Expansion of pre-existing, lymph node-localized CD8+ T cells during supervised treatment interruptions in chronic HIV-1 infection

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To date, most studies have focused on the characterization of HIV-1–specific cellular immune responses in the peripheral blood (PB) of infected individuals. Much less is known about the comparative magnitude and breadth of responses in the lymphoid tissue. This study analyzed HIV-1–specific CD8+ T cell responses simultaneously in PB and lymph nodes (LNs) of persons with chronic HIV-1 infection and assessed the dynamics of these responses during antiretroviral treatment and supervised treatment interruption (STI). In untreated chronic infection, the magnitude of epitope-specific CD8+ T cell activity was significantly higher in LNs than in PB. Responses decreased in both compartments during highly active antiretroviral therapy, but this decline was more pronounced in PB. During STI, HIV-1–specific CD8+ T cell responses in PB increased significantly. Enhancement in breadth and magnitude was largely due to the expansion of pre-existing responses in the LNs, with new epitopes infrequently targeted. Taken together, these data demonstrate that HIV-1–specific CD8+ T cells are preferentially located in the LNs, with a subset of responses exclusively detectable in this compartment. Furthermore, the enhanced CD8+ T cell responses observed during STI in chronically infected individuals is largely due to expansion of pre-existing virus-specific CD8+ T cells, rather than the induction of novel responses.


Introduction

Since the first clinical evidence of AIDS was reported two decades ago, HIV-1 has killed over 20 million people worldwide, and an estimated 40 million people are infected with HIV-1. The development of highly active antiretroviral therapy (HAART) has led to substantial advances in the management of HIV-1–infected individuals. However, this therapy is limited due to resistance development, toxicity, high costs, and difficulties with delivery in resource-poor areas. The majority of infected individuals who live in sub-Saharan Africa and Southeast Asia have no access to antiretroviral treatment. Alternative therapeutic approaches, such as immunization or immunotherapeutic interventions, are therefore needed to control the rapidly spreading HIV-1 epidemic worldwide.

The basis for the rational development of immunotherapeutic interventions is a detailed understanding of HIV-1 pathogenesis and the correlates of protective immunity. Accumulating data have shown a central role for HIV-1–specific cytotoxic T lymphocytes (CTLs) and T helper cells in controlling viral replication (1–8). Studies in the SIV/macaque model provide compelling data in support of this, showing that removal of CD8+ T cells (and therefore CTLs) in vivo results in a rapid increase in the steady-state level of SIV viremia (9, 10). Recent studies using supervised treatment interruption (STI) as an immunotherapeutic intervention have shown that HIV-1–specific cellular immune responses can be enhanced in infected individuals (11–17). In individuals treated during acute HIV-1 infection, this enhancement of HIV-1–specific CTL responses was associated with at least transient immune control of viremia (12). All these studies have focused on the characterization of HIV-1–specific immune responses in the peripheral blood (PB) of infected individuals.

Much less is known about the role of virus-specific cellular immune responses in the lymphoid tissues, which harbor more than 95% of the body’s lymphocytes and
represent the major sites of virus replication and primary and secondary immune responses (18). In HIV-1-infected individuals, massive infiltration of secondary lymphoid organs with activated CD8+ T cells has been reported (19–23). HIV-1–specific CD8+ T cells colocalize with HIV-1–producing cells in the lymphoid tissue and may mediate antiviral activity (24–26). However, neither the relationship between virus-specific CTL responses in the lymphoid tissue and the PB in untreated infection, nor the dynamics of CTL responses in these two compartments during immunotherapeutic interventions, is known.

In this study, we assessed virus-specific CD8+ T cell responses in the PB and lymph nodes (LNs) of HIV-1-infected individuals at the single-epitope level. Responses in these two compartments were compared during untreated chronic infection, and the impact of antiretroviral treatment and STI on these CD8+ T cell responses was determined in longitudinal studies.

