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# Decreased CXCR3$^+$ CD8 T Cells in Advanced Human Immunodeficiency Virus Infection Suggest that a Homing Defect Contributes to Cytotoxic T-Lymphocyte Dysfunction$^\nabla$

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**To exert their cytotoxic function, cytotoxic T-lymphocytes (CTL) must be recruited into infected lymphoid tissue where the majority of human immunodeficiency virus (HIV) replication occurs. Normally, effector T cells exit lymph nodes (LNs) and home to peripheral sites of infection. How HIV-specific CTL migrate into lymphoid tissue from which they are normally excluded is unknown. We investigated which chemokines and receptors mediate this reverse homing and whether impairment of this homing could contribute to CTL dysfunction as HIV infection progresses. Analysis of CTL chemokine receptor expression in the blood and LNs of untreated HIV-infected individuals with stable, chronic infection or advanced disease demonstrated that LNs were enriched for CXCR3$^+$ CD8 T cells in all subjects, suggesting a key role for this receptor in CTL homing to infected lymphoid tissue. Compared to subjects with chronic infection, however, subjects with advanced disease had fewer CXCR3$^+$ CD8 T cells in blood and LNs. CXCR3 expression on bulk and HIV-specific CD8 T cells correlated positively with CD4 count and negatively with viral load. In advanced infection, there was an accumulation of HIV-specific CD8 T cells at the effector memory stage; however, decreased numbers of CXCR3$^+$ CD8 T cells were seen across all maturation subsets. Plasma CXCL9 and CXCL10 were elevated in both infected groups in comparison to the levels in uninfected controls, whereas lower mRNA levels of CXCR3 ligands and CD8 in LNs were seen in advanced infection. These data suggest that both CXCR3$^+$ CD8 T cells and LN CXCR3 ligands decrease as HIV infection progresses, resulting in reduced homing of CTL into LNs and contributing to immune dysfunction.**

Effector CD8 T cells play a critical role in host defense against viral infections, including human immunodeficiency virus (HIV) infection (4, 31, 43). In most untreated HIV-infected persons, however, increases in viral load and decreases in CD4 count ultimately lead to AIDS despite the persistence of broadly directed anti-HIV cytotoxic T-lymphocyte (CTL) responses, suggesting that CTL become functionally impaired as HIV infection progresses (2, 32, 45, 63). The nature of this functional impairment remains poorly understood, and yet is critically important to rational vaccine design and immunotherapeutic strategies.

Effective anti-viral CD8 T-cell responses depend on the ability of CTL to traffic into tissue sites of active viral replication (7, 8, 37). Chemokines and their receptors direct the movement of lymphocytes between the circulatory system and specific tissues throughout the body and thus play a critical role in coordinating the adaptive immune response (10). The prevailing paradigm of T-cell trafficking suggests that naïve T cells home to lymphoid tissues guided by chemokines CCL19 and CCL21 and their receptor, CCR7. In contrast, effector T cells exit lymphoid tissues and home to peripheral sites of infection guided by inflammatory chemokines and chemokine receptors, such as CXCR3 and CCR5 (38). In HIV infection, however, lymphoid tissues are a major site of viral replication (12, 28, 44), demanding that HIV-specific CTL traffic counter to their usual pattern—i.e., to be effective, anti-HIV effector CD8 T cells must be recruited into, and/or be retained within, infected lymphoid tissue, a compartment from which they are normally excluded. Adoptive transfer of autologous, ex vivo-expanded, HIV-specific CTL into HIV-infected individuals has demonstrated that effector cells can indeed enter infected lymph nodes and seem to mediate antiviral activity once there (5, 6). How CTL accomplish this "reverse migration," however, remains unknown.

Increasing evidence suggests that HIV infection leads to pronounced changes in the chemotactic and cellular environment of the lymphoid tissue where the virus replicates (47). In acute and early HIV and simian immunodeficiency virus (SIV) infection, up regulation of inflammatory chemokines and marked accumulations of activated CD8 T cells occur in infected lymph nodes (33, 42, 58). Interestingly, two studies in-

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vestigating lymph node chemokine production reported a decline in lymph node inflammatory chemokine production in advanced HIV infection (6, 57), which may compromise effector cell trafficking. CTL expression of inflammatory chemokine receptors may also become compromised as HIV infection progresses. HIV-specific CD8 T cells in advanced infection may be impaired in their ability to fully mature into terminally differentiated effector cells (9, 23). CD8 T-cell chemokine receptor expression changes as T cells mature from naïve to effector phenotypes. Therefore, if HIV-specific CTL are unable to fully mature, their expression of inflammatory chemokine receptors may be insufficient to support adequate trafficking into infected lymph nodes.

We hypothesized that, as HIV disease progresses, CTL lose the ability to traffic into infected lymph nodes, due either to reduced lymph node chemokine levels or reduced CTL chemokine receptor expression, and that this trafficking defect contributes to their dysfunction in advanced HIV infection. We anticipated that lymph node chemokine expression and/or CTL chemokine receptor expression in chronic HIV infection, when there is at least partial immune control of viral replication, despite ongoing immune activation and detectable viremia, would be significantly different from that in end-stage HIV infection when immune control is lost. To test these hypotheses, we therefore compared CTL chemokine receptor expression and chemokine levels in both the blood and lymph nodes from two groups of HIV-infected individuals: (i) persons in the asymptomatic, chronic phase of HIV infection with stable viral loads and relatively high CD4 counts; and (ii) persons with more advanced HIV infection/AIDS with high viral loads and lower CD4 counts. The results of these comparisons support the concept that disruption in CD8 T-cell trafficking into infected lymph nodes may be due to loss of CXCR3 expression on these cells, as well as decreased lymph node CXCR3 ligand expression, and may contribute to the functional impairment of CTL in advanced HIV infection.

## MATERIALS AND METHODS

**Study subjects.** Thirty-two HIV-infected study subjects followed at the Massachusetts General Hospital (Boston, MA) were recruited for this study, providing either peripheral blood samples alone ($n = 20$) or paired lymph node and blood samples ($n = 9$). Three additional HIV-infected blood and lymph node samples were obtained from a postmortem procurement service (NDRI, Philadelphia, PA). Postmortem samples were kept on ice and processed immediately upon receipt of tissue. Clinical and immunologic characteristics are summarized in Table 1. HIV-infected individuals with stable, chronic infection had CD4 counts of >300 cells/ml and HIV viral loads of <100,000 copies/ml that had not changed more than 100 cells/ml or 0.5 log copies/ml, respectively, in the previous 3 months. Study subjects with advanced HIV infection had CD4 counts of <300 cells/ml and HIV viral loads of >100,000 copies/ml. All study subjects were off of highly active antiretroviral therapy for at least six months, and CD4 count and viral load measurements were performed within 1 month of the study visit. The Institutional Review Board approved this study, and the tenets of the Helsinki protocol were followed. Informed consent was obtained from all study participants.

**Lymph node FNA.** Experiments to establish the adequacy of fine needle aspiration (FNA) compared to that of excisional lymph node biopsy were performed on discarded surgical specimens from three healthy subjects. FNA was performed using five passes with a 23-gauge needle, and the remaining node was processed for comparison. We found that both flow cytometry and quantitative PCR (qPCR) data were equivalent for lymph node tissue obtained by FNA or excisional biopsy (see Fig. 5). We therefore used FNA to obtain lymph node tissue of HIV-infected subjects by using four to five passes with a 23-gauge needle on the largest palpable lymph node ($n = 9$). Active bacterial infection in a region drained by the lymph node used for FNA was ruled out in each study subject, and subjects with a history of malignancy were excluded.

