

# CTL Fail to Accumulate at Sites of HIV-1 Replication in Lymphoid Tissue<sup>1,2</sup>

Elizabeth Connick,<sup>3\*</sup> Teresa Mattila,<sup>†</sup> Joy M. Folkvord,<sup>\*</sup> Rick Schlichtemeier,<sup>\*</sup> Amie L. Meditz,<sup>\*</sup> M. Graham Ray,<sup>\*</sup> Martin D. McCarter,<sup>‡</sup> Samantha MaWhinney,<sup>§</sup> Aaron Hage,<sup>†</sup> Cara White,<sup>†</sup> and Pamela J. Skinner<sup>†</sup>

The inability of HIV-1-specific CTL to fully suppress virus replication as well as the failure of administration of exogenous CTL to lower viral loads are not understood. To evaluate the hypothesis that these phenomena are due to a failure of CTL to localize at sites of HIV-1 replication, we assessed the distribution of HIV-1 RNA and HIV-1-specific CTL identified by HIV-1 peptide/HLA class I tetrameric complexes (tetramers) within lymph nodes of 14 HIV-1-infected individuals who were not receiving antiretroviral therapy. A median of 0.04% of follicular compared with 0.001% of extrafollicular CD4<sup>+</sup> cells were estimated to be producing HIV-1 RNA, a 40-fold difference ( $p = 0.0001$ ). Tetramer-stained cells were detected by flow cytometry in disaggregated lymph node cells from 11 subjects and constituted a significantly higher fraction of CD8<sup>+</sup> cells in lymph node (mean, 2.15%) than in PBMC (mean, 1.52%;  $p = 0.02$ ). In situ tetramer staining in three subjects' lymph nodes, in which high frequencies of tetramer-stained cells were detected, revealed that tetramer-stained cells were primarily concentrated in extrafollicular regions of lymph node and were largely absent within lymphoid follicles. These data confirm that HIV-1-specific CTL are abundant within lymphoid tissues, but fail to accumulate within lymphoid follicles where HIV-1 replication is concentrated, suggesting that lymphoid follicles may be immune-privileged sites. Mechanisms underlying the exclusion of CTL from lymphoid follicles as well as the role of lymphoid follicles in perpetuating other chronic pathogens merit further investigation. *The Journal of Immunology*, 2007, 178: 6975–6983.

Human immunodeficiency virus-1-specific CD8<sup>+</sup> CTL are thought to play a critical role in controlling HIV-1 replication in vivo. The appearance of HIV-1-specific CTL during acute HIV-1 infection coincides with declines in viremia (1–3), suggesting that CTL may be critical determinants of the initial control of virus replication. Removal of CD8<sup>+</sup> cells from SIV-infected macaques leads to increased plasma viremia, further supporting the hypothesis that CTL suppress HIV-1 replication (4, 5). The CTL response to HIV-1 is unique because in some cases virus-specific cytolysis is detectable in PBMC in the absence of in vitro stimulation (6, 7), a phenomenon that has not been frequently reported in other chronic viral infections. Furthermore, high frequencies of HIV-1-specific CD8<sup>+</sup> T cells have been detected in PBMC from both acutely and chronically infected individuals using HLA class I tetrameric complexes loaded with HIV-1 epitopic

peptides (tetramers) (8–10). Nevertheless, despite evidence that HIV-1-specific CTL are abundant and capable of cytolytic function, they are unable to fully suppress viral replication in vivo resulting in the progressive depletion of CD4<sup>+</sup> T cells and, ultimately, death in untreated individuals. Quite perplexingly as well, in multiple studies the administration of exogenous CTL has failed to result in significant decreases in plasma HIV-1 RNA concentrations (11–14). It is not clear why CTL are unable to control HIV infection.

The majority of HIV-1 replication occurs in CD4<sup>+</sup> T cells in lymphoid tissues (15, 16). HIV-1 replication is not evenly distributed within lymphoid tissues, but is concentrated within lymphoid follicles where CD4<sup>+</sup> cells constitute a minority of cells (17–19). We previously reported that a CD4<sup>+</sup> cell within a lymphoid follicle was on average 31-fold more likely to be productively infected compared with a CD4<sup>+</sup> cell in the extrafollicular region of lymph node (17). We also demonstrated that multiple antiretroviral effector proteins as well as CD8<sup>+</sup> cells are relatively deficient within lymphoid follicles compared with extrafollicular regions of lymph node (17), suggesting that lymphoid follicles may be relatively immune-privileged sites. The existence of an immune-privileged site in lymphoid tissue where HIV-1-specific CTL do not circulate could potentially explain both the inability of endogenous CTL to fully suppress HIV-1 replication as well as the failure of exogenous CTL to decrease viral load. To date, however, the distribution of HIV-1-specific CTL within lymphoid tissues has not been determined, despite their hypothesized importance in controlling HIV-1 replication. The purpose of the present study was to evaluate the magnitude and distribution within lymphoid tissue of HIV-1-specific CTL. We hypothesized that HIV-1-specific CTL would be primarily concentrated in the extrafollicular regions of lymphoid tissues and not in lymphoid follicles where HIV-1 replication is most strongly concentrated.

\*Division of Infectious Diseases, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262; <sup>†</sup>Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN 55108; <sup>‡</sup>Department of Surgery, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262; and <sup>§</sup>Department of Preventive Medicine and Biometrics, University of Colorado at Denver and Health Sciences Center, Denver, CO 80262

Received for publication August 17, 2006. Accepted for publication March 5, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by Public Health Services Grants R29AI-42499, U01AI-41536, PO1-AI-55356, and P30-AI-054907 from the National Institutes of Health.

<sup>2</sup> These data were presented in part at the 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 22–25, 2005 (Abstract 464).

<sup>3</sup> Address correspondence and reprint requests to Dr. Elizabeth Connick, Division of Infectious Diseases, University of Colorado at Denver and Health Sciences Center, Box B168, 4200 East 9th Avenue, Denver, CO 80262. E-mail address: liz.connick@uchsc.edu

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/\$2.00

## Materials and Methods

### Human subjects

HIV-1-infected individuals who were not receiving antiretroviral therapy were recruited to donate inguinal lymph nodes. None of these subjects had an active opportunistic infection or malignancy or a sexually transmitted disease other than HIV-1. Informed consent was obtained from all participants in accordance with the Colorado Multiple Institutional Review Board.

### Clinical specimens

Inguinal lymph node excisional biopsies were performed under local anesthesia at the University of Colorado Hospital as previously described (20). To efficiently identify eligible subjects, before lymph node excision, all subjects were screened for HLA types for which HIV-1 epitope-specific tetramers were available using Abs directed against A2, A3, B7, and B8 (One Lambda). Of note, according to the manufacturer, the B7 Ab also detects individuals with HLA B27 and B42. Only individuals whose cells stained positively with one of these HLA Abs were included in the present study. One portion of the lymph node was placed in RPMI 1640 with sodium heparin (18.7 U/ml) and shipped overnight to the laboratory of Dr. P. Skinner. A second portion of the lymph node was snap-frozen in OCT compound (VWR) and stored at  $-70^{\circ}\text{C}$ . A third portion of lymph node was minced in PBS (Invitrogen Life Technologies) to obtain a single-cell suspension and was either used directly in phenotypic or functional assays or was cryopreserved for later studies. Peripheral blood specimens were obtained on the same date and used to determine CD4<sup>+</sup> T cell counts by flow cytometry and plasma HIV-1 RNA concentration (HIV-1 Monitor Assay; Roche Diagnostics). PBMC were isolated as well for tetramer staining and functional assays. Subsequent to lymph node excision, all subjects had class I HLA typing performed at the University of Colorado Hospital HLA Typing Laboratory using standard molecular techniques.

### In situ hybridization for HIV-1 RNA and immunohistochemical staining for CD20 in lymph nodes

In situ hybridization for HIV-1 RNA was performed on 6- $\mu\text{m}$  tissue sections using digoxigenin-labeled antisense riboprobes for *env*, *gag*, and *nef*, which were detected using NBT/5-bromo-4-chloro-3-indolyl phosphate as previously described (17, 20). This technique identifies cells that are actively transcribing HIV-1, but not extracellular virions encapsulated in envelope glycoprotein. As a negative control, sections from a subset of subjects were hybridized with digoxigenin-labeled sense probes for *env*, *gag*, and *nef* using the same procedure. Immunohistochemical double staining for the B cell marker CD20 was performed as previously described (17) to morphologically identify lymphoid follicles. For each subject, in situ hybridization for HIV-1 RNA and double staining for anti-CD20 was performed in a minimum of three tissue sections, each between 12 and 60  $\mu\text{m}$  apart. The number of virus-producing cells within and outside of lymphoid follicles, defined by anti-CD20 staining, was determined by visual inspection and manually counting the positively stained cells on each section. The area of follicular and extrafollicular lymph node tissue was determined by quantitative image analysis using a computerized image analysis system (Leica Q5001W Image Analysis). The total number of virus-producing cells in follicular and extrafollicular regions was divided by the total area of the follicular or extrafollicular tissue, respectively, to determine the frequency of virus-producing cells per  $\text{mm}^2$  of follicular and extrafollicular tissue, as previously described (17). The total tissue area evaluated per subject ranged from 53 to 565  $\text{mm}^2$ , with a median value of 186  $\text{mm}^2$ .