Methods
Study subjects. Fifteen asymptomatic, chronically HIV-1–infected Caucasian males who were naive to antiretroviral therapy were enrolled in this study. For all studied individuals, matched PBMCs and LNMCs from the same timepoint were available for analysis prior to initiation of highly active antiretroviral treatment (HAART). At the time of the first analysis of HIV-1–specific immune responses, mean viral load was 318,767 ± 685,699 copies HIV-1 RNA/ml, and mean CD4+ T cell counts were 562 cells/µl in the study subjects. Second LNMC and PBMC samples were available from five of the 15 individuals after 12 months of treatment with HAART. All patients had undetectable viremia (less than 25 HIV-1 RNA copies/ml at the time of the second LN biopsy. In situ hybridization revealed no or minimal residual HIV-1 RNA in LNs (27). The mean CD4+ T cell count at that time had increased from 562 cells/µl before therapy to 931 cells/µl. Six of the 15 study subjects, including the five individuals who gave a second LNMC sample, underwent a single STI. All six experienced viral load rebound within 1.5 to 6 weeks (median 3 weeks) to pretreatment levels (447,438 ± 910,960 copies HIV-1 RNA/ml before treatment vs. 227,750 ± 366,923 copies/ml after STI, P = 0.54). A third PBMC sample from these individuals was available for analysis at peak viremia after STI (median 4 weeks). HLA class I typing was performed using flow cytometric analyses after staining with the corresponding mAb.

Preparation of cells. Axillary LN biopsies were performed under local anesthesia after written informed consent. LN samples were placed in normal saline at 4°C and immediately transferred to the laboratory for further processing. Mononuclear cells were mechanically liberated from lymphoid tissue, and LNMCs were frozen in FCS plus 10% DMSO. Fresh PBMCs were separated from PB by density gradient centrifugation using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Missouri, USA) and frozen in FCS plus 10% DMSO.

Synthetic HIV-1 peptides. Peptides corresponding to described optimal HIV-1 clade B CTL epitopes (28) were synthesized on an automated peptide synthesizer (model 396 MBS and Omega; Advance ChemTech, Louisville, Kentucky, USA). A total of 107 different optimal CTL epitopes described for the HLA class I alleles expressed in the study cohort were tested, including epitopes contained within all HIV-1 proteins except HIV-1 Vpu. Each individual was tested for responses directed against all optimal CTL epitopes described for his corresponding HLA class I type (28).

ELISpot assay. HIV-1–specific CD8+ T-cell responses were quantified by ELISpot assay as previously described (29, 30). In brief, cells were thawed and incubated for 8–10 hours in R10 medium (RPMI 1640 medium plus 10% FCS) at 37°C in 5% CO2. After counting, viable cells were plated out at 50,000 to 100,000 PBMCs or LNMCs per well, with peptides at a final concentration of 10⁻⁹ M in 96-well polyvinylidene difluoride-backed plates (MAIP S45; Millipore Corp., Bedford, Massachusetts, USA). Plates were precoated with 0.5 µg/ml anti–IFN-γ mAb (1-DIK; Mabtech AB, Stockholm, Sweden) overnight at 4°C. For negative controls, 100,000 PBMCs and LNMCs were incubated with R10 alone. The plates were incubated overnight (14–16 hours) at 37°C in 5% CO2 and washed. Next, biotinylated anti–IFN-γ mAb 7-B6-1 (Mabtech AB) was added at 0.5 µg/ml and incubated at room temperature for 90 minutes. After washing, 100 µl of streptavidin-conjugated alkaline phosphatase (diluted 1:2,000; Mabtech AB) was added per well, and incubation continued at room temperature for 45 minutes. Individual IFN-γ-producing cells were detected as dark spots after a 20- to 30-minute color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad Laboratories, Hercules, California, USA). The number of specific IFN-γ-secreting T cells was counted by direct visualization, calculated by subtracting the negative-control value, and expressed as spot-forming cells (SFCs) per million input cells. Negative controls always had less than 30 SFCs per million input cells. In addition, for each studied sample, the percentage of CD4+ and CD8+ T cells was determined by flow cytometry, and magnitudes of responses were normalized to SFCs per million CD8+ T cells.

Flow-cytometric detection of antigen-induced intracellular IFN-γ-secretion. Intracellular cytokine staining assays were performed as described previously, with minor modifications (7, 31, 32). Briefly, 0.5 × 10⁶ to 1 × 10⁶ PBMCs or LNMCs were incubated with 4 µM peptide and 1 µg/ml of each of the anti-CD28 and anti-CD49d mAb’s (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) at 37°C in 5% CO2 for 1 hour, before the addition of 10 µg/ml of brefeldin A (Sigma Chemical Co.). Following a further 6-hour incubation at 37°C in 5% CO2, the cells were placed at 4°C overnight. Cells were then washed and stained with the surface Ab’s anti-CD8-PerCP and anti-CD4-APC (Becton Dickinson Immunocytometry Systems) at 4°C for 30 minutes.
CD8+ T cells were significantly lower in the LN samples to address potential differences in HIV-1–specific CD8+ untreated individuals with chronic HIV-1 infection. SFCs/million LNMCs, SFCs/million PBMCs compared with 1,114 ± 756 vs. 50.3% ± 9.5% CD8+ PBMCs, 1,938 ± 1,766 SFCs/million CD8+ PBMCs, than in the PB samples (38.1% ± 11.9% CD8+ LNMCs, 5–32). In each case, the same epitopes were tested using LNCs and PBMCs.