**Flow cytometry.** Antibodies recognizing CXCR3 (1C6), CCR5 (3A9), CCR7 (3D12), CD27 (MT271), CD45RA (L48), CD3 (UCHT1), CD4 (SK3), and CD8 (SK1) were purchased from BD PharMingen (San Diego, CA). The following HIV type 1 human leukocyte antigen (HLA) tetramers were used where applicable (see Table 1): HLA-B8-restricted FLKEKGGL (nef), HLA-B8-restricted EIYKRWII (p24), HLA-B57-restricted KAFSPEVIPMF (p24), HLA-A2-restricted SLYNTVATL (p17), HLA-A2-restricted ILKEPVHGV (RT), HLA-A3-restricted QVPLRPMTYK (nef), HLA-A11-restricted QVPLRPMTYK (nef), and HLA-A11-restricted AIFQSSMTK (RT) (Immunomics, San Diego, CA). Dead cells were identified and excluded from analysis by using a reduced-biohazard cell viability kit from Molecular Probes (Eugene, OR). Fluorescence-activated cell sorter analysis was performed on fresh or cryopreserved peripheral blood mononuclear cells isolated by centrifugation through a density gradient on a Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Mononuclear cells from lymph node tissue were obtained by percutaneous FNA ($n = 9$) and stained directly or by postmortem lymph node excision ($n = 3$), in which case they were processed through a 70-μm filter prior to staining. Data were acquired on an LSRII and analyzed with FlowJo software (Tree Star, Stanford, CA).

Control experiments performed on peripheral blood from healthy volunteers ($n = 4$) compared chemokine receptor expression levels between whole blood, mononuclear cells separated by Ficoll-Hypaque density centrifugation, and cryopreserved cells. No significant differences were observed (data not shown).

**qPCR.** Total RNA was extracted from snap-frozen lymph node tissue using either the RNeasy or RNeasy Micro kit (QIAGEN, Valencia, CA). After DNase I (Invitrogen, Carlsbad, CA) treatment, total RNA from each sample was used as a template for the reverse-transcription reaction. qPCR was performed as previously described with an MX4000 multiplex quantitative PCR system (Stratagene, La Jolla, CA) (40). Briefly, all samples were reverse transcribed under the same conditions (25°C for 10 min, 48°C for 30 min) and from the same reverse-transcription master mix to minimize differences in reverse-transcription efficiency. The qPCR reaction mixture contained 3 μg of cDNA, 12.5 μl 2× SYBR green master mix (Stratagene), and 500 nmol of sense and antisense primers. Emitted fluorescence for each reaction was measured during the annealing/extension phase, and amplification plots were analyzed with the MX4000 software, version 3.0 (Stratagene). Quantity values (i.e., copies) for gene expression were generated by comparing the fluorescence generated by each sample with standard curves of known quantities and the calculated number of copies divided by the number of copies of the constitutively active gene encoding glyceraldehyde-3-phosphate dehydrogenase.

**Plasma chemokine measurements.** Plasma CCL2, CCL5, CXCL8, CXCL9, and CXCL10 levels were assessed by using a cytometric bead array system from BD Biosciences (San Jose, CA) as described previously (14). For each plasma sample and cytokine standard mixture, 50 μl of the sample or the standard mixture was added to a mixture of 50 μl each of capture antibody-bead reagent and detector antibody-phycoerythrin reagent. The mixture was subsequently incubated for 3 h at room temperature and washed to remove unbound detector antibody-phycoerythrin reagent before data acquisition using a BD FACSCalibur flow cytometer. CXCL11 levels were quantified using the Quantikine human CXCL11 immunoassay kit (R&D Systems, Minneapolis, MN). All plasma samples were processed within 6 h of phlebotomy and kept at −80°C until use.

**Statistical analysis.** Student's $t$ test analyses and Spearman's rank correlations were performed with GraphPad Prism software (San Diego, CA). Values of $P \leq 0.05$ were considered significant. Data are presented as the means ± standard errors of the results.

## RESULTS

**Decreased CXCR3 on circulating bulk and HIV-specific CD8 T cells in subjects with advanced infection.** We first examined chemokine receptor expression on bulk and antigen-specific CD8 T cells from peripheral blood samples of untreated HIV-infected individuals belonging to two distinct clinical categories (Table 1). Subjects with stable, chronic infection ($n = 15$) had CD4 counts of >300 cells/ml (mean, 406) and HIV viral loads of <100,000 copies/ml (mean, 52,308), whereas those with advanced infection ($n = 17$) had CD4 counts of <300 cells/ml (mean, 105) and viral loads of ≥100,000 (mean, 323,000). Major histocompatibility complex class I tetramers were used to identify HIV-specific CD8 T cells in individuals with known responses as

TABLE 1. Summary of clinical and virological parameters of blood and lymph tissue samples from HIV-infected study subjects[a]