### Estimation of frequencies of virus-producing CD4<sup>+</sup> cells within lymphoid tissue

Frequencies of CD4<sup>+</sup> cells within lymphoid tissue were estimated by evaluating for each subject three full cross-sections of lymph node, each 60–180  $\mu\text{m}$  apart, that were double stained for CD4 and CD20 as we have previously described (17). Ten randomly selected fields, each 0.74  $\text{mm}^2$ , were evaluated on each tissue section. Using quantitative image analysis, the area of the follicular and extrafollicular regions was determined as well as the area of each of these regions that stained positively for CD4. The average area of a CD4<sup>+</sup> staining cell was determined by measuring the length and width of 10 positively stained cells on each section and using the formula for the area of an ellipse:  $\pi \times (\text{mean length}/2) \times (\text{mean width}/2)$ . The percentage of the tissue area that stained positively for CD4 was divided by the average area of a CD4<sup>+</sup> cell to estimate the number of CD4<sup>+</sup> cells per  $\text{mm}^2$ . Frequencies of CD4<sup>+</sup> cells that were producing HIV-1 RNA were calculated by dividing the frequency of HIV-1 RNA-producing cells per  $\text{mm}^2$  by the estimated number of CD4<sup>+</sup> cells per  $\text{mm}^2$ .

Table I. HIV-1 CD8<sup>+</sup> T cell epitopes evaluated by tetramer staining

HLA Restriction	Epitope Location	Amino Acid Sequence	Abbreviation
HLA-A*0201	HIV.p17.77–85	SLYNTVATL	A2 <i>gag</i>
HLA-A*0201	HIV.rt.476–484	ILKEPVGHV	A2 <i>pol</i>
HLA-A3	HIV.p17.18–26	KIRLRPGGK	A3 <i>gag</i>
HLA-A3	HIV.nef.71–80	QVPLRPMTYK	A3 <i>nef</i>
HLA-B7	HIV.nef.128–137	TPGPGVRYPL	B7 <i>nef</i>
HLA-B8	HIV.p24.259–267	EIYKRWI I	B8 <i>gag</i>
HLA-B8	HIV.nef.89–97	FLKEKGGGL	B8 <i>nef</i>

### Tetramer staining of PBMC and disaggregated lymph node cells

PBMC and disaggregated lymph node cells were stained with selected tetramers loaded with CD8<sup>+</sup> T cell HIV-1 epitopic peptides (Table I) that matched subjects' HLA class I molecules. Tetramers were selected for analysis based upon prior reports in the literature (21–25) as well as unpublished experience within our laboratories that suggested there was a high likelihood for HIV-1-infected individuals to harbor such cells. Tetramers were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility (Emory University, Atlanta, GA), except for those restricted by HLA-A\*0201, which were purchased from Beckman Coulter. PBMC were stained with PE- or allophycocyanin-labeled tetramer reagent for 30 min at room temperature with FITC-labeled anti-CD3 Ab and either PE- or allophycocyanin-labeled CD8 Ab (Beckman Coulter) followed by two washes. All samples were analyzed on a FACSCalibur (BD Biosciences) with CellQuest software. Results were expressed as the percentage of tetramer-stained cells in the CD8<sup>+</sup> population. Approximately 100,000 gated events were acquired in each analysis. Results were considered positive for tetramer-stained cells if the fraction of tetramer-stained cells that coexpressed CD8 was  $\geq 0.1\%$  of CD8<sup>+</sup> cells and a distinct population of cells, characteristic of tetramer staining (10), was observed.

### In situ tetramer staining

In situ tetramer staining was performed on fresh lymph node specimens shipped overnight and stained as previously described (26). Biotinylated HLA A2 *gag*, A2 *pol*, or an irrelevant melanoma (ELAGIGLTV) peptides were synthesized at Beckman Coulter Immunomics. Biotinylated HLA A3 *gag*, A3 *nef*, B8 *gag*, B8 *nef*, and B7 *nef* tetramers were produced at the NIAID Tetramer Facility. HLA/peptide tetramers were produced by adding six aliquots of FITC-labeled ExtraAvidin (Sigma-Aldrich) to biotinylated HLA/ $\beta_2$ -microglobulin/peptide monomers over the course of 8 h to a final molar ratio of 4.5:1. Fresh lymph node tissues were cut into 0.5-cm pieces and embedded in 4% low melt agarose. Tissue blocks were placed in a vibratome bath containing 0–2°C PBS supplemented with heparin (100  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich) (PBS-H),<sup>4</sup> and 200- $\mu\text{m}$  thick sections were generated. Fresh sections were stained free floating in 1 ml of solution with one to four sections per well in 24-well tissue culture plates. Incubations were conducted at 4°C on a rocking platform. Tetramers at a concentration of 0.5  $\mu\text{g}/\text{ml}$  with 2% normal goat serum (NGS) and 0.5  $\mu\text{g}/\text{ml}$  mouse anti-human CD8 Abs (DakoCytomation; clone DK25) or anti-human CD20 Abs (Nova Costra; clone L26) were diluted in PBS-H and incubated overnight. Sections were washed with chilled PBS-H and then fixed with freshly made 4% paraformaldehyde for 2 h at room temperature. Sections were again washed with PBS-H and then incubated with rabbit anti-FITC Abs (BioDesign) diluted 1/10,000 in PBS-H with 2% NGS and incubated 1–3 days. Sections were washed three times with PBS-H for at least 20 min and then incubated with Cy3-conjugated goat anti-rabbit Abs and Alexa 488-conjugated goat anti-mouse Abs (Jackson ImmunoResearch Laboratories) both diluted 1/1,000 in PBS-H with 2% NGS for 1–3 days. Finally, sections were washed three times for at least 20 min in PBS-H, postfixed with 4% paraformaldehyde for 1 h, and then mounted on slides with warmed glycerol gelatin (Sigma-Aldrich) containing 4 mg/ml *n*-propyl gallate (Fluka). Stained sections were analyzed using a Bio-Rad 1000 confocal microscope. The individual who analyzed in situ tetramer-stained specimens was blinded to the results of tetramer staining on PBMC and disaggregated lymph node cells.

<sup>4</sup> Abbreviations used in this paper: PBS-H, PBS supplemented with heparin; NGS, normal goat serum; B-LCL, B-lymphoblastoid cell line; CI, confidence interval; KSHV, Kaposi's sarcoma herpes virus.

Table II. Demographic and clinical characteristics of study subjects

Subject ID	Age (years)	Sex	Plasma HIV-1 RNA (log <sub>10</sub> copies/ml)	CD4 <sup>+</sup> T Cells/mm <sup>3</sup>	HLA A and B Alleles	
32	48	Female	3.71	775	A0301, 1101	B0702, 3501
61 <sup>a</sup>	42	Male	3.29	821	A0301, 2902	B1402, 4501
62 <sup>a</sup>	32	Male	4.35	305	A0201, -	B0702, 1524
64	25	Female	3.44	467	A0201, -	B2705, 5101
67 <sup>a</sup>	45	Female	4.37	406	A0201, -	B2705, 5101
79 <sup>a</sup>	42	Female	4.74	653	A0201, 0301	B1402, 3906
80	43	Male	3.94	628	A2402, 2902	B0801, 5801
81	29	Male	4.23	697	A0101, 0206	B0801, 3906
82	41	Female	3.57	835	A0201, 0205	B1501, 5301
84	39	Female	3.83	358	A3101, 3201	B1501, 3801
86	31	Female	4.01	460	A0301, 6601	B0702, 5802
87 <sup>a</sup>	26	Female	4.36	256	A0201, 3002	B1801, 3901
90	34	Female	4.86	332	A2301, 2601	B0801, 5802
92	40	Female	4.02	671	A0201, 0301	B2705, 4101

<sup>a</sup> Subjects who previously had received antiretroviral therapy. CD4<sup>+</sup> T cell counts indicated in the table were also the nadir CD4<sup>+</sup> T cell counts for subjects 62, 79, and 87. Nadir CD4<sup>+</sup> T cell counts were 636 cells/mm<sup>3</sup> for subject 61 and 398 cells/mm<sup>3</sup> for subject 67.