HIV-1–specific CD8+ T cell responses were detectable in all 15 individuals in PBMCs and LNCs. Magnitudes of total HIV-1–specific CD8+ T cell responses were similar in PBMCs and LNCs (1,020 ± 1,011 SFCs/million PBMCs compared with 1,114 ± 756 SFCs/million LNCs, P = 0.8). As the percentages of CD8+ T cells were significantly lower in the LN samples than in the PB samples (38.1% ± 11.9% CD8+ LNCs vs. 50.3% ± 9.5% CD8+ PBMCs, P < 0.01), the normalized magnitude of total HIV-1–specific responses per million CD8+ T cells was significantly higher in LNs than in PB (3,293 ± 2,467 SFCs/million CD8+ LNCs vs. 1,938 ± 1,766 SFCs/million CD8+ PBMCs, P < 0.01).

At the single CTL epitope level, HIV-1–specific CD8+ T cells in PB correlated significantly to responses in LNs, whether analyzed per number of total input cells (Figure 1a) or per number of input CD8+ T cells (Figure 1b). These correlations remained significant when limited to the 68 epitopes recognized in both LN and PB samples (r = 0.38, P = 0.0014 for total input cells; r = 0.38, P = 0.0015 after normalization to input CD8+ T cells). As described for total HIV-1–specific CTL responses, the magnitude of epitope-specific responses per million CD8+ T cells was significantly higher in LNs than in PB (Figure 2a), but the breadth of responses was similar in both compartments (Figure 2b). An average of four optimal CTL epitopes (median 5, range 1–9) per individual was targeted in the PB, compared with an average of five epitopes (median 5, range 1–9) in the LNs (P = 0.5). The hierarchy in magnitude of responses to individual CTL epitopes within a single individual was similar in LNs and PB, as shown for four representative individuals in Figure 2c. However, of the 15 persons studied, five had detectable responses in the LN specimen that were undetectable in the PB sample. Another way to express this is that of the 68 epitope-specific responses detectable in the entire cohort, CD8+ T cell responses to a total of eight epitopes were detectable only in the LN specimen, but not in PB, as shown in Figure 1 and for individual B in Figure 2c. In contrast, all responses detected in the PB were also detected in the LN samples.

In a small subset of individuals (n = 3) in whom sufficient cells were available from PB and LNs, direct comparison of epitope-specific IFN-γ production of CD8+ T cells by intracellular cytokine staining also showed stronger CD8+ T cell responses in the LNCs than in PBMCs (data not shown), supporting the initial findings of stronger HIV-1–specific CD8+ T cell responses in the LNs. No significant correlation between the magnitude or breadth of HIV-1–specific CD8+ T cell responses and HIV-1 viral load was observed in the LNs or PB.

Taken together, these data indicate that the results of analysis of HIV-1–specific CD8+ T cells in the PB largely mirror the responses in the LNs. However, virus-specific CD8+ T cells appear to be enriched in the LNs, a major site of viral replication.
Impact of antiretroviral treatment on the dynamics of HIV-1–specific CD8+ T cell responses in LNs and PB. We next evaluated the impact of HAART on the magnitude and breadth of HIV-1–specific CD8+ T cell responses in LNs and PB. This analysis was performed on five of the 15 individuals described above for whom second LNMC and PBMC samples were available after 1 year of effective treatment with HAART. In all study subjects, plasma viral load was suppressed to below the level of detection (25 copies HIV-1 RNA/ml) on HAART. Treatment with HAART reduced HIV-1–specific CD8+ T cell responses in both compartments, but the decline in responses to individual CD8+ T cell epitopes was more pronounced in the PB (from 550 ± 661 to 227 ± 387 SFCs/million CD8+ PBMCs, \( P = 0.04 \)) than in the LNs (from 792 ± 949 to 479 ± 476 SFCs/million CD8+ LNMCs, \( P = 0.14 \)) (Figure 3). In addition, the number of detectable CTL epitopes declined in the PBMCs from an average of five epitopes per individual (median 5, range 3–6) to an average of two epitopes (median 1, range 1–6) after 1 year of treatment with HAART, representing a total loss of 13 of 25 (52%) previously detectable CD8+ T cell responses in the individuals studied (Figure 3a). In contrast, only five of 26 (19%) responses to previously detectable CTL epitopes were lost in the LNs on treatment, reducing the average number of epitopes targeted per individual from five (median 6, range 3–6) to four (median 5, range 2–6) (Figure 3b). The positive correlation between HIV-1–specific CD8+ T cell responses in PBMCs and LNMCs was maintained during treatment (\( r = 0.72, P < 0.0001 \) for total input cells and \( r = 0.72, P < 0.0001 \) after normalization for input CD8+ T cells).

