CD8 vs. CXCR5– CD8 T cells. (*B*) Representative histogram plots and scatter plots showing the expression of the indicated markers on naïve CD8 T cells (CD95–, CD45RA+), CXCR5– and CXCR5+ Gag CM9 tetramer+ CD8 T cells, and Tfh cells. $*P < 0.05$.

expression microarray analysis on sorted SIV-specific CXCR5+ and CXCR5– CD8 T cells from SIV-infected RMs with low VL (Fig. 4). For this analysis, we sorted cells from either SIVmac251-infected or SIVE660-infected animals. Interestingly, the CXCR5+ CD8 T cells revealed a distinct gene expression pattern compared with CXCR5– CD8 T cells. Unlike the CXCR5– CD8 T cells, the CXCR5+ CD8 T cells showed higher signal intensities of genes associated with Tfh CD4 T cells, such as the master transcription factor Bcl-6, NFATC1, NFATC2, CD200, and CTLA-4, as well as markers associated with Th2 CD4 T cells, such as IL-4R (CD124), CCR4, STAT6, and IL-10 (Fig. 4*A*). The signal intensities for effector molecules typically observed in cytotoxic CD8 T cells such as granzyme A, B, and K were lower on SIV-specific CXCR5+ CD8 T cells compared with their CXCR5– counterparts, confirming our ex vivo phenotypic analysis of these effector molecules on the subsets (Fig. 3*B*). Thus, it is not surprising that CXCR5+ SIV-specific CD8 T cells express lower levels of cytolytic molecules ex vivo, as these cells retain a unique profile similar to Tfh cells and data have shown that overexpression of Bcl-6 in CD8 T cells results in reduced granzyme B expression (30). However, it is important to note that CXCR5+ CD8 T cells rapidly up-regulate expression of some of these genes following stimulation (Fig. 3*D*); thus, the activation state and exposure to antigen may strongly influence the effector state of CXCR5+ SIV-specific CD8 T cells. Further studies need to be conducted to understand what drives these differential phenotypes in these antiviral CXCR5+ CD8 T cells.

CXCR5+ CD8 T cells also showed higher signal intensities for molecules associated with costimulation/antigen presentation such as CD40, CD83, 41BBL, and MAMU-DRA (Fig. 4*A*) and inhibitory receptors CD200 and SPRY2 but lower intensities for inhibitory receptors CD160 and CD244 (Fig. 4*A*). The functional consequence of the expression of these molecules is yet to be determined. Additionally, CXCR5+ CD8 T cells showed higher signal intensities for the antiapoptotic gene Bcl-2 and lower intensity for the proapoptotic genes annexin1 and -2, suggestive of their better survival potential during chronic SIV infection compared with CXCR5– CD8 T cells (Fig. 4*A*).

The expression of transcriptional regulators such as TCF4, TCF24, SOX4, CULT1, MEF2C, and CDCA7 was also higher in CXCR5+ CD8 T cells. However, the expression of ID2, the