### Assays for HIV-1-specific cytolytic activity

Freshly isolated lymph node cells and PBMC were assayed for HIV-1-specific cytolytic activity in <sup>51</sup>Cr release assays as we have previously described (2), using as target cells autologous B-lymphoblastoid cell lines (B-LCL) pulsed with HIV-1 CD8<sup>+</sup> T cell epitopic peptides. B-LCL were established by EBV transformation of PBMC (7). For selected subjects known to harbor tetramer-stained cells before lymph node excision, B-LCL were labeled with <sup>51</sup>Cr and incubated for 1 h with the appropriate HIV-1 epitopic peptide (100 μg/ml) (Table I) synthesized by Multiple Peptide Systems. As a control for A2-restricted peptides, <sup>51</sup>Cr-labeled B-LCL were incubated with the irrelevant A2-restricted melanoma peptide. For HIV-1 peptides restricted by other HLA molecules, <sup>51</sup>Cr-labeled B-LCL alone were used as a control. Freshly isolated lymph node cells or PBMC were incubated for 6 h with HIV-1 peptide-pulsed <sup>51</sup>Cr-labeled B-LCL or control B-LCL, at E:T cell ratios of 200:1 (if sufficient cells were available), 100:1, 50:1, and 25:1. Maximum <sup>51</sup>Cr release was determined by detergent lysis of target cells with 5% Triton X-100 and spontaneous release was determined by incubating target cells in the absence of effector cells. Supernatants (0.05 ml) were harvested and the number of cpm was determined on a γ counter (Beckman Instruments). Percent cytotoxicity for peptide-pulsed B-LCL and control B-LCL was determined as: (mean observed cpm – mean spontaneous cpm)/(mean maximum cpm – mean spontaneous cpm) (2). The tetramer stained cell-to-target cell ratio was determined by multiplying the E:T cell ratio by the percentage of lymph node cells or PBMC that stained with tetramers as determined by flow cytometry.

### Statistical analysis

All statistical analyses assumed a two-sided significance level of 0.05. Analyses were conducted using SAS (SAS Institute) and Splus (Insightful Corporation) software. Reported mean values for frequencies of HIV-1 RNA-producing cells per mm<sup>2</sup> by lymph node region were obtained using Poisson regression with generalized estimating equations accounting for within-subject correlation. For this regression, the count of HIV-1 RNA-producing cells within the lymph node region was the dependent variable with the corresponding log<sub>e</sub> area included as an offset. Tetramer analyses used generalized least squares regression to account for within-subject correlations. Before analysis, data were plotted to ascertain normality. Correlations were conducted using Spearman ρ.

## Results

### Demographic and clinical characteristics of study subjects

Fourteen HIV-1-infected individuals screened positively with Abs directed at selected HLA molecules and were included in this study. Demographic and clinical characteristics of these individuals as well as their HLA A and B alleles as determined by molecular HLA typing are shown in Table II. Subject 64 was recruited 4 mo after documented primary HIV-1 infection. All other subjects had been infected for more than 1 year. None were receiving antiretro-

roviral therapy at the time of the lymph node excision and the majority were antiretroviral therapy naive. Five subjects had received some form of antiretroviral therapy in the past, but had discontinued therapy a median of 2 years (range, 8 mo to 5 years) before lymph node excision. The median CD4<sup>+</sup> T cell count was 467 cells/mm<sup>3</sup> and the median plasma HIV-1 RNA concentration was 4.01 log<sub>10</sub> copies/ml. None of the subjects had a history of an opportunistic infection or a CD4<sup>+</sup> T cell nadir below 250 cells/mm<sup>3</sup>.

### Frequencies of HIV-1 RNA-producing cells detected in lymph node

To characterize the distribution of HIV-1-producing cells within lymphoid tissue, we performed in situ hybridization on frozen lymph node sections using antisense riboprobes for *env*, *gag*, and *nef*. Frequencies of HIV-1 RNA-producing cells per mm<sup>2</sup> lymph node tissue ranged from 0.05 to 0.52 with a median of 0.21. Control sections treated with sense probes were uniformly negative. Frequencies of HIV-1 RNA-producing cells within lymph node correlated with plasma HIV-1 RNA concentration (Spearman ρ = 0.59; *p* = 0.035), suggesting that these cells are representative of the major virus-producing cells in the body. A median of 17% (range, 3–50%) of subjects' lymph node tissue consisted of lymphoid follicles, defined morphologically by anti-CD20 staining (17). A median of 60% (range, 29–87%) of HIV-1 RNA-producing cells as determined by in situ hybridization were located within lymphoid follicles. Frequencies of HIV-1 RNA-producing cells per mm<sup>2</sup> tissue were uniformly higher within lymphoid follicles compared with extrafollicular regions, as previously reported by our group (17). A mean of 0.69 (95% confidence interval (CI): 0.55, 0.87) HIV-1 RNA-producing cells per mm<sup>2</sup> tissue were detected within lymphoid follicles compared with 0.09 (95% CI: 0.05, 0.16) cells per mm<sup>2</sup> in extrafollicular regions of lymph node (*p* < 0.0001).

The number of CD4<sup>+</sup> cells per mm<sup>2</sup> lymph node tissue was calculated based upon immunohistochemical staining and estimates in each subject of the average area of a CD4<sup>+</sup> staining cell (Table III). The median density of CD4<sup>+</sup> cells in follicular regions was 21% (range, 15–50%) of that in extrafollicular regions of lymphoid tissue, and is consistent with the known anatomy of lymph nodes as well as previous reports from our laboratory (17). Based on prior observations that the vast majority of HIV-1-producing cells in lymphoid tissues in vivo are CD4<sup>+</sup> cells (15, 16, 27), the

Table III. Estimated densities of CD4<sup>+</sup> cells and frequencies of HIV-1-producing CD4<sup>+</sup> cells in lymph nodes of HIV-1-infected subjects ordered by ascending plasma viral load

Subject ID	Estimated Number of CD4 <sup>+</sup> Cells/mm <sup>2a</sup>			Estimated Number of HIV-1-Producing Cells/10 <sup>6</sup> CD4 <sup>+</sup> Cells <sup>b</sup>		
	Lymph node	Inside follicle	Outside follicle	Lymph node	Inside follicle	Outside follicle
61	13,890	3,685	16,310	16	594	3
64	7,335	1,470	9,713	12	382	2
82	6,254	1,558	7,350	24	391	7
32	7,825	1,769	9,822	13	220	1
84	5,476	1,171	6,333	13	123	8
80	5,739	1,524	6,995	35	286	14
86	2,563	1,379	2,766	123	414	79
92	9,170	2,054	11,185	17	307	8
81	4,408	1,340	4,973	104	1,644	40
62	10,304	4,180	11,602	5	261	2
87	5,178	1,414	6,468	101	590	33
67	8,085	2,282	10,019	44	617	16
79	3,999	1,009	5,052	120	1,265	50
90	3,822	1,127	4,575	84	922	30
Median	5,997	1,497	7,173	30	403	11

<sup>a</sup> The number of CD4<sup>+</sup> cells per mm<sup>2</sup> tissue was calculated by dividing the percentage of the tissue area that stained positively for CD4 by the average area of a CD4<sup>+</sup> staining cell in the same tissue section. Differences between follicular and extrafollicular tissues were statistically significant ( $p = 0.0001$ ).

<sup>b</sup> The number of HIV-1-producing CD4<sup>+</sup> T cells per 10<sup>6</sup> CD4<sup>+</sup> cells was determined by dividing the frequency of HIV-1-producing cells per mm<sup>2</sup> tissue as determined by in situ hybridization by the estimated number of CD4<sup>+</sup> T cells per mm<sup>2</sup> tissue. Differences between follicular and extrafollicular tissues were statistically significant ( $p = 0.0001$ ).

frequency of HIV-1-producing CD4<sup>+</sup> cells in follicular and extrafollicular regions of lymph node was estimated for each subject by combining the results from in situ hybridization for HIV-1 RNA with the results from the CD4<sup>+</sup> cell quantification (Table III). A median of 30 per 10<sup>6</sup> or 0.003% of lymph node CD4<sup>+</sup> cells were estimated to be producing HIV-1 RNA. Frequencies of infected CD4<sup>+</sup> cells were ~40 times higher in lymphoid follicles (median, 0.04% of CD4<sup>+</sup> cells) than in extrafollicular regions (median, 0.001% of CD4<sup>+</sup> cells;  $p = 0.0001$ ).