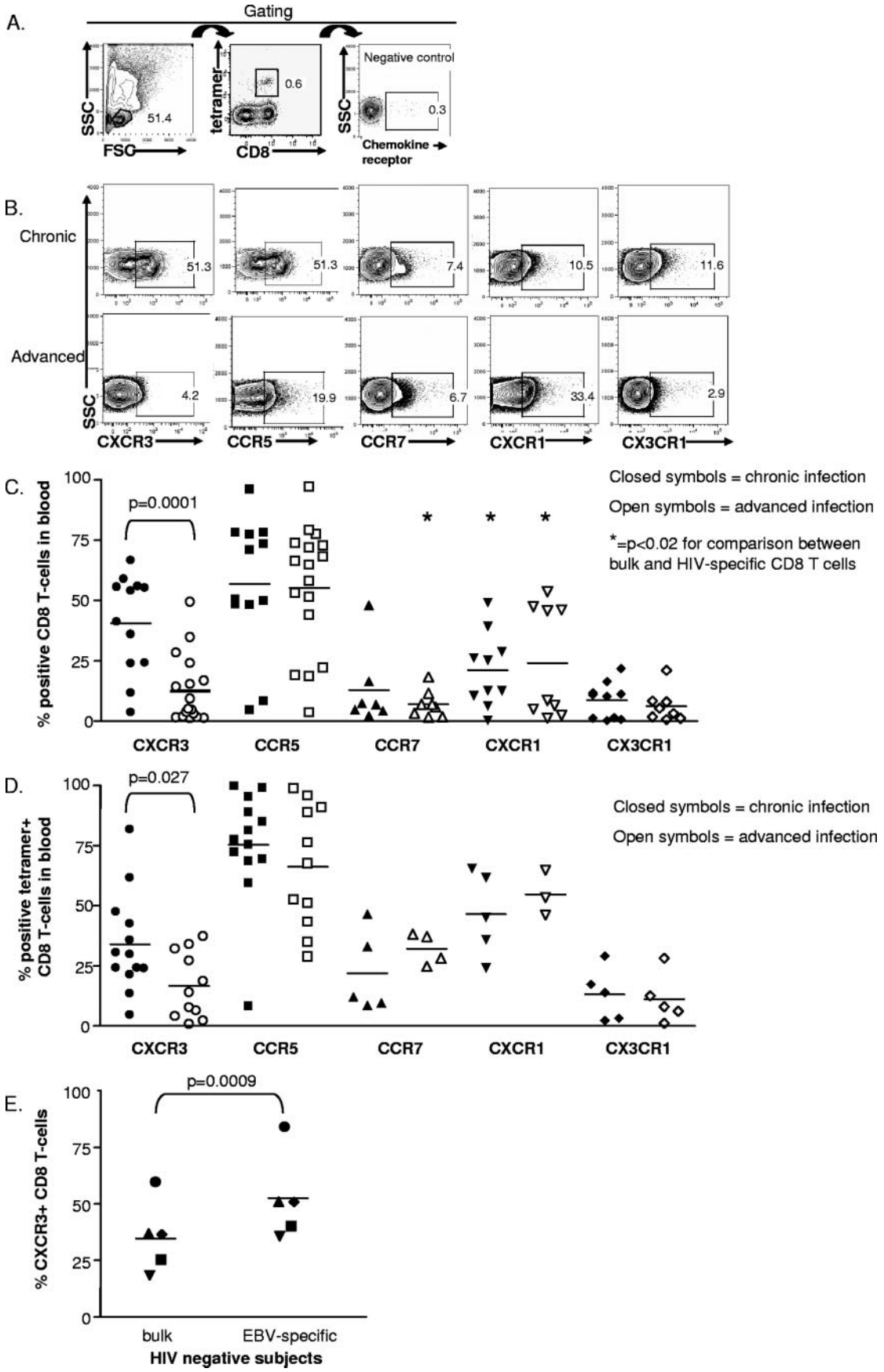
| Patient[b] | Count (cells/ml) of: | | Viral load (copies/ml)[c] | Epitope(s) and HLA restriction(s) |
|---|---|---|---|---|
| | CD4 | CD8[c] | | |
| CI1 | 411 | 1617 | 11,300 | FLKEKGGL (B8) |
| CI2 | 408 | 1611 | 52,000 | FLKEKGGL (B8) |
| CI3 | 350 | NA | 37,000 | FLKEKGGL (B8) |
| CI4 | 368 | 1548 | 37,030 | SLYNTVATL (A2), ILKEPVHGV (A2) |
| CI5 | 637 | 882 | 5,000 | QVPLRPMTYK (A11), AIFQSSMTK (A11) |
| CI6 | 597 | NA | 80,890 | QVPLRPMTYK (A3) |
| CI7 | 316 | 1078 | 83,400 | FLKEKGGL (B8), EIYKRWII (B8) |
| CI8 | 320 | NA | 49,900 | FLKEKGGL (B8), EIYKRWII (B8) |
| CI9 | 400 | 812 | 98,000 | SLYNTVATL (A2) |
| **CI10** | **338** | **804** | 87,000 | |
| **CI11** | **348** | **778** | 75,100 | |
| **CI12** | **355** | **980** | 62,000 | |
| **CI13** | **369** | **911** | 13,200 | |
| **CI14** | **455** | **602** | 17,200 | |
| **CI15** | **415** | **1196** | 75,600 | |
| Average | 406 | 1068 | 52,308 | |
| AD1 | 60 | NA | 160,000 | SLYNTVATL (A2) |
| AD2 | 24 | 583 | 750,000 | KAFSPEVIPMF (B57) |
| AD3 | 58 | NA | 278,000 | FLKEKGGL (B8) |
| AD4 | 17 | NA | 500,001 | FLKEKGGL (B8), EIYKRWII (B8) |
| AD5 | 231 | 1210 | 287,000 | SLYNTVATL (A2) |
| AD6 | 220 | 755 | 269,000 | QVPLRPMTYK (A3) |
| AD7 | 282 | 1575 | 565,000 | KAFSPEVIPMF (B57) |
| AD8 | 129 | 457 | 292,000 | FLKEKGGL (B8), EIYKRWII (B8) |
| AD9 | 182 | 516 | 138,000 | QVPLRPMTYK (A3) |
| AD10 | 151 | 907 | 173,000 | SLYNTVATL (A2) |
| AD11 | 243 | 1189 | 200,000 | FLKEKGGL (B8), EIYKRWII (B8) |
| **AD12** | **1** | **NA** | 100,000 | |
| **AD13** | **9** | **NA** | NA | |
| **AD14** | **20** | **NA** | NA | |
| **AD15** | **108** | **1186** | 112,000 | |
| **AD16** | **95** | **1950** | 332,000 | |
| **AD17** | **5** | **401** | 689,000 | |
| Average | 105 | 975 | 323,000 | |

[a] Bold font indicates that paired peripheral blood mononuclear cells and lymph node tissue were available from these individuals.
[b] CI indicates patients with chronic infection; AD indicates patients with advanced infection.
[c] NA, not available.

determined by enzyme-linked immunospot assay analyses. Of note, the breadth and magnitude of these HIV-specific responses, determined by enzyme-linked immunospot assay, did not differ between subjects with chronic versus advanced infection (15, 20). All tetramer-positive responses ($n = 27$) were gated on a minimum of 25,000 events and ranged from 0.4 to 5.9% of total CD8 T cells. Frozen peripheral blood mononuclear cells were used when fresh blood was not available. Control experiments in healthy volunteers determined that CD8 T-cell chemokine receptor expression did not differ between whole blood mononuclear cells obtained by Ficoll-Histopaque density centrifugation or cryopreserved samples (data not shown).

Due to limitations in cell numbers, flow cytometric studies of chemokine receptor expression were restricted to those receptors previously described as important in effector CD8 T-cell trafficking and/or HIV infection: CXCR3, CCR5, CCR7, CXCR1, and CX3CR1 (13, 36). Representative flow cytometry data demonstrating the gating used for chemokine receptor analyses of CD8 T cells are shown in Fig. 1A. Typical staining patterns for the chemokine receptors studied from subjects with chronic (patient CI1) and advanced (patient AD1) HIV infection are shown in Fig. 1B. Cumulative data (Fig. 1C and D) demonstrate that individuals with advanced infection had 3.5-fold-lower CXCR3 expression on bulk CD8 T cells ($P = 0.0001$) and over twofold-lower CXCR3 expression on HIV-specific CD8 T cells ($P = 0.027$) than those with chronic infection. Peripheral blood CD8 T-cell counts were available for a subset of study subjects (Table 1), and in these cases, we calculated the number of circulating CXCR3$^+$ CD8 T cells. As was seen when looking at the percentages of CXCR3$^+$ CD8 T cells, we saw significantly lower numbers of bulk and HIV-specific CXCR3$^+$ CD8 T cells in subjects with advanced infection than chronic infection ($150 \pm 39$ versus $342 \pm 42$ cells/ml, $P = 0.013$, and $4.3 \pm 1.1$ versus $1.4 \pm 0.4$ cells/ml, $P = 0.017$, respectively). All of the other chemokine receptors studied did not differ between the two groups for either bulk or HIV-specific CD8 T cells.

When comparing chemokine receptor expression between bulk and HIV-specific CD8 T cells, several significant differences were noted (asterisks in Fig. 1C). Subjects with advanced


A.
Gating
SSC
FSC
51.4
tetramer
CD8
0.6
Negative control
SSC
Chemokine receptor
0.3
B.
Chronic
Advanced
SSC
51.3
51.3
7.4
10.5
11.6
4.2
19.9
6.7
33.4
2.9
CXCR3
CCR5
CCR7
CXCR1
CX3CR1
C.
% positive CD8 T-cells in blood
p=0.0001
Closed symbols = chronic infection
Open symbols = advanced infection
*=p<0.02 for comparison between bulk and HIV-specific CD8 T cells
CXCR3
CCR5
CCR7
CXCR1
CX3CR1
D.
% positive tetramer+ CD8 T-cells in blood
p=0.027
Closed symbols = chronic infection
Open symbols = advanced infection
CXCR3
CCR5
CCR7
CXCR1
CX3CR1
E.
% CXCR3+ CD8 T-cells
p=0.0009
bulk
EBV-specific
HIV negative subjects