Taken together, these studies demonstrate that a subset of epitope-specific CD8+ T cell responses is detectable in the LNs, but not in the PB. This was observed during chronic HIV-1 infection, prior to the initiation of treatment, for a total of eight of 68 detectable epitopes (12%) in the 15 individuals studied, and was even more pronounced (eight of 21 epitopes, or 38%) in the five individuals studied after 1 year of treatment. These data indicate that LNs may serve as a reservoir for virus-specific CD8+ T cell responses when antigen load is effectively suppressed during treatment with HAART.

Expansion of HIV-1–specific CD8+ T cell responses during STI. Recent studies have demonstrated the enhancement of HIV-1–specific CD8+ T cell responses in individuals who underwent STI (11–17). However, it is not fully understood whether this enhancement of responses is due to the expansion of pre-existing responses to levels that allow their detection using ex vivo assays or whether it represents the priming of novel specificities. In order to address this question, we followed six individuals from this cohort (including the five individuals described above who gave a second LN sample after 1 year of treatment, and one additional individual for whom only a baseline LN sample was available) longitudinally during a single STI. All six of these chronically infected individuals experienced a rebound in viremia, within a median of 3 weeks after interruption of treatment, to pretreat-
ment values (447,438 ± 910,960 copies HIV-1 RNA/ml before treatment vs. 227,750 ± 366,923 copies/ml after STI, \( P = 0.54 \)). The re-exposure to antigen was associated with an enhancement of HIV-1–specific CD8+ T cell responses in all six individuals. HIV-1–specific CD8+ T cell response to individual CTL epitopes increased significantly (\( P = 0.001 \)) compared with CD8+ T cell responses after 1 year of treatment (Figure 4), and reached even higher levels than prior to initiation of treatment (\( P < 0.05 \)). This enhancement in the magnitude of HIV-1–specific CD8+ T cell responses was associated with the development of responses to 22 epitopes in the six individuals studied that were not detectable while on therapy (median 3.5 novel epitopes per individual, range 1–6 epitopes) (Figure 4). These responses to newly targeted epitopes included responses to 13 epitopes recognized in the PB at baseline, prior to the initiation of HAART, as well as responses to nine new epitopes (median 1 epitope per individual, range 0–4) that were detectable for the first time in the PB after STI (Figure 4).

Interestingly, four of these nine newly targeted epitopes were already detectable in the LN specimen at the time of initial treatment (Figure 4). Additionally, in the five individuals for whom a second LN and PB sample was available after 1 year of treatment with HAART, all the responses to epitopes that were lost during HAART in the PB, but persisted in the LNs at that time (n = 8/21, Figure 3), became detectable again in the PB after treatment interruption.

Taken together, these data demonstrate that the enhancement of HIV-1–specific CD8+ T cell responses during STIs is largely the result of the expansion of pre-existing responses. Epitope-specific CD8+ T cells may persist in the lymphoid tissue during antiretroviral treatment, even if these specific CD8+ T cells are not detectable in the PB using the same methods. After re-exposure to antigen, these responses are among the first to be detectable in the PB, and are largely responsible for the enhancement of CD8+ T cell responses observed during STI.