#### Tetramer staining of PBMC and disaggregated lymph node cells

To characterize HIV-1-specific CTL in PBMC and disaggregated lymph node cells, flow cytometric assays were performed using 7 HIV-1 tetramers restricted by four different HLA molecules. A total of 37 tetramer-staining assays were performed on paired PBMC and disaggregated lymph node cells in the 14 subjects recruited to this study (Table IV). Tetramer-stained cells were detected in 15 PBMC assays and 16 disaggregated lymph node cell assays, and these were concordant in terms of the presence or absence of tetramer-stained cells in all instances in which results were available for both compartments. Tetramer-stained cells were detected by all tetramers used except for the A3 *gag* tetramer. Tetramer-stained cells were detected by the A3 *gag* tetramer and confirmed in functional assays in a patient not included in the present study (data not shown), demonstrating that the tetramer was not defective. Neither plasma HIV-1 RNA concentration nor frequencies of HIV-1 RNA-producing cells in lymph node were a significant predictor of percentage of lymph node tetramer-stained cells ( $p = 0.84$  and  $p = 0.31$ , respectively). The mean percentage of lymph node CD8<sup>+</sup> cells that stained positively with tetramers was 2.15% (95% CI: 1.20, 3.10) and was significantly higher than that in PBMC (mean, 1.52%; 95% CI: 0.54, 2.50) with a mean difference of 0.70% (95% CI: 0.16, 1.23;  $p = 0.02$ ).

Table IV. Tetramer staining of PBMC, disaggregated lymph node cells, and lymph node sections<sup>a</sup>

Subject ID	Epitope	% Tetramer <sup>+</sup> /CD8 <sup>+</sup> Cells		In Situ Tetramer Staining
		PBMC	Lymph node	
32	A3/ <i>gag</i>	Negative	Negative	Negative nd
	A3/ <i>nef</i>	Negative	Negative	
	B7/ <i>nef</i>	nd <sup>b</sup>	<b>0.2</b>	
61	A3/ <i>gag</i>	Negative	Negative	Negative
	A3/ <i>nef</i>	Negative	Negative	Negative
62	A2/ <i>gag</i>	<b>4.1</b>	<b>5.3</b>	<b>Positive</b>
	A2/ <i>pol</i>	Negative	Negative	Negative
64	A2/ <i>gag</i>	<b>2.7</b>	<b>4.8</b>	<b>Positive</b>
	A2/ <i>pol</i>	Negative	Negative	Negative
67	A2/ <i>gag</i>	Negative	Negative	Negative
	A2/ <i>pol</i>	Negative	Negative	Negative
	B7/ <i>nef</i> <sup>c</sup>	<b>0.4</b>	<b>1.9</b>	<b>Positive</b>
79	A2/ <i>gag</i>	<b>0.4</b>	<b>1.2</b>	<b>Positive</b>
	A2/ <i>pol</i>	<b>0.1</b>	<b>0.4</b>	nd
80	A3/ <i>gag</i>	Negative	Negative	Negative
	A3/ <i>nef</i>	Negative	Negative	Negative
	B8/ <i>gag</i>	<b>0.4</b>	<b>1.4</b>	nd
81	B8/ <i>nef</i>	Negative	Negative	Negative
	B7/ <i>nef</i> <sup>c</sup>	Negative	Negative	Negative
	B8/ <i>gag</i>	<b>0.8</b>	<b>1.0</b>	<b>Positive</b>
82	B8/ <i>nef</i>	<b>1.9</b>	<b>3.2</b>	<b>Positive</b>
	A2/ <i>gag</i>	Negative	Negative	Negative
84	A2/ <i>pol</i>	<b>0.4</b>	<b>0.8</b>	Negative
	B7/ <i>nef</i> <sup>c</sup>	Negative	Negative	Negative
86	A3/ <i>gag</i>	Negative	Negative	Negative
	A3/ <i>nef</i>	<b>2.6</b>	<b>3.6</b>	nd
	B7/ <i>nef</i>	<b>1.0</b>	<b>2.1</b>	<b>Positive</b>
	A2/ <i>gag</i>	Negative	Negative	Negative
87	A2/ <i>pol</i>	Negative	Negative	Negative
	B7/ <i>nef</i> <sup>c</sup>	Negative	Negative	nd
90	B8/ <i>gag</i>	<b>0.7</b>	<b>0.7</b>	nd
	B8/ <i>nef</i>	<b>7.0</b>	<b>5.1</b>	nd
	A2/ <i>gag</i>	Negative	Negative	Negative
92	A2/ <i>pol</i>	<b>0.2</b>	<b>0.4</b>	Negative
	A3/ <i>gag</i>	Negative	Negative	Negative
	A3/ <i>nef</i>	<b>0.1</b>	<b>0.2</b>	nd
	B7/ <i>nef</i> <sup>c</sup>	Negative	Negative	Negative

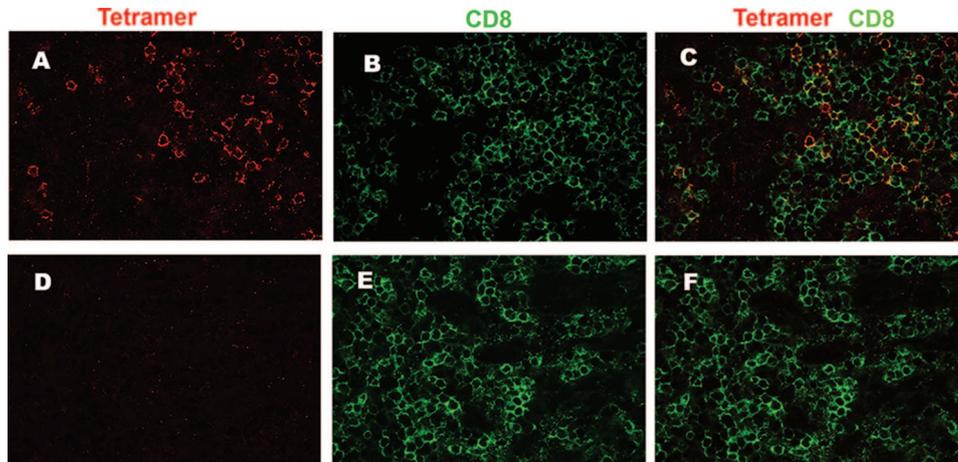
<sup>a</sup> Positive responses are highlighted in bold type.

<sup>b</sup> nd indicates that an assay was not done due to insufficient amounts of tissues or cells, or shipping problems.

<sup>c</sup> Subject was tested for B7/*nef*-specific cells because screening Ab tests with the B7 Ab were positive, but HLA B7 was not confirmed on molecular HLA typing (Table II).

#### In situ tetramer staining of lymph nodes

To determine the spatial localization of HIV-1-specific CD8<sup>+</sup> T cells in lymph nodes of HIV-1-infected subjects, in situ tetramer staining was performed on fresh lymph node sections. HIV-1-specific cells were detected within intact lymph node specimens by in situ tetramer staining in 7 of 13 subjects analyzed (Table IV). In situ tetramer-stained sections that were counterstained with CD8 Abs showed that most tetramer-stained cells coexpressed CD8 (Fig. 1, A–C). Negative controls for the in situ tetramer staining included sections stained with tetramers loaded with irrelevant peptides and HLA-mismatched patient tissue stained with tetramers loaded with HIV peptides, and results were similar to that seen in tissues from patients that did not show detectable tetramer staining (Fig. 1, D–F). Positive results from in situ tetramer staining were confirmed in all instances by tetramer staining of disaggregated lymph node cells (Table IV). In three instances, tetramer-stained cells were detected in disaggregated lymph node cells, but in situ studies did not demonstrate tetramer staining above background levels. The percentages of tetramer-stained cells in these



**FIGURE 1.** HLA class I tetramer (red; *left panels*) and CD8 (green; *middle panels*) staining and merged images (*right panels*) in lymph node sections from two HIV-1-infected individuals. Lymph node from subject 62 (A–C) stained with A2 *gag* tetramer and CD8 Ab demonstrated coexpression of CD8 by the majority of tetramer-stained cells, as shown in the merged image (C). Lymph node from subject 67 (D–F) stained with A2 *gag* tetramer and CD8 Ab demonstrated no evidence of tetramer-stained cells, although CD8<sup>+</sup> cells were present. Images are confocal Z-scans collected 9  $\mu$ m into the tissue sections with a  $\times 60$  objective using the same confocal parameters.