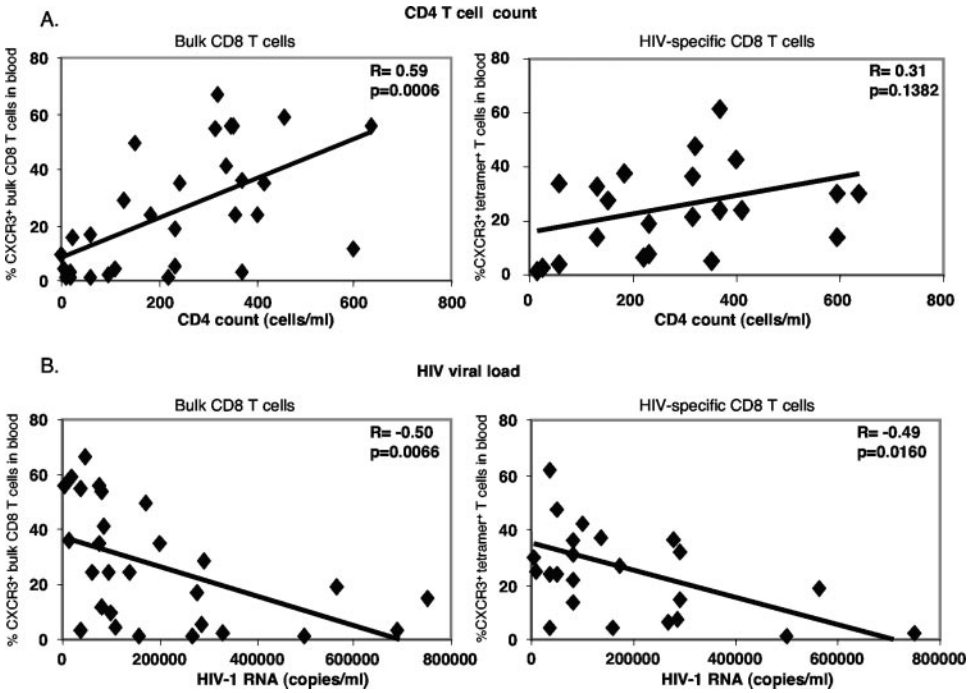



FIG. 2. CD8 T-cell CXCR3 expression correlates positively with peripheral CD4+ T-cell count and negatively with HIV viral load. The levels of CXCR3 expression on bulk CD8 T cells or HIV-specific CD8 T cells as a function of peripheral CD4 T cells (A) or HIV viral load (B) are shown.

infection had over threefold-higher levels of CCR7+ HIV-specific than bulk CD8 T cells ($P = 0.00004$). Similarly, both groups of study subjects had significantly more CXCR1+ HIV-specific than bulk CD8 T cells (chronic infection, $P = 0.008$; advanced infection, $P = 0.015$).

As a comparison, we examined CXCR3 expression on bulk and EBV-specific CD8 T cells from the peripheral blood of HIV-uninfected healthy volunteers (Fig. 1E). Similar to the results of previously published reports (24, 55, 62), a higher percentage of antigen-specific CD8 T cells than bulk CD8 T cells expressed CXCR3 (52% versus 35%, $P = 0.0009$). This enrichment of CXCR3 on antigen-specific CD8 T cells was not seen in HIV-specific CD8 T cells from HIV-infected subjects with either chronic or advanced disease.

**Circulating CXCR3+ CD8 T cells correlate positively with CD4 count and negatively with HIV viral load.** We next examined the relationship of chemokine receptor expression with the two main predictors of HIV disease progression, CD4+ T-cell count and plasma viral load (Fig. 2). We found that the percentage of CXCR3+ bulk CD8 T cells strongly correlated with the CD4+ T-cell count ($R = 0.59$, $P = 0.0006$). A similar positive correlation that approached statistical significance was also seen between the percentage of CXCR3+ HIV-specific CD8 T cells and the CD4+ T-cell count. In contrast, both bulk and HIV-specific CXCR3+ CD8 T-cell percentages correlated inversely with plasma viral load ($R = -0.50$, $P = 0.0066$, and $R = -0.49$, $P = 0.0160$, respectively). No other chemokine receptor percentages on bulk or antigen-specific CD8 T cells correlated with CD4+ T-cell count or plasma viral load (data not shown). These data indicate a clear association between decreased CXCR3+ CD8 T cells and parameters of disease progression in HIV infection.

**Decreased terminally differentiated CD8 T cells in advanced HIV infection.** To better understand the nature of the difference in CD8 T-cell CXCR3 expression between subjects with chronic versus advanced infection, we examined the maturation phenotypes of bulk and HIV-specific CD8 T cells from these two groups. CD27 and CD45RA were used to define naïve (CD27+ CD45RA+), central memory (CM; CD27+ CD45RA−), effector memory (EM; CD27− CD45RA−), and terminally differentiated CD45RA-re-expressing EM (EMRA; CD27− CD45RA+) subsets (Fig. 3A). Among bulk CD8 T cells, the relative proportions of these subsets did not differ significantly between the two groups (Fig. 3B). However, when we examined the maturation phenotypes of HIV-specific CD8 T cells, we found twofold-lower percentages of EM CD8 T cells ($P = 0.003$) and correspondingly twofold-higher percentages of EMRA CD8 T cells ($P = 0.050$) in the subjects with

FIG. 1. Chemokine receptor expression on bulk and HIV-specific CD8 T cells in chronic and advanced HIV infection. (A) Contour plots demonstrating gates used for lymphocytes, CD8 T cells and tetramer+ CD8 T cells, and chemokine receptors. SSC, side scatter; FSC, forward scatter. (B) Representative examples of cell-surface expression of chemokine receptors in study subjects with either chronic (top row) or advanced (bottom row) HIV infection. SSC, side scatter. (C and D) The levels of chemokine receptor expression on bulk CD8 T cells (C) and HIV-specific CD8 T cells (D) were compared between HIV-positive study subjects with chronic and advanced infection. (E) Levels of CXCR3 expression on bulk and EBV-specific CD8 T cells from five HIV-negative subjects.