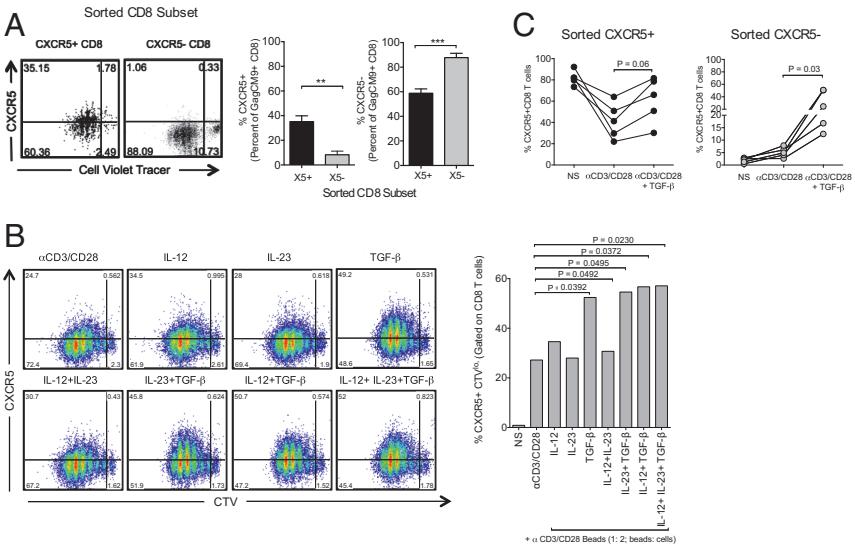


**Fig. 5.** Induction of CXCR5+ CD8 T cells can be enhanced in vitro. (*A*) CXCR5 expression on purified CXCR5+ or CXCR5– Gag CM9 tetramer+ CD8 T cells on day 5 following stimulation with anti-CD3 and anti-CD28 antibodies. (*B*) CXCR5 expression on total CD8 T cells on day 5 following stimulation with anti-CD3 and anti-CD28 antibodies in the presence of indicated cytokines ($n = 3$). (*C*) CXCR5 expression on sorted CXCR5+ and CXCR5– CD8 T cells on day 3 following stimulation with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β ($n = 3$). $**P < 0.01$, $***P < 0.001$.

transcription factor associated with terminal effectors (31), was lower in CXCR5+ CD8 T cells, suggesting that the CXCR5+ CD8 T cells have transcriptional signatures associated with memory precursor cells that survive and give rise to the pool of long-lived memory CD8 T cells. In addition, CXCR5+ CD8 T cells showed higher expression of chemokines such as CCL5 and CXCL10 and cytokines IL-2 and CSF-1 compared with CXCR5– CD8 T cells. We further confirmed the expression of some of these genes on a protein level using flow cytometry (Fig. 4*B*). We also measured the level of PD-1 expression and, consistent with the LCMV model, found that CXCR5+ CD8 T cells express marginally higher levels of PD-1 compared with CXCR5– CD8 T cells (Fig. S4*F*). Collectively, these results demonstrate that SIV-specific CXCR5+ CD8 T cells possess a unique gene expression pattern that is distinct compared with SIV-specific CXCR5– CD8 T cells.

**Induction of CXCR5+ CD8 T Cells Can Be Enhanced in Vitro.** In an effort to understand the modulation of CXCR5 expression following TCR-driven CD8 T-cell proliferation, we sorted CXCR5+ and CXCR5– CD8 T cells from the LNs of chronically infected RMs and stimulated them in vitro with anti-CD3/CD28 (Fig. 5*A* and Fig. S5). Both CXCR5+ and CXCR5– CD8 T cells proliferated equally, but interestingly the CXCR5+ CD8 T cells differentiated into both CXCR5+ and CXCR5– CD8 T-cell subsets. In contrast, CXCR5– CD8 T cells largely remained CXCR5– (Fig. S5*A*). This was also true for Gag CM9 Tet+ CD8 T cells in the culture (Fig. 5*A*). Therefore, CXCR5+ CD8 T cells may potentially gain access to the GC through increased expression of the follicular homing receptor CXCR5 and, then once activated and in response to antigen, can differentiate into a population of CXCR5– CD8 T cells retaining an effector CD8 T-cell profile. One question that remains is what signals may drive the induction or expression of CXCR5 on these SIV-specific CD8 T cells. A recent study found that TGF-β was critical in providing the initial signals required for Tfh differentiation (32). Therefore, we cultured anti-CD3/CD28–stimulated PBMCs in vitro in the presence of TGF-β and found that TGF-β can also significantly increase the expression of CXCR5 on proliferating RM CD8 T cells (Fig. 5*B*). TGF-β in combination with IL-12 and IL-23 induced higher CXCR5 expression compared with cells stimulated with IL-12 and IL-23 alone. Similar analysis on sorted CXCR5+ and CXCR5– CD8 T cells confirmed that TGF-β could enhance CXCR5 expression on CXCR5– CD8 T cells as well (Fig. 5*C*).