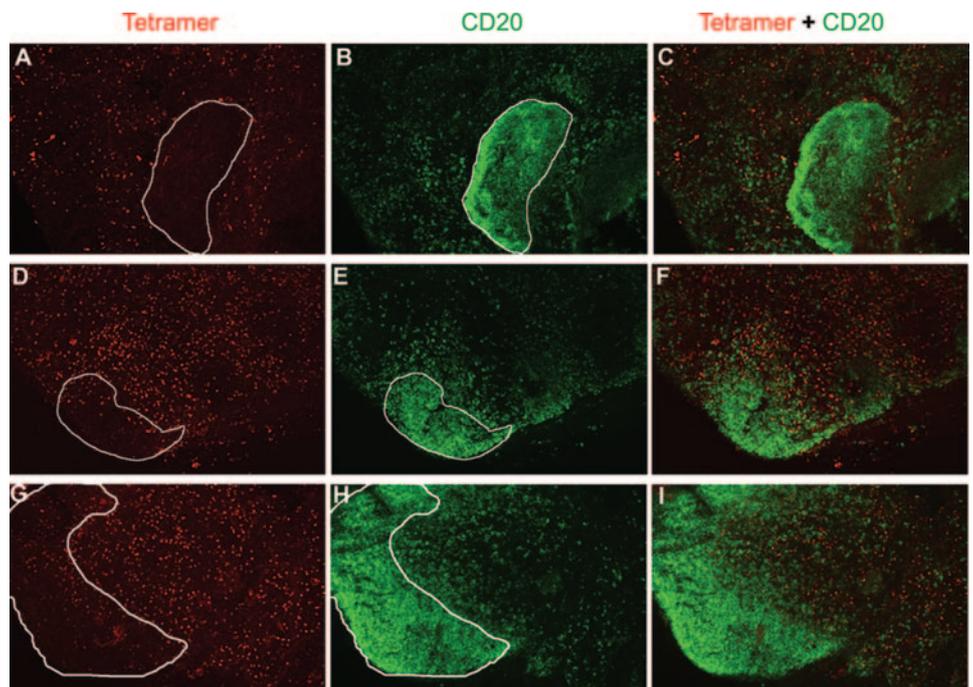
three cases were  $<1\%$  of CD8<sup>+</sup> cells, suggesting that in situ tetramer staining is less sensitive than flow cytometric analysis of disaggregated lymph node cells.

To characterize the distribution of HIV-1-specific CTL within and outside of lymphoid follicles, double staining with anti-CD20 Abs and tetramers was performed. Tissues from three subjects showed abundant tetramer-stained cells as well as multiple follicles within stained tissue sections and were used for this analysis. Subjects 62 and 64 harbored tetramer-stained cells against an HLA A\*0201-restricted *gag* epitope, whereas subject 81 harbored tetramer-stained cells against an HLA B8-restricted *nef* epitope. Substantially, fewer tetramer-stained cells were localized inside follicles compared with extrafollicular regions in lymph node sections from these subjects (Fig. 2). Many follicles were almost entirely devoid of any tetramer-stained cells (Fig. 2, A–C). In follicles that showed some tetramer-stained cells, the tetramer-stained cells fre-

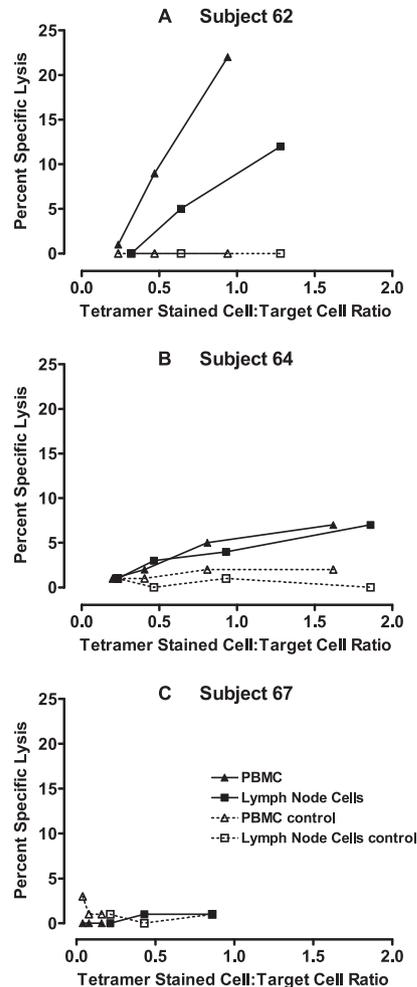
quently localized near one edge of the follicle with the remaining areas of the follicle devoid of tetramer-stained cells (Fig. 2, D–F).

#### *HIV-1 epitope-specific cytolytic activity mediated by tetramer-stained cells*

To evaluate the cytolytic function of tetramer-stained cells, HIV-1 epitope-specific cytotoxicity was assessed in <sup>51</sup>Cr release assays using PBMC and disaggregated lymph node cells and autologous B-LCL pulsed with HIV-1 peptides (Fig. 3). These assays were performed in three subjects known to harbor tetramer stained cells and for whom autologous B-LCL were available at the time of lymph node excision. Subjects 62 and 64 both had tetramer-stained cells directed against the A2 *gag* epitope, and subject 67 had tetramer-stained cells directed against the B7-restricted *nef* epitope (Table IV). Controls for these assays consisted of autologous B-LCL pulsed with an irrelevant A2-restricted melanoma peptide for



**FIGURE 2.** HLA class I tetramer (red; *left panels*) and CD20 (green; *middle panels*) staining and merged images (*right panels*) in representative lymph node sections from three HIV-1-infected individuals. Lymphoid follicles, defined morphologically by CD20 staining, are outlined in white. Lymph node sections from subject 64 (A–C) and subject 62 (D–F) were stained with A2 *gag* tetramer and lymph node sections from subject 81 (G–I) were stained with B8 *nef* tetramer. Lymph node sections from each subject showed markedly fewer tetramer-stained cells localized within follicles compared with extrafollicular regions. Images are confocal Z-scans collected using a  $\times 10$  objective.



**FIGURE 3.** Cytolysis mediated by PBMC (triangles) and lymph node cells (squares) against B-LCL pulsed with epitopic peptides (closed symbols) or controls (open symbols) from three HIV-1-infected subjects. PBMC and lymph node cells from subjects 62 (A) and 64 (B) demonstrated concentration-dependent cytolysis against autologous B-LCL pulsed with an A2-restricted *gag* peptide compared with autologous B-LCL pulsed with an irrelevant peptide. Neither PBMC nor lymph node cells from subject 67 (C) demonstrated cytolysis against a B7-restricted *nef* peptide compared with autologous B-LCL alone, although tetramer-to-target cell ratios tested were much lower in this subject than in the previous two subjects.

the A2 *gag* assays, or B-LCL alone for the B7 *nef* assays. As shown in Fig. 3, A and B, concentration-dependent cytolytic activity was mediated by the PBMC and lymph node cells of both subjects 62 and 64 against autologous B-LCL pulsed with the HIV *gag* peptide at tetramer-to-target cell ratios between 1:1 and 2:1, but not against autologous B-LCL pulsed with an irrelevant melanoma peptide. Subject 67 (Fig. 3C) did not demonstrate any significant cytolysis of either HIV-1 peptide-pulsed target cells or control cells. However, in subject 67 the tetramer-stained cell-to-target cell ratios were all <1:1, and therefore too low to determine whether these cells were capable of mediating cytolytic activity comparable to the tetramer-stained cells of the other two subjects. Importantly, in vitro studies of HIV-1-specific CTL clones usually use substantially higher E:T cell ratios, often as much as 10:1 or 20:1 to detect cytolytic activity (2, 28). Thus, cytolytic activity was detected in lymph node cells at low E:T cell ratios in the present study and there was no evidence of gross cytolytic dysfunction.