**Discussion**

Most human studies of virus-specific cellular immune responses have been performed on lymphocytes derived from the PB, and very little is known about the relative distribution of epitope-specific CD8+ T cells directed against HIV-1 in the PB compared with the CD8+ T cells directed against HIV-1 in the secondary lymphoid tissues. Here we use a sensitive IFN-γ ELISpot assay and a large panel of peptides corresponding to optimal CTL epitopes to examine the magnitude and specificity of HIV-1–specific CD8+ T cells in the PB and axillary LNs of 15 individuals with untreated chronic HIV-1 infection. In addition, the dynamics of epitope-specific CD8+ T cell responses were assessed longitudinally in a subset of individuals following the initiation of antiretroviral treatment, as well as during STI. We demonstrate that HIV-1–specific CD8+ T cells are enriched in the LNs in chronic infection, with a subset of responses that are detectable exclusively in this compartment. The preferential location of virus-specific CD8+ T cells in LNs was most pronounced during effective antiretroviral therapy, when there is a relative absence of antigen in the PB. Moreover, new HIV-1–specific CD8+ T cells that arose in the PB during treatment cessation in chronic infection largely reflect antigen-specific CD8+ T cells that persist in LNs, rather than the induction of novel responses.

LNs represent one of the main reservoirs for HIV-1, and substantially more HIV-1 replication takes place in the LNs than in the PB in infected individuals (18, 33, 34). Within lymphoid organs, massive infiltration of clonally expanded CD8+ T cells has been observed (19–23, 26, 35). In this study we used a large panel of described optimal HIV-1–specific CTL epitopes to characterize virus-specific CD8+ T cell responses. Even if this approach may underestimate total HIV-1–specific CD8+ T cell responses compared with the assessment of responses directed against the entire expressed HIV-1 genome (30, 36, 37), our data show polyclonal responses to multiple HIV-1 epitopes in both compartments. The magnitude and specificity of CD8+ T cell responses in the PB correlated significantly with responses detected in LNs from the same timepoint in the untreated chronically infected individuals studied. Moreover, the hierarchy in immunodominance of these responses was similar in both compartments. The magnitude of CD8+ T cell responses was found to be significantly higher in the LNs, and in every case where differences in the breadth of responses were detected, the broader response was seen in the LNs. Of the 15

![Figure 3](image-url)

**Figure 3**
Decline of HIV-1–specific CD8+ T cell responses to individual CTL epitopes upon treatment with HAART in the five individuals studied in the PB (left) and LNs (right). Epitope-specific CD8+ T cell responses are shown prior to the initiation of HAART (pre-HAART) and after 12 months on HAART (on HAART). Magnitudes of responses are given as SFCs/million CD8+ T cells, and each line demonstrates the evolution of responses in an individual CTL epitope. Mean magnitude of HIV-1–specific CD8+ T cell responses ± SD are shown before HAART and after 12 months on HAART, as is the total number of recognized CTL epitopes (numbers inside boxes) in the five individuals. Statistical significance of differences between the magnitude of responses to individual CTL epitopes before HAART and after 12 months on HAART were calculated by two-tailed Student t test.
individuals, the magnitude of responses to targeted epitopes in the six individuals is shown in each box. Statistical significance of differences between the magnitude of responses to individual CTL epitopes was calculated by two-tailed Student’s t test.

Pre-HAART

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Figure 4
Mean epitope-specific CD8+ T cell frequencies plus SD in the PB (gray bars) and LNs (black bar) prior to initiation of HAART, as well as mean epitope-specific CD8+ T cell frequencies plus SD in the PB (gray bars) after 12 months on HAART and at the time of peak viremia after STI in the six individuals studied. Mean magnitudes of epitope-specific responses are given as SFCs/million cells. The total number of targeted epitopes in the six individuals is shown in each box. Statistical significance of differences between the magnitude of responses to individual CTL epitopes was calculated by two-tailed Student’s t test.

In conclusion, this study demonstrates that HIV-1–specific CD8+ T cells are preferentially located in the LNs (compared with PB) including a subset of responses that is detectable exclusively in the secondary lymphoid tissue. Expansion of HIV-1–specific CD8+ T cells pre-existing in the LNs contributes importantly to the enhancement of virus-specific CTL responses observed in the PB of chronically infected individuals when treatment is discontinued. The simple expansion of pre-existing CD8+ T cell responses that failed to control viral replication prior to the initiation of antiretroviral treat-
ment may have contributed to the limited success of STI in these individuals with chronic HIV-1 infection. This emphasizes the need for more potent immunotherapeutic interventions to induce antiviral immunity in chronically infected individuals to levels that may allow for immune control of HIV-1 replication.

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