## Discussion

In this study, we focused on investigating the dynamics of follicular homing antiviral CD8 T cells during chronic SIV infection in vaccinated and unvaccinated RMs that showed a varying degree of SIV control. Our findings revealed the rapid expansion of a subset of highly functional antiviral CXCR5+ follicular CD8 T cells with potential to migrate to B-cell follicles/GCs in the LNs and possess a unique phenotype in RMs. Importantly, the expansion of SIV-specific CXCR5+ CD8 T cells in the blood and LNs was associated with vaccine-mediated control of pathogenic SIV infection, suggesting that it is important to generate these cells by vaccination to enhance control of chronic immunodeficiency virus infections.

In this study, we focused our analyses on SIV-specific CD8 T-cell responses in Mamu A*01 animals. We chose to use Mamu A*01 animals because CXCR5 expression is down-regulated following peptide stimulation (see Fig. S1*E*), and the use of Mamu A*01 animals allowed us to use the immunodominant Gag CM9 tetramer to measure the majority of SIV-specific CD8 T cell response without stimulation. In addition, we note that even in unvaccinated SIV-infected animals with varying degrees of viral control, higher CXCR5 expression on Gag CM9 Tet+ CD8 T cells in blood is associated with improved viral control during the chronic phase, highlighting their contribution to enhanced viral control (Fig. S5*B*).

Our results showed that SIV-specific CXCR5+ CD8 T cells can restrict expansion of SIV antigen-pulsed Tfhs from SIV-infected macaques in vitro, supporting the potential for these cells to limit Tfh expansion in vivo. However, the mechanisms by which these cells are able to restrict Tfh expansion require further investigation. In the current study, several lines of evidence suggested that CXCR5+ CD8 T cells may potentially limit the expansion of antigen-pulsed Tfh through perforin- and granzyme B-dependent mechanisms, as these cells strongly up-regulated these molecules in culture and transferred granzyme B to Tfh. Our attempts to perform a CD8 T-cell-killing assay ex vivo were not successful, as this assay requires a large number of sorted SIV-specific CXCR5+ CD8 T cells.

It is interesting to note that SIV-specific CXCR5+ CD8 T cells express transcripts/proteins associated with Tfh and Th2 cells. This raises the possibility that cytokines and signals that shape a T-helper differentiation program may also help differentiation of CXCR5+ CD8 T cells. We did find that TGF-β, which has been shown to promote Tfh differentiation in human cells, also enhanced CXCR5 expression on CD8 T cells (discussed further below). Another interesting finding was that the CXCR5+ CD8 T cells gave rise to both CXCR5+ and CXCR5– CD8 T cells when stimulated through TCR, supporting their self-renewal and stem cell-like properties. These data also suggest a supporting role that CXCR5+ CD8 T cells may potentially play in regulating an extrafollicular effector CXCR5– CD8 T-cell response during chronic HIV/SIV infection.

It is very important to understand the mechanisms underlying the induction of CXCR5+ CD8 T cells by vaccination and during infection to develop immune-based strategies that can substantially reduce the HIV reservoir and generate effective control of viral replication in the absence of ART. Our in vitro studies showed that TGF-β may play a significant role in inducing these cells. Previous studies showed that TGF-β levels increase significantly following SIV (33) and HIV infections (34). Moreover, pathogenic SIV infection in rhesus macaques is characterized by activation of the smad7 pathway, an inhibitor of the TGF-β1 signaling cascade, whereas nonpathogenic SIV infection in African green monkeys is characterized by prolonged smad4 signaling, a mediator that enhances TGF-β1 signaling, and an absence of smad7 signaling (33). These data suggest that immunoregulatory mechanisms that occur very early during acute infection may potentially influence the phenotypic outcome of antiviral CD8 T cells. Further investigation into early immune signatures will help to address what mechanisms influence the in vivo generation of these CXCR5+ antiviral CD8 T cells. These in vitro studies suggest that the TGF-β pathway could be modulated in vivo to augment induction of antigen-specific CXCR5+ CD8 T cells.