## Discussion

The inability of HIV-1-specific CTL to fully suppress virus replication as well as the failure of administration of exogenous CTL to produce declines in plasma viral load have never been adequately explained. We evaluated the magnitude and distribution of HIV-1-specific CTL detected by tetramers within lymph nodes of untreated, chronically HIV-1-infected individuals to assess the hypothesis that HIV-1-specific CTL fail to localize at sites of virus replication, which could explain these perplexing findings. The present study confirms previous observations that HIV-1 replication is concentrated primarily within lymphoid follicles (17–19, 29, 30) and provides the first estimates of the percentage of CD4<sup>+</sup> cells that are productively infected within these two compartments. Tetramer-stained cells were abundant, constituting >5% of lymph node CD8<sup>+</sup> cells in some cases, and were a significantly larger fraction of lymph node than PBMC CD8<sup>+</sup> cells. In situ analyses revealed that tetramer-stained cells failed to accumulate within lymphoid follicles where virus replication was concentrated, but were located primarily in the extrafollicular regions of lymph node. These findings confirm and extend our previous observations that multiple antiretroviral effector proteins are relatively deficient within lymphoid follicles compared with extrafollicular regions of lymph node (17) and lend credence to the hypothesis that a deficiency in immune responses in lymphoid follicles promotes virus replication at those sites.

Between 0.0005 and 0.01% of lymph node CD4<sup>+</sup> cells were estimated to be productively infected in the present study. These estimates were based upon prior observations that the vast majority of productively infected cells within lymphoid tissues during asymptomatic HIV-1 infection are CD4<sup>+</sup> cells (27) and are similar to estimates previously reported in the literature for individuals at this stage of disease (31, 32). A median of 0.04% of follicular CD4<sup>+</sup> cells were estimated to harbor HIV-1 RNA, ~40-fold more than the percentage of HIV-1 RNA-producing CD4<sup>+</sup> cells in extrafollicular tissues. To our knowledge, this is the first study to quantify the percentage of productively infected CD4<sup>+</sup> cells within lymphoid tissue subcompartments and further strengthens the observation that HIV-1 replication is concentrated primarily within lymphoid follicles of lymphoid tissues (17–19, 29, 30). A previous study found that 59% of the HIV-1 RNA-producing cells in lymphoid tissues in early stages of HIV-1 infection express HLA-DR and 43% express Ki67 (33), which are markers of cellular activation and proliferation, respectively. The phenotype of follicular CD4<sup>+</sup> cells that are replicating HIV-1 has never been reported. It is probable, however, that a significant fraction of productively infected follicular CD4<sup>+</sup> cells express Ki67 and HLA-DR, because the majority of HIV-1-producing cells are within lymphoid follicles and follicular dendritic cells previously have been shown to induce activation and proliferation of CD4<sup>+</sup> T cells (34). The presence of large amounts of highly infectious HIV-1 virions on the surface of follicular dendritic cells (31, 35), as well as the ability of follicular dendritic cells to activate and induce proliferation of CD4<sup>+</sup> T cells are likely key determinants of the concentration of HIV-1 RNA production within lymphoid follicles.

We demonstrated in the present study that tetramer-stained cells were more concentrated in lymph node than peripheral blood, consistent with a previous study that observed higher concentrations of HIV-1 tetramer-stained cells in gut-associated lymphoid tissues than PBMC (36, 37). However, another earlier study did not detect significant differences between concentrations of tetramer-stained cells in PBMC and peripheral lymph node cells (24). It is unclear whether the discrepancy in results between the previous lymph

node study and the present one relates to technical differences in how tetramer-stained cells were measured, or whether there were clinical differences among subjects. The present study, nonetheless, adds to the literature demonstrating that HIV-1-specific CD8<sup>+</sup> cells readily accumulate within lymphoid tissues of HIV-1-infected individuals and provides persuasive evidence against the hypothesis that HIV-1-specific cells are selectively excluded from lymphoid tissues (38).

It has been suggested that ongoing virus replication in untreated HIV-1-infected individuals is due to dysfunction of virus-specific CTL (39). Virus-specific IFN- $\gamma$  responses do not consistently reflect cytolytic capabilities (40) and HIV-1-specific tetramer-stained cells demonstrate impairments of cytokine release (41) and cytolytic function (25). Furthermore, a failure of perforin to be coexpressed with granzyme A within lymphoid tissues of HIV-1-infected individuals has been interpreted as evidence of a cytolytic defect (42). In the present study, we evaluated HIV-1-specific activity using conventional <sup>51</sup>Cr release assays rather than IFN- $\gamma$  responses or immunohistochemical staining for perforin and granzyme A because, although <sup>51</sup>Cr release assays are substantially less sensitive, they definitively demonstrate cytolytic capability. We observed epitope-specific, concentration-dependent cytolytic activity in lymph node cells and PBMC from two of three subjects assayed at quite low tetramer-to-target cell ratios. These data are consistent with a previous report of cytolytic activity mediated by spleen and tonsil cells from two HIV-1-infected individuals (43). Collectively, these data do not support the hypothesis that HIV-1-specific CD8<sup>+</sup> T cells in lymphoid tissues are grossly impaired in terms of cytolytic function.

Ours is the first study to characterize the distribution of endogenous HIV-1-specific CTL within lymphoid tissues. We found that HIV-1-specific CTL localized primarily in extrafollicular regions of lymph node, away from sites of virus replication, in all three subjects analyzed. A previous study that evaluated the spatial localization within lymph nodes of gene-marked CTL infused into three HIV-1-seropositive subjects also found that they localized primarily in extrafollicular regions (44), suggesting that trafficking of gene-marked CTL is similar to that of endogenous CTL. Although limited numbers of subjects were evaluated, the consistency of this finding in both studies strengthens the conclusion that HIV-1-specific CTL localize within lymphoid tissues, but fail to accumulate within lymphoid follicles.

The interaction of chemokines with their cognate receptors is thought to be the major determinant of localization of cells within lymphoid tissues resulting in distinct microenvironments. The chemokine receptor CCR7, which is found primarily on naive and central memory T lymphocytes (45), induces the migration of these cells from peripheral blood into lymph node and promotes their localization within the extrafollicular or T cell zones of lymph node where the CCR7 ligands, CCL19 and CCL21, are concentrated. Follicular CD4<sup>+</sup> T cells constitute a specialized subset of Th cells that provide critical signals to B cells through costimulatory molecules and cytokines that induce Ab class switching and proliferation (46, 47). Migration of this specialized subset of CD4<sup>+</sup> T cells into lymphoid follicles requires both up-regulation of CXCR5, the receptor for the B cell-attracting chemokine 1 (CXCL13), as well as down-regulation of CCR7 (48). Importantly, few CD8<sup>+</sup> T cells express CXCR5, suggesting differential regulation of this receptor in T cell subsets (48). We previously reported that CD8<sup>+</sup> T cells are primarily concentrated within extrafollicular regions of lymph node in both HIV-1-seropositive and -seronegative individuals (17) and limited CXCR5 expression on CD8<sup>+</sup> cells may explain this. Teleologically, it makes sense that lymphoid follicles might exclude CD8<sup>+</sup> T cells, because they

could interfere with the process of affinity maturation of Ab-producing cells. Thus, the failure of HIV-1-specific CD8<sup>+</sup> T cells to localize within lymphoid tissues likely results from the normal biology of the lymphoid tissue microenvironment and not a specific derangement induced by HIV-1.

The finding that HIV-1 replication is concentrated within lymphoid follicles, but that virus-specific CTL do not accumulate at those sites, indicates that lymphoid follicles are immune-privileged sites. The model of immune privilege within lymphoid follicles suggested by the findings of this study helps to explain why untreated individuals demonstrate ongoing HIV-1 replication despite large numbers of functional HIV-1-specific CTL. It also explains the paradox that depletion of CD8<sup>+</sup> T cells results in dramatic increases in plasma viral load (4, 5), but administration of exogenous CTL fails to produce significant declines in viral load (11–14), a paradox that cannot be explained by immune dysfunction. According to the model proposed here, endogenous CTL can never fully suppress HIV-1 replication because they do not accumulate in lymphoid follicles where HIV-1 replication is focused. Depletion of CD8<sup>+</sup> T cells from lymphoid tissue allows virus replication to spread to extrafollicular areas where the majority of CD4<sup>+</sup> T cells are located resulting in increased viral loads. The administration of exogenous CTL, however, fails to produce significant declines in virus replication because exogenous CTL are likewise unable to accumulate in lymphoid follicles and endogenous CTL are already highly effective in suppressing HIV-1 replication in the extrafollicular regions. This paradigm also explains why HIV-1-specific CD8<sup>+</sup> T cell responses do not correlate inversely with plasma viral load in the present study and other studies of lymphoid tissues (36, 37, 49–51), as well as the vast majority of studies of peripheral blood (6, 8, 52–55). In this model, the correlates of plasma HIV-1 RNA concentration would be factors that are present within the follicular milieu, such as chemokine receptor expression on host CD4<sup>+</sup> T cells (56) and virus replicative capacity (57), as has been observed. Because HIV-1-specific CD8<sup>+</sup> T cells are largely excluded from follicles, neither their magnitude nor their breadth would be expected to correlate with plasma HIV-1 concentrations and, by inference, to disease progression.