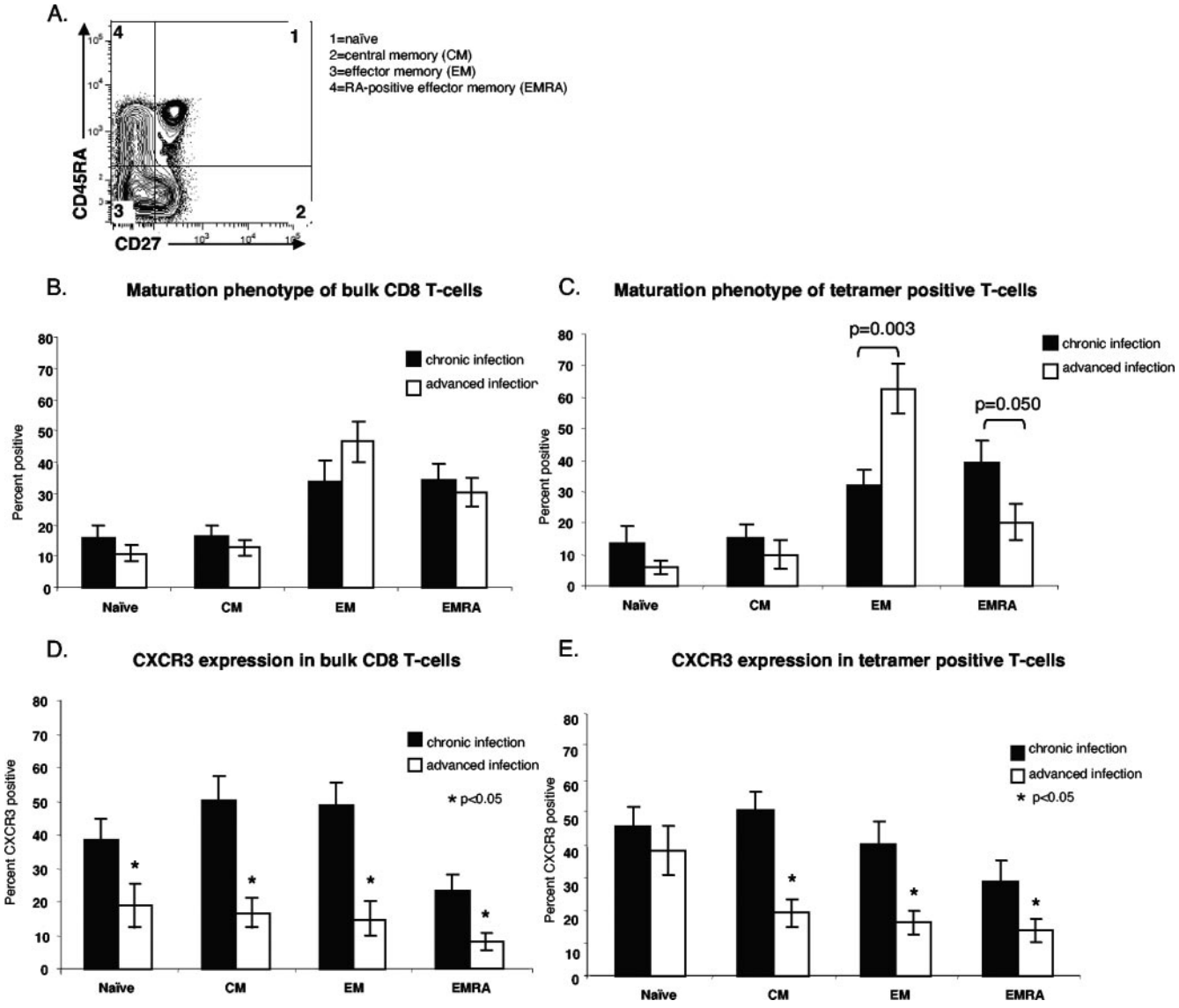



FIG. 3. Maturation phenotype and CXCR3 expression on bulk and HIV-specific CD8 T cells. (A) Representative contour plot demonstrating gates used for definition of four maturation subsets: naïve ($CD27^+$ $CD45RA^+$), CM ($CD27^+$ $CD45RA^-$), EM ($CD27^-$ $CD45RA^-$), and EMRA ($CD27^-$ $CD45RA^+$) CD8 T cells. (B and C) Distribution of bulk CD8 T cells (B) and HIV-specific CD8 T cells (C) according to maturation phenotype. (D and E) Lower levels of CXCR3 were seen across all maturation subsets of bulk (D) and HIV-specific (E) CD8 T cells from subjects with advanced versus chronic infection, suggesting that the maturation defect seen in subjects with advanced infection did not account for the decreased CXCR3 expression on CD8 T cells.

chronic infection compared to those with advanced disease (Fig. 3C). These observations are consistent with the previously reported block in CD8 T-cell maturation seen in HIV infection (9, 23). Of note, the small but detectable percentages of "naïve" HIV-specific CD8 T cells in chronic (13.7% ± 5.3%) and advanced (6.0% ± 2.0%) infection reflect the difficulty in finding phenotypic markers that provide a perfect separation of memory CD8 T cells in the setting of generalized immune activation (17), rather than truly antigen-naïve T cells.

**Decreased CXCR3 on bulk and HIV-specific CD8 T cells across all maturation subsets in advanced infection.** To determine if the reduced CXCR3 expression we had observed on CD8 T cells in advanced infection was related to the differences in maturation phenotype of these cells, we examined CXCR3 expression levels on bulk and HIV-specific CD8 T cells in naïve, CM, EM, and EMRA subsets. $CXCR3^+$ CD8 T cells were uniformly two- to threefold lower in subjects with advanced infection than in subjects with chronic infection across all maturation subsets: naïve, $P = 0.035$; CM, $P = 0.013$; EM, $P = 0.003$; and EMRA, $P = 0.013$ (Fig. 3D). Similarly, on HIV-specific CD8 T cells, subjects with advanced infection had statistically significant lower percentages of CXCR3 across all subsets, except naïve cells, than subjects with chronic infection: CM, $P = 0.009$; EM, $P = 0.008$; and EMRA, $P = 0.046$ (Fig. 3E). Thus, we conclude that, although we observed differences in the maturation phenotypes of bulk and HIV-specific CD8 T

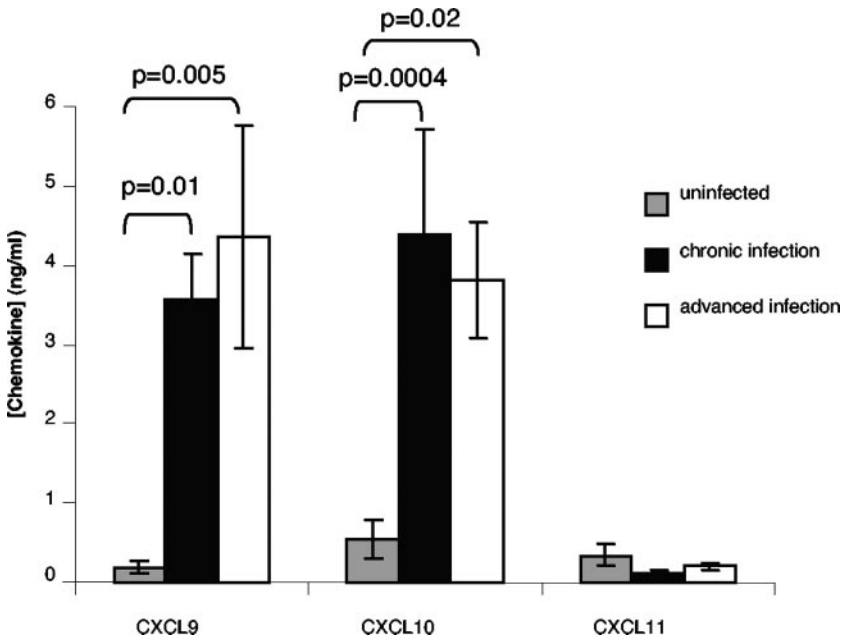



FIG. 4. Plasma levels of CXCR3 ligands in healthy controls ($n = 5$) and subjects with chronic HIV infection ($n = 8$) and advanced HIV infection ($n = 10$) as measured by enzyme-linked immunosorbent assay or cytometric bead assay.

cells in subjects with chronic versus advanced infection, this did not account for the lower percentages of CXCR3 expression observed on CD8 T cells seen as HIV infection progresses.

**Elevated plasma levels of CXCR3 ligands in both subjects with chronic and advanced HIV infection.** Cell surface expression of chemokine receptors can be down regulated by the engagement of cognate ligands (50), and data suggest that HIV infection is associated with increased levels of circulating inflammatory chemokines (47). We therefore wanted to exclude differences in plasma chemokine levels as a cause of the observed differences in CXCR3$^+$ CD8 T cells seen in our two clinical cohorts. We measured plasma levels of multiple chemokines, including the three known CXCR3 ligands, CXCL9 (monokine induced by gamma interferon [Mig]), CXCL10 (gamma interferon-induced protein of 10 kDa [IP-10]), and CXCL11 (interferon-inducible T-cell α chemoattractant [I-TAC]) in uninfected control subjects ($n = 5$) and in subjects with chronic ($n = 8$) and advanced ($n = 10$) HIV infection. As shown in Fig. 4, CXCL9 and CXCL10 levels were similar in both chronic (3.82 ± 0.74 ng/ml and 4.37 ± 1.3 ng/ml, respectively) and advanced (4.35 ± 1.4 ng/ml and 3.82 ± 0.74 ng/ml, respectively) HIV infection, but were significantly increased compared to the levels in uninfected controls (0.19 ± 0.08 ng/ml and 0.05 ± 0.02 ng/ml, respectively). CXCL11 levels were comparatively low and did not differ between any of the groups. Although the other chemokines tested, CCL2 (macrophage inflammatory protein-1 α [MIP-1α]), CCL5 (RANTES) and CXCL8 (IL-8), were also upregulated in HIV infection, there were no significant differences between the plasma levels in subjects with chronic or advanced disease (data not shown).