The recently described follicular CXCR5+ CD8 T cells from chronic LCMV and HIV infections (21, 22) have been shown to express transcription factor BCL-6 similar to what we observed in our SIV-specific CXCR5+ CD8 T cells, and selectively entered into B-cell follicles eradicating both infected Tfh and B cells (21). LCMV-specific CXCR5+ CD8 T cells described by Im et al. (23) express TCF-1 and act as stem cells during LCMV infection. Interestingly, these cells reside in the T-cell zones and not in follicles. Further investigation into early immune signatures will help to address what mechanisms influence the in vivo generation of these CXCR5+ antiviral CD8 T cells, but our data in combination with these recently published studies highlight the importance of CXCR5+ CD8 T cells and underscore the need to develop immune-based strategies capable of generating these cells in vivo as a means of targeting and eliminating HIV-infected CD4 T cells that contribute to ongoing viral production and persistence.

In conclusion, our results show a population of SIV-specific CXCR5+ CD8 T cells induced early after infection with potential to migrate to the GC within lymphoid sites and contribute to control of pathogenic SIV. The potential for these cells to infiltrate sites of ongoing viral replication and viral persistence and the ability to induce these cells by vaccination provide a tremendous opportunity to develop and optimize therapeutic strategies that can successfully target and reduce the viral reservoir in lymphoid

tissues. Our findings have important implications for functional cure efforts for HIV.

## Materials and Methods

**Immunizations and Infections.** Young adult (1.5–4.5 y) male Indian rhesus macaques (*Macca mulatta*) from the Yerkes breeding colony were cared for under the guidelines established by the Animal Welfare Act and the National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (35) using protocols approved by the Emory University Institutional Animal Care and Use Committee. All RMs sampled were unvaccinated or vaccinated with DNA/MVA SIV vaccine (DM). Vaccination consisted of two DNA primes on weeks 0 and 8 and two MVA boosts on weeks 16 and 24. Both DNA and MVA immunogens expressed SIV239 Gag, Pol, and Env, as described previously (36). Vaccinated animals either received unadjuvanted DNA/MVA vaccine (36), CD40L adjuvanted DNA/MVA vaccine (described previously in ref. 36), or unadjuvanted DNA/MVA vaccine with rapamycin. Rapamycin was administered intramuscularly from day –3 to day 28 during each of the DNA and MVA immunizations. The dose of Rapamycin on day 0 of each cycle was 50 μg/kg and was adjusted to reach a serum concentration of 5–15 ng/mL. This resulted in the use at a dose between 10 and 50 μg/kg. All animals were challenged intrarectally weekly with SIVmac251 starting 21–24 wk after the final MVA immunization with a dose of 647 $TCID_{50}$ ($1.25 \times 10^7$ copies of viral RNA) until they were infected. All animals were infected by seven challenges under these conditions. N. Miller, NIH, Bethesda, MD, provided the challenge stock.

**Phenotyping and Intracellular Cytokine Staining.** Phenotying and intracellular cytokine staining were performed as described previously (37). See *SI Materials and Methods* for details.

**Immunofluorescence Staining and in Situ Tetramer Staining.** These procedures were performed as described previously (17). See *SI Materials and Methods* for details.

**Microarray Analysis.** See *SI Materials and Methods* for details. Microarray results have been deposited in the Gene Expression Omnibus database (accession no. GSE74751). The data can be accessed using the following link: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=epmdikuulfmbhmj&acc=GSE74751.

**Statistical Analysis.** Statistical analyses were performed using Prism (version 5.0d; GraphPad Software Inc.). Statistical significance (*P* values) was obtained using nonparametric Mann–Whitney test (for comparisons between groups/subsets), nonparametric paired *t* test (for comparisons between subsets within the same animal), or Spearman rank test (for correlations). Statistical analyses of cytokine coexpression profiles were performed by partial permutation tests using SPICE software (NIAID, NIH) as previously described (15). For microarray analysis, background adjustment, normalization, and median polish summarization of CEL files were performed with the robust multichip average (RMA) algorithm using the affy Bioconductor package. The quality of hybridized chips was assessed after normalization by examining their NUSE and RLE plots. Downstream analyses were performed using Partek Genomics Suite software version 6.5 (Partek Inc.). *P* values for the contrasts between CXCR5+ and CXCR5– CD8 T cells in the low-VL RMs were determined by paired *t* test. To be consistent, we used robust nonparametric tests that do not depend on assumptions of data distribution. A two-sided *P* value with less than 0.05 was considered as significant. *P* values were not corrected for multiple comparisons.

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