Several directions for future research are suggested by the finding that HIV-1-specific CTL fail to accumulate within lymphoid follicles. First, the most convincing evidence that lymphoid follicles evade immune surveillance by host CTL would be to demonstrate that redistribution of CTL to lymphoid follicles results in improved virus suppression. To accomplish this, it is essential to better understand the mechanisms that might induce migration of CTL into follicles. The failure of HIV-1-specific CD8<sup>+</sup> T cells to accumulate within lymphoid follicles reported in this study was relative, but not absolute, as some tetramer-staining cells were detected in those regions. Evaluation of chemokine receptor expression, particularly CXCR5, on those HIV-1-specific cells that were detected within lymphoid follicles and comparison to the chemokine receptor expression of tetramer-staining cells outside of follicles could provide important insight into the mechanisms that might be involved and suggest potential strategies to redirect CTL into follicles. Other pathogens that infect cells that normally reside within lymphoid follicles may exploit the immune privilege of lymphoid follicles as well. Indeed, we previously reported that in asymptomatic individuals coinfecting with Kaposi's sarcoma herpes virus (KSHV) and HIV-1, KSHV latency-associated nuclear Ag is found primarily in lymphoid follicles (58), indicating that KSHV also colonizes this niche. Investigation of the relative distribution of other chronic pathogens and pathogen-specific CTL within lymphoid tissues is critical to evaluate the hypothesis that

multiple pathogens exploit the immune privilege of lymphoid follicles. Ultimately, a better understanding of the mechanisms that underlie the exclusion of CTL from lymphoid follicles as well as the possible role of lymphoid follicles in other diseases could lead to novel immunotherapeutic strategies to redirect CTL to lymphoid follicles and improve control of HIV-1 and many other seemingly intractable chronic infections.

## Acknowledgments

We express our gratitude to the subjects who participated in this study. We also thank Karen Whalen for nursing assistance during surgical procedures; the NIAID Tetramer Facility for provision of tetramers; Leica Corporation for the loan of the microscope and quantitative image analysis equipment; the University of Colorado Center for AIDS Research Immunology Core for facilitation of the flow cytometry and in situ hybridization for HIV-1 RNA studies; and Steven Johnson, William Burman, Patty Caraway, Beverly Barber, Monica Carten, Beverly Putnam, Pat Gourley, Ron Schimmel, and Jeff Logan, for assistance in recruiting subjects to this study.

## Disclosures

The authors have no financial conflict of interest.

## References

- Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 1994. Virus-specific CD8<sup>+</sup> cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68: 6103–6110.
- Connick, E., D. G. Marr, X. Q. Zhang, S. J. Clark, M. S. Saag, R. T. Schooley, and T. J. Curiel. 1996. HIV-specific cellular and humoral immune responses in primary HIV infection. *AIDS Res. Hum. Retroviruses* 12: 1129–1140.
- Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68: 4650–4655.
- Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, et al. 1999. Control of viremia in simian immunodeficiency virus infection by CD8<sup>+</sup> lymphocytes. *Science* 283: 857–860.
- Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, et al. 1999. Dramatic rise in plasma viremia after CD8<sup>+</sup> T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* 189: 991–998.
- Ferbas, J., A. H. Kaplan, M. A. Hausner, L. E. Hultin, J. L. Matud, Z. Liu, D. L. Panicali, H. Nerng-Ho, R. Detels, and J. V. Giorgi. 1995. Virus burden in long-term survivors of human immunodeficiency virus (HIV) infection is a determinant of anti-HIV CD8<sup>+</sup> lymphocyte activity. *J. Infect. Dis.* 172: 329–339.
- Walker, B. D., S. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durmo, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch, and R. T. Schooley. 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature* 328: 345–348.
- Gray, C. M., J. Lawrence, J. M. Schapiro, J. D. Altman, M. A. Winters, M. Crompton, M. Loi, S. K. Kundu, M. M. Davis, and T. C. Merigan. 1999. Frequency of class I HLA-restricted anti-HIV CD8<sup>+</sup> T cells in individuals receiving highly active antiretroviral therapy (HAART). *J. Immunol.* 162: 1780–1788.
- Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
- Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279: 2103–2106.
- Koenig, S., A. J. Conley, Y. A. Brewah, G. M. Jones, S. Leath, L. J. Boots, V. Davey, G. Pantaleo, J. F. Demarest, C. Carter, et al. 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat. Med.* 1: 330–336.
- Lieberman, J., P. R. Skolnik, G. R. Parkerson III, J. A. Fabry, B. Landry, J. Bethel, and J. Kagan. 1997. Safety of autologous, ex vivo-expanded human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte infusion in HIV-infected patients. *Blood* 90: 2196–2206.
- Mitsuyasu, R. T., P. A. Anton, S. G. Deeks, D. T. Scadden, E. Connick, M. T. Downs, A. Bakker, M. R. Roberts, C. H. June, S. Jalali, et al. 2000. Prolonged survival and tissue trafficking following adoptive transfer of CD4ζ gene-modified autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cells in human immunodeficiency virus-infected subjects. *Blood* 96: 785–793.
- Brodie, S. J., D. A. Lewinsohn, B. K. Patterson, D. Jiyamapa, J. Krieger, L. Corey, P. D. Greenberg, and S. R. Riddell. 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat. Med.* 5: 34–41.
- Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* 362: 355–358.
- Embertson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* 362: 359–362.
- Folkvord, J. M., C. Armon, and E. Connick. 2005. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. *AIDS Res. Hum. Retroviruses* 21: 363–370.
- Spiegel, H., H. Herbst, G. Niedobitek, H. D. Foss, and H. Stein. 1992. Follicular dendritic cells are a major reservoir for human immunodeficiency virus type 1 in lymphoid tissues facilitating infection of CD4<sup>+</sup> T-helper cells. *Am. J. Pathol.* 140: 15–22.
- Tenner-Racz, K., and P. Racz. 1995. Follicular dendritic cells initiate and maintain infection of the germinal centers by human immunodeficiency virus. *Curr. Top. Microbiol. Immunol.* 201: 141–159.
- Folkvord, J. M., D. M. Anderson, J. Arya, S. MaWhinney, and E. Connick. 2003. Microanatomic relationships between CD8<sup>+</sup> cells and HIV-1-producing cells in human lymphoid tissue in vivo. *J. Acquir. Immune Defic. Syndr.* 32: 469–476.
- Oxenius, A., D. A. Price, P. J. Easterbrook, C. A. O'Callaghan, A. D. Kelleher, J. A. Whelan, G. Sontag, A. K. Sewell, and R. E. Phillips. 2000. Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes. *Proc. Natl. Acad. Sci. USA* 97: 3382–3387.
- Kostense, S., S. A. Otto, G. J. Knol, E. H. Manting, N. M. Nanlohy, C. Jansen, J. M. Lange, M. H. van Oers, F. Miedema, and D. van Baarle. 2002. Functional restoration of human immunodeficiency virus and Epstein-Barr virus-specific CD8<sup>+</sup> T cells during highly active antiretroviral therapy is associated with an increase in CD4<sup>+</sup> T cells. *Eur. J. Immunol.* 32: 1080–1089.
- Goulder, P. J., Y. Tang, C. Brander, M. R. Betts, M. Altfeld, K. Annamalai, A. Trocha, S. He, E. S. Rosenberg, G. Ogg, et al. 2000. Functionally inert HIV-specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children. *J. Exp. Med.* 192: 1819–1832.
- Ellefsen, K., A. Harari, P. Champagne, P. A. Bart, R. P. Sekaly, and G. Pantaleo. 2002. Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections. *Eur. J. Immunol.* 32: 3756–3764.
- Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, et al. 2000. HIV-specific CD8<sup>+</sup> T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* 192: 63–75.
- Skinner, P. J., M. A. Daniels, C. S. Schmidt, S. C. Jameson, and A. T. Haase. 2000. Cutting edge: in situ tetramer staining of antigen-specific T cells in tissues. *J. Immunol.* 165: 613–617.
- Schacker, T., S. Little, E. Connick, K. Gebhard, Z. Q. Zhang, J. Krieger, J. Pryor, D. Havlir, J. K. Wong, R. T. Schooley, et al. 2001. Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection. *J. Infect. Dis.* 183: 555–562.
- Yang, O. O., P. T. Sarkis, A. Trocha, S. A. Kalams, R. P. Johnson, and B. D. Walker. 2003. Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL. *J. Immunol.* 171: 3718–3724.
- Tenner-Racz, K., H. J. Stellbrink, J. van Lunzen, C. Schneider, J. P. Jacobs, B. Raschdorff, G. Grosschupff, R. M. Steinman, and P. Racz. 1998. The unenlarged lymph nodes of HIV-1-infected, asymptomatic patients with high CD4 T cell counts are sites for virus replication and CD4 T cell proliferation: the impact of highly active antiretroviral therapy. *J. Exp. Med.* 187: 949–959.
- Hufert, F. T., J. van Lunzen, G. Janosy, S. Bertram, J. Schmitz, O. Haller, P. Racz, and D. von Laer. 1997. Germinal centre CD4<sup>+</sup> T cells are an important site of HIV replication in vivo. *AIDS* 11: 849–857.
- Haase, A. T. 1999. Population biology of HIV-1 infection: viral and CD4<sup>+</sup> T cell demographics and dynamics in lymphatic tissues. *Annu. Rev. Immunol.* 17: 625–656.
- Zhang, Z. Q., D. W. Notermans, G. Sedgewick, W. Cavert, S. Wietgrefe, M. Zupancic, K. Gebhard, K. Henry, L. Boies, Z. Chen, et al. 1998. Kinetics of CD4<sup>+</sup> T cell repopulation of lymphoid tissues after treatment of HIV-1 infection. *Proc. Natl. Acad. Sci. USA* 95: 1154–1159.
- Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, et al. 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4<sup>+</sup> T cells. *Science* 286: 1353–1357.
- Burton, G. F., B. F. Keele, J. D. Estes, T. C. Thacker, and S. Gartner. 2002. Follicular dendritic cell contributions to HIV pathogenesis. *Semin. Immunol.* 14: 275–284.
- Heath, S. L., J. G. Tew, J. G. Tew, A. K. Szakal, and G. F. Burton. 1995. Follicular dendritic cells and human immunodeficiency virus infectivity. *Nature* 377: 740–744.
- Altfeld, M., J. van Lunzen, N. Frahm, X. G. Yu, C. Schneider, R. L. Eldridge, M. E. Feeney, D. Meyer-Olson, H. J. Stellbrink, and B. D. Walker. 2002. Expansion of pre-existing, lymph node-localized CD8<sup>+</sup> T cells during supervised treatment interruptions in chronic HIV-1 infection. *J. Clin. Invest.* 109: 837–843.
- Shacklett, B. L., C. A. Cox, J. K. Sandberg, N. H. Stollman, M. A. Jacobson, and D. F. Nixon. 2003. Trafficking of human immunodeficiency virus type 1-specific CD8<sup>+</sup> T cells to gut-associated lymphoid tissue during chronic infection. *J. Virol.* 77: 5621–5631.
- Chen, G., P. Shankar, C. Lange, H. Valdez, P. R. Skolnik, L. Wu, N. Manjunath, and J. Lieberman. 2001. CD8 T cells specific for human immunodeficiency virus,

- Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. *Blood* 98: 156–164.
39. Lieberman, J., P. Shankar, N. Manjunath, and J. Andersson. 2001. Dressed to kill: a review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. *Blood* 98: 1667–1677.
  40. Betts, M. R., and R. A. Koup. 2004. Detection of T-cell degranulation: CD107a and b. *Methods Cell Biol.* 75: 497–512.
  41. Goepfert, P. A., A. Bansal, B. H. Edwards, G. D. Ritter, Jr., I. Tellez, S. A. McPherson, S. Sabbaj, and M. J. Mulligan. 2000. A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce  $\gamma$  interferon. *J. Virol.* 74: 10249–10255.
  42. Andersson, J., H. Behbahani, J. Lieberman, E. Connick, A. Landay, B. Patterson, A. Sonnerborg, K. Lore, S. Uccini, and T. E. Fehniger. 1999. Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection. *AIDS* 13: 1295–1303.
  43. Hosmalin, A., A. Samri, M. J. Dumaurier, Y. Dudoit, E. Oksenhendler, M. Karmochkine, B. Autran, S. Wain-Hobson, and R. Cheynier. 2001. HIV-specific effector cytotoxic T lymphocytes and HIV-producing cells colocalize in white pulps and germinal centers from infected patients. *Blood* 97: 2695–2701.
  44. Brodie, S. J., B. K. Patterson, D. A. Lewinsohn, K. Diem, D. Spach, P. D. Greenberg, S. R. Riddell, and L. Corey. 2000. HIV-specific cytotoxic T lymphocytes traffic to lymph nodes and localize at sites of HIV replication and cell death. *J. Clin. Invest.* 105: 1407–1417.
  45. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
  46. Akiba, H., K. Takeda, Y. Kojima, Y. Usui, N. Harada, T. Yamazaki, J. Ma, K. Tezuka, H. Yagita, and K. Okumura. 2005. The role of ICOS in the CXCR5<sup>+</sup> follicular B helper T cell maintenance in vivo. *J. Immunol.* 175: 2340–2348.
  47. Kim, J. R., H. W. Lim, S. G. Kang, P. Hillsamer, and C. H. Kim. 2005. Human CD57<sup>+</sup> germinal center-T cells are the major helpers for GC-B cells and induce class switch recombination. *BMC Immunol.* 6: 3.
  48. Schaerli, P., K. Willmann, A. B. Lang, M. Lipp, P. Loetscher, and B. Moser. 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* 192: 1553–1562.
  49. Kuroda, M. J., J. E. Schmitz, W. A. Charini, C. E. Nickerson, C. I. Lord, M. A. Forman, and N. L. Letvin. 1999. Comparative analysis of cytotoxic T lymphocytes in lymph nodes and peripheral blood of simian immunodeficiency virus-infected rhesus monkeys. *J. Virol.* 73: 1573–1579.
  50. Kuroda, M. J., J. E. Schmitz, A. Seth, R. S. Veazey, C. E. Nickerson, M. A. Lifton, P. J. Dailey, M. A. Forman, P. Racz, K. Tenner-Racz, and N. L. Letvin. 2000. Simian immunodeficiency virus-specific cytotoxic T lymphocytes and cell-associated viral RNA levels in distinct lymphoid compartments of SIVmac-infected rhesus monkeys. *Blood* 96: 1474–1479.
  51. Moniuszko, M., D. Bogdan, R. Pal, D. Venzon, L. Stevceva, J. Nacsa, E. Trynieszewska, Y. Edghill-Smith, S. M. Wolinsky, and G. Franchini. 2005. Correlation between viral RNA levels but not immune responses in plasma and tissues of macaques with long-standing SIVmac251 infection. *Virology* 333: 159–168.
  52. Addo, M. M., X. G. Yu, A. Rathod, D. Cohen, R. L. Eldridge, D. Strick, M. N. Johnston, C. Corcoran, A. G. Wurcel, C. A. Fitzpatrick, et al. 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J. Virol.* 77: 2081–2092.
  53. Altfeld, M., E. S. Rosenberg, R. Shankarappa, J. S. Mukherjee, F. M. Hecht, R. L. Eldridge, M. M. Addo, S. H. Poon, M. N. Phillips, G. K. Robbins, et al. 2001. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J. Exp. Med.* 193: 169–180.
  54. Kalams, S. A., S. P. Buchbinder, E. S. Rosenberg, J. M. Billingsley, D. S. Colbert, N. G. Jones, A. K. Shea, A. K. Trocha, and B. D. Walker. 1999. Association between virus-specific cytotoxic T-lymphocyte and helper responses in human immunodeficiency virus type 1 infection. *J. Virol.* 73: 6715–6720.
  55. Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* 75: 11983–11991.
  56. Reynes, J., P. Portales, M. Segondy, V. Baillat, P. Andre, B. Reant, O. Avinens, G. Couderc, M. Benkirane, J. Clot, et al. 2000. CD4<sup>+</sup> T cell surface CCR5 density as a determining factor of virus load in persons infected with human immunodeficiency virus type 1. *J. Infect. Dis.* 181: 927–932.
  57. Campbell, T. B., K. Schneider, T. Wrin, C. J. Petropoulos, and E. Connick. 2003. Relationship between in vitro human immunodeficiency virus type 1 replication rate and virus load in plasma. *J. Virol.* 77: 12105–12112.
  58. Campbell, T. B., K. A. Staskus, J. Folkvord, I. E. White, J. Neid, X. Q. Zhang, and E. Connick. 2005. Persistence of Kaposi sarcoma-associated herpesvirus (KSHV)-infected cells in KSHV/HIV-1-coinfected subjects without KSHV-associated diseases. *J. Infect. Dis.* 191: 367–371.