**Enrichment of CXCR3$^+$ CD8 T cells in HIV-infected lymph nodes.** Because CXCR3 is known to guide effector T cells to peripheral sites of inflammation, we hypothesized that the decreased percentages of circulating CXCR3$^+$ CD8 T cells we observed in advanced HIV infection might be attributable to an accumulation of these cells in lymphoid tissue where HIV is replicating at the highest levels. Initial experiments using tissue from healthy subjects compared the sample composition of FNA to that of excisional biopsy. Discarded surgical specimens from three HIV-negative subjects were obtained, and lymph nodes were identified within the tissue. FNA was performed, and then the remaining node was processed for comparison. We found that both flow cytometry data and qPCR data were equivalent for lymph node tissue obtained by FNA or excisional biopsy (Fig. 5). We therefore obtained paired peripheral blood and lymph node samples from subjects with chronic ($n = 6$) or advanced ($n = 6$) HIV infection.

Representative flow cytometry analysis of the distribution of CXCR3$^+$ CD8 T cells in the blood and lymph nodes of subjects with chronic (patient CI10) and advanced (patient AD12) HIV infection are shown in Fig. 6A, and cumulative data are shown in Fig. 6B. Higher percentages of CXCR3$^+$ CD8 T cells were seen in the lymph nodes than in blood from subjects with both chronic ($P = 0.005$) and advanced ($P = 0.006$) infection, suggesting that CXCR3 plays a critical role in CD8 T-cell homing to lymph nodes throughout the entire course of HIV infection. We then compared the percentages of CXCR3$^+$ CD8 T cells in both blood and lymphoid tissue of subjects with chronic versus advanced infection. As noted earlier, individuals with advanced disease had decreased levels of CXCR3$^+$ CD8 T cells in blood compared to those with chronic infection ($P = 3 \times 10^{-6}$). We found that, compared to subjects with chronic infection, those with advanced infection had over twofold lower numbers of CXCR3$^+$ CD8 T cells in the lymph nodes ($P = 0.002$), despite the fact that their lymph nodes were enriched for CXCR3$^+$ CD8 T cells. In contrast, percentages of other chemokine receptors on CD8 T cells, including CCR7, CCR5, CXCR1, and CX3CR1, did not differ between lymph node and

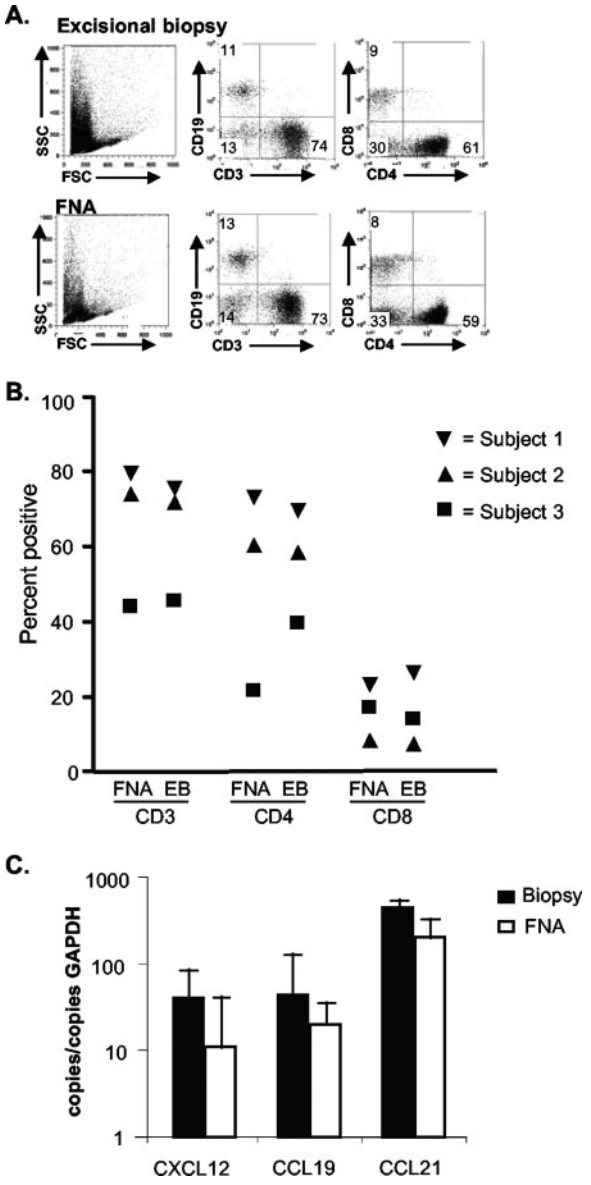



FIG. 5. Comparison of results from lymph node samples obtained by either excisional biopsy or FNA. (A) Representative dot plots demonstrating scatter properties and mononuclear staining for samples obtained by excisional biopsy and FNA of the same lymph node are shown. SSC, side scatter; FSC, forward scatter. (B) Cumulative flow cytometry data on the distribution of CD3, CD4, and CD8 T cells in FNA or excisional biopsy (EB) samples from three subjects. (C) Homeostatic chemokine levels as measured by qPCR in lymph node samples obtained by FNA or excisional biopsy from three subjects.

blood in subjects with either chronic or advanced HIV infection (data not shown).

**Decreased lymph node CXCR3 ligand and CD8 mRNA levels in subjects with advanced HIV infection.** To determine if a decreased level of CXCR3$^+$ CD8 T cells in the lymph nodes in subjects with advanced HIV infection was representative of an overall defect in T-cell trafficking to lymph nodes in these study subjects, we analyzed lymph node tissue for CD8 and chemokine mRNA expression by qPCR. Compared to subjects with chronic infection, those with advanced disease had 10-fold-lower levels of CD8 mRNA expression in their lymph nodes ($P = 0.015$, Fig. 7A), suggesting they have diminished CTL trafficking to their infected lymph nodes. The levels of CXCR3 ligands CXCL9 and CXCL10 were also significantly lower in subjects with advanced versus chronic infection ($P = 0.002$ for CXCL9 and $P = 0.03$ for CXCL10; Fig. 7B), possibly contributing to the failure of recruitment of CXCR3$^+$ CD8 T cells into infected lymph nodes. The levels of CCR5 ligands were also lower in subjects with advanced infection, but this difference was not statistically significant. In contrast, the levels of homeostatic chemokines CCL19 and CCL21 were similar in both groups (data not shown).

## DISCUSSION

Our understanding of how HIV evades the immune system remains incomplete and is currently one of the greatest challenges to the design of an effective vaccine. We hypothesized that impaired effector CD8 T-cell trafficking into lymph nodes plays a role in the immune dysfunction seen in advanced HIV infection. Because a longitudinal study of serial paired lymph node and blood samples from the same patients over many years as HIV infection progresses to AIDS was not feasible, we performed a cross-sectional analysis of chemokine receptor expression in peripheral blood and lymph node CD8 T cells from individuals with either stable, chronic infection or advanced HIV/AIDS. We found that lymph nodes in all subjects were enriched for CXCR3$^+$ CD8 T cells compared to the levels in blood, suggesting that this chemokine receptor plays a key role in guiding CTL into infected lymph nodes throughout the course of HIV infection. Interestingly, subjects with advanced infection had significantly fewer CXCR3$^+$ CD8 T cells in both blood and lymph nodes than those with chronic infection, and CXCR3 expression correlated positively with CD4 count and inversely with viral load, consistent with CXCR3 contributing to the ability of CTL to limit viral replication and, ultimately, disease progression. In addition, we found that levels of CD8 mRNA were 10-fold lower in the lymph nodes of individuals with advanced versus chronic infection, despite these two groups having nearly identical peripheral CD8 T-cell counts. The findings of globally reduced CXCR3 expression on CD8 T cells and of lower lymph node CD8 levels in individuals with advanced HIV infection suggest that CTL homing to infected lymph nodes becomes impaired as infection progresses, and that this impairment may contribute to the immune dysfunction seen in AIDS.

Our data point to a key role for CXCR3 in CD8 T-cell trafficking into HIV-infected lymph nodes. In the absence of infection, CCR7 rather than CXCR3 is the critical chemokine receptor involved in guiding naïve and effector memory T cells into lymph nodes (10, 19). CXCR3$^+$ T cells consequently would not be expected to be enriched in the lymph node in uninfected individuals. Equivalent percentages of CXCR3$^+$ T cells have been demonstrated to be present in the lymph node and the blood of uninfected rhesus macaques (49). In contrast, CXCR3$^+$ T cells are enriched in the lymph nodes of SIV-infected macaques (49), consistent with what we now report in humans. The mechanism by which effector CD8 T cells enter infected lymph nodes in HIV infection is uncertain, yet critically important, given that lymphoid tissue is the largest reservoir for replicating virus. In HIV infection, published data

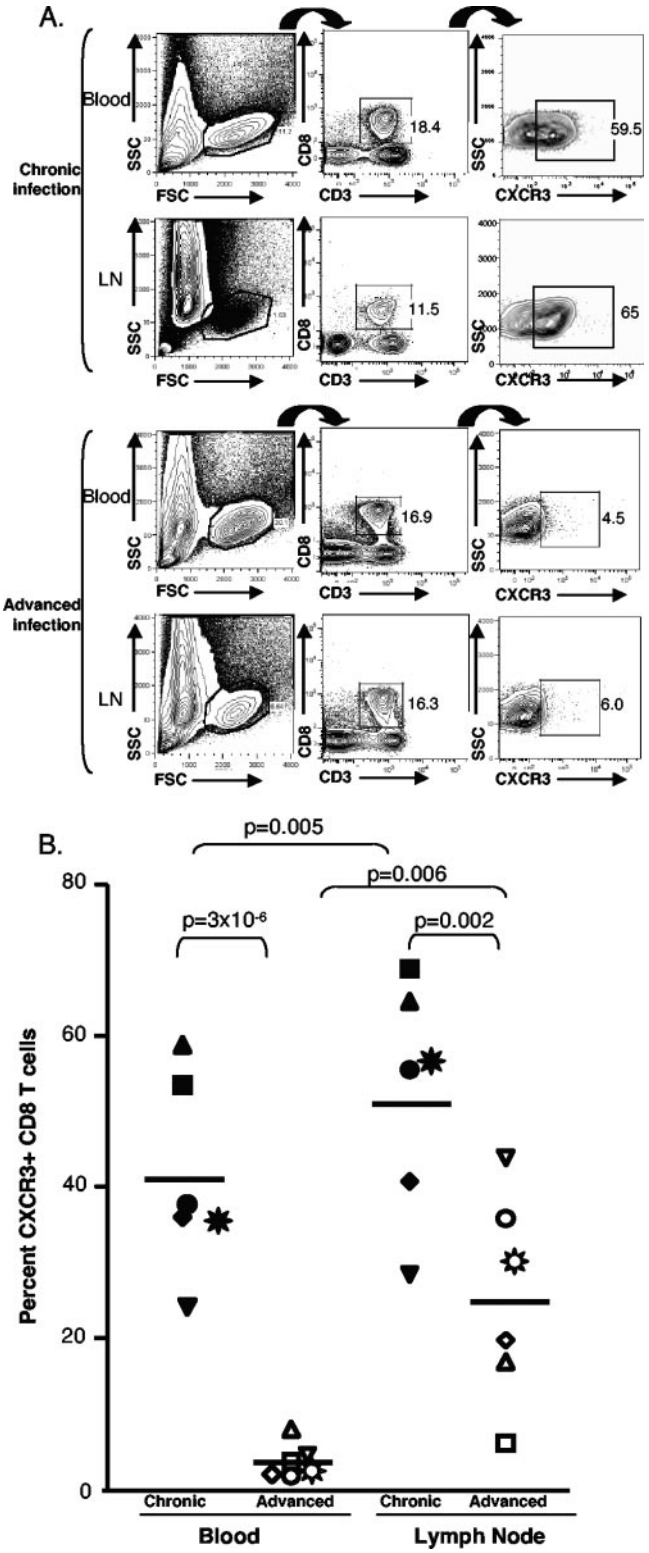



FIG. 6. CXCR3 levels on bulk CD8 T cells from paired blood and lymph node samples in subjects with chronic or advanced HIV infection. (A) Contour plots of lymphocyte gating (left panels), CD8 T-cell staining (middle panels) of the lymphocyte gated population, and CXCR3 expression of gated CD8 T cells (right panels) in the blood and lymph nodes of representative study subjects with chronic or advanced HIV infection. SSC, side scatter; FSC, forward scatter. (B) Percentages of CD8 T cells expressing CXCR3 in paired blood and lymph node samples of individuals with chronic ($n = 6$) and advanced ($n = 6$) HIV infection.

suggest lymph nodes are converted from homeostatic tissues to sites of inflammation due to the high degree of viral replication (51), and, consistent with our findings, characterizations of CD8 T cells from HIV-infected lymph nodes have described the majority of cells as having an effector/memory phenotype—i.e., $CCR7^-$ and/or $CD45RO^+$ (17, 56). CCR7 thus does not seem to play a prominent role in lymph node trafficking in HIV infection. In contrast, we found that in both chronic and advanced HIV infection, $CXCR3^+$ CD8 T cells were significantly enriched in the lymph nodes compared to their levels in blood, suggesting that CXCR3, a receptor that typically guides effector CD8 T cells to inflamed peripheral tissues (16, 22, 25–27, 29, 30, 41, 60, 64), also directs HIV-specific CTL to infected lymphoid tissue. In further support of this hypothesis, several earlier reports have demonstrated that inflammatory chemokines, including CXCR3 ligands CXCL9 and CXCL10, are up regulated in the lymph nodes during HIV infection (18, 33, 59).

Many previous studies have focused on chemokine receptors CCR5 and CXCR4 in HIV infection because they serve as coreceptors for viral entry, whereas less is known about CXCR3 in HIV infection. Our finding that CXCR3 is found on 30 to 40% of both bulk and HIV-specific CD8 T cells in the blood of persons with stable, chronic HIV infection is consistent with the results of previously published reports (24, 55). Whereas trafficking to lymphoid tissue has not been previously investigated, existing data suggest that in HIV infection, as in other infections, CXCR3 is involved in guiding CD8 T cells to peripheral sites of inflammation. One report found an enrichment of $CXCR3^+$ CD8 T cells in the cerebral spinal fluid of untreated HIV-infected patients with early disease (mean CD4 count, 412 cells/ml, and log viral load, 4.23 copies/ml) with a concomitant elevation of cerebral spinal fluid CXCL10 levels (55). Another study described an increase in $CXCR3^+$ CD8 T cells in the lungs of HIV-infected subjects with alveolitis (1). Furthermore, in acutely SIV-infected macaques, CXCR3 was important for trafficking both to lymph nodes and the small intestine (11). Our current studies demonstrate that CXCR3 is also uniquely important for the homing of CTL to infected lymphoid tissue in HIV infection. These data are additionally supported by work demonstrating that in Epstein-Barr viral infection, up to 90% of antigen-specific CD8 T cells in infected tonsillar tissue are $CXCR3^+$ (62).

In addition to revealing the importance of CXCR3 for CD8 T-cell homing to lymph nodes in HIV infection, our study found evidence for a CTL trafficking defect in subjects with advanced infection. Compared to individuals with chronic infection, those with advanced infection had 10-fold-lower levels of CD8 mRNA in their lymph nodes, even though their peripheral blood CD8 cell counts were no different. We envisioned two alternative mechanisms to account for this impairment of CTL trafficking into lymph nodes as HIV disease progresses. The first hypothesis is that the expression of the relevant chemokine receptor(s) required for the recruitment of CD8 T cells into infected lymph nodes decreases as HIV dis-

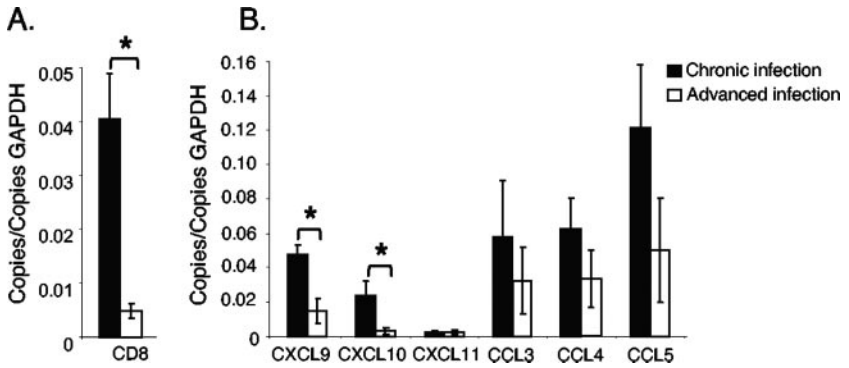



FIG. 7. Expression levels of CD8 and chemokine mRNA in lymph node tissue from subjects with chronic or advanced HIV infection. Lymph node CD8 (A) and inflammatory chemokine (B) mRNA copy numbers normalized to copy numbers of glyceraldehyde-3-phosphate dehydrogenase mRNA from study subjects with chronic ($n = 6$) or advanced ($n = 6$) HIV infection.

ease progresses to AIDS. The second hypothesis is that decreased lymph node chemokine production results in a dampened signal that is less effective at attracting CTL into the infected lymph nodes. Our data suggest that CD8 T-cell recruitment into the lymph nodes is impaired in advanced HIV infection primarily because the level of CXCR3 expression on circulating CD8 T cells is so profoundly reduced. As noted previously, we found enrichment of $CXCR3^+$ CD8 T cells in lymph nodes versus blood in advanced infection, suggesting that these $CXCR3^+$ CD8 T cells are able to successfully home to lymph nodes in this phase of infection. However, due to the significant reduction of $CXCR3^+$ CD8 T cells in the circulatory system of subjects with advanced HIV infection, the total number of effector CD8 T cells getting into infected lymph nodes is markedly reduced. This trafficking defect may lead to impaired killing of HIV-infected cells in the lymph nodes. Some studies suggest that as HIV infection progresses, CD4 T cells are increasingly depleted from lymph nodes (52, 53). It remains to be determined whether the greatest reservoirs of virus production lie outside the lymph nodes in AIDS. If so, we may be overstating the importance of CD8 T-cell trafficking to lymph nodes late in HIV infection.

Although reduced CXCR3 expression on CD8 T cells seems to be the primary cause of the observed trafficking defect in advanced HIV infection, lower levels of lymph node CXCR3 ligands could also have secondarily contributed to the reduced lymph node levels of CD8 mRNA seen in advanced HIV infection. We found that both CXCL9 and CXCL10 were significantly reduced in the lymph nodes of those with advanced versus chronic HIV infection. While infiltration by effector CD8 T cells and upregulation of inflammatory chemokines have been observed in the lymph nodes of untreated HIV-infected persons, the manner in which these inflammatory processes change over time remains unknown (47). Persistent activation leads to disruption of the normal functional organization of lymphoid tissue (21). In fact, one of the earliest changes in HIV-infected lymph nodes is the disruption of normal follicular architecture (54, 61), presumably related to the influx of CD8 T cells. These cytotoxic T cells alter the normal chemokine environment. Over time, increasing collagen deposition and fibrosis of lymphoid tissue occurs, which may further impair critical lymph node functions, such as local chemokine production (53). In our study, the inflammatory chemokines CXCL9, CXCL10, CCL3, CCL4, and CCL5 were all decreased in subjects with advanced versus chronic infection, suggesting that there may be a waning of chemokine signal in lymph nodes that contributes to the impaired ability of effector T cells to reach this site of viral replication. Due to the small amount of tissue obtained by lymph node FNA, however, we were unable to quantitatively assess samples for collagen, CD4 T cells, dendritic cells, or macrophages.

The mechanism by which the percentage of CD8 T cells that express CXCR3 decreases as chronic HIV infection progresses to AIDS, thereby impairing effector cell trafficking to infected lymph nodes, remains unclear. Circulating levels of inflammatory chemokines are elevated in HIV infection (3, 39), and these ligands could cause internalization and down regulation of their cognate receptors (50). Compared to the levels in uninfected controls, HIV-infected individuals had significant elevations of both CXCL9 and CXCL10, but there were no significant differences in chemokine levels between chronic and advanced infection. Increased plasma levels of CXCR3 ligands, therefore, are unlikely to contribute to the observed decrease in $CXCR3^+$ CD8 T cells in advanced HIV infection. High levels of plasma CXCL9 and CXCL10 were seen in subjects with advanced infection despite decreased levels of these chemokines in the lymph nodes. Infected lymphocytes and monocytes have been shown in vitro to produce CXCL9 and CXCL10 (46), and these cells in the circulatory system or in nonlymphoid tissue compartments may be the origin of the elevated CXCL9 and CXCL10 seen in our HIV-infected subjects. Along these lines, it is possible that $CXCR3^+$ CD8 T cells are located in a noninvestigated anatomic site where the ligands are highly expressed.

We also considered whether our results demonstrating a marked decrease in circulating $CXCR3^+$ CD8 T cells as HIV infection progresses might be a function of an alteration in effector cell maturation phenotype. Previous studies have demonstrated a failure of HIV-specific CD8 T cells to fully mature to terminally differentiated effector cells in comparison with the maturation of virus-specific CD8s in other viral infections (9) or in comparison with the maturation of HIV-specific CD8 T cells in individuals who can control HIV viremia when off of highly active antiretroviral therapy (23). Along these lines, the chemokine receptor CXCR1 is preferentially expressed on ter-

minally differentiated effector cells and its absence in most HIV-infected individuals, except those able to control HIV replication during treatment interruptions, is attributable to the aforementioned differences in CD8 T-cell maturation phenotype in these different cohorts (24). When comparing our two groups of study subjects, we found that those with advanced infection did have a significantly lower percentage of fully mature HIV-specific CD8 T cells than those with chronic infection, and correspondingly a higher percentage of HIV-specific CD8 T cells at the pre-terminally differentiated state. Nevertheless, when we examined CXCR3 expression across naïve, CM, EM, and terminally differentiated, EMRA CD8 T cells, decreased expression was equally distributed across all maturation subsets among individuals with advanced disease. The decreased CXCR3 expression we observed in subjects with advanced infection could not, therefore, be explained by the maturation defect also found in this patient population.

Alternatively, the reduced CXCR3 expression on CD8 T cells in subjects with advanced HIV infection may be related to direct effects of high-level viral replication and/or CD4 T-cell depletion. CXCR3 is known to be up regulated on resting T cells by interleukin-2 (35), so it is conceivable that the impaired cytokine production (34, 57) seen in late stage HIV infection contributes to decreased CXCR3 expression. Additionally, while 4-h in vitro peptide stimulation increases CXCR3 on CD8 T cells (24), 24 h of stimulation profoundly down regulates CXCR3 (48). The effects of persistent antigenic stimulation, as seen in HIV infection, on levels of CXCR3$^+$ CD8 T cells in vivo remain unknown. Elucidating the mechanism by which CXCR3$^+$ CD8 T cells decrease in advanced HIV infection will be the focus of future investigations.

In summary, we have identified defects in CD8 T-cell CXCR3 expression and lymph node mRNA levels of both CD8 and inflammatory chemokines in subjects with advanced HIV infection, suggesting that HIV infection leads to impaired homing of antiviral CD8 T cells. Our data suggest that CXCR3 and its ligands may be important for the effective delivery of antiviral CD8 T cells into infected lymph nodes and subsequent viral control. Vaccine and immunotherapeutic strategies should aim to generate adaptive immune responses that express CXCR3 and can therefore effectively home to sites of viral replication.

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