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Maintenance of Large Numbers of Virus-Specific CD8\(^+\) T Cells in HIV-Infected Progressors and Long-Term Nonprogressors

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The virus-specific CD8\(^+\) T cell responses of 21 HIV-infected patients were studied including a unique cohort of long-term nonprogressors with low levels of plasma viral RNA and strong proliferative responses to HIV Ags. HIV-specific CD8\(^+\) T cell responses were studied by a combination of standard cytotoxic T cell (CTL) assays, MHC tetramers, and TCR repertoire analysis. The frequencies of CD8\(^+\) T cells specific to the majority of HIV gene products were measured by flow cytometric detection of intracellular IFN-γ in response to HIV-vaccinia recombinant-infected autologous B cells. Very high frequencies (0.8–18.0%) of circulating CD8\(^+\) T cells were found to be HIV specific. High frequencies of HIV-specific CD8\(^+\) T cells were not limited to long-term nonprogressors with restriction of plasma virus. No correlation was found between the frequency of HIV-specific CD8\(^+\) T cells and levels of plasma viremia. In each case, the vast majority of cells (up to 17.2%) responded to gag-pol. Repertoire analysis showed these large numbers of Ag-specific cells were scattered throughout the repertoire and in the majority of cases not contained within large monoclonal expansions. These data demonstrate that high numbers of HIV-specific CD8\(^+\) T cells exist even in patients with high-level viremia and progressive disease. Further, they suggest that other qualitative parameters of the CD8\(^+\) T cell response may differentiate some patients with very low levels of plasma virus and nonprogressive disease. The Journal of Immunology, 2000, 165: 1082–1092.

Further understanding of the components, targets, and magnitude of an effective immune response to HIV are important steps toward the development of effective prophylactic vaccines or immunotherapies. Induction and maintenance of a primed cellular immune response to HIV may present an important mechanism of defense that may alter the course of HIV infection. A number of recent studies have provided direct evidence for the role of CD8\(^+\) T cells in restriction of lentiviral replication in vivo. CD8\(^+\) T cell depletion by exogenous mAbs has been shown to abrogate restriction of virus replication in both simian/human immunodeficiency virus-infected or SIV-infected monkeys (1–3). In addition, it has been shown that animals infected with live attenuated SIV vaccines are able to resist SIV challenge through Ab- and chemokine-independent mechanisms (4). It now appears clear that CD8\(^+\) T cells are an important component to restriction of virus replication induced by chronic virus infections in each of these model systems and likely play a similar role in the restriction of HIV replication in humans. For this reason, considerable attention has been focused on the HIV-specific CD8\(^+\) T cell responses of patients who are felt to have immune system-mediated restriction of virus replication (5–12). Although patients with normal CD4\(^+\) T cell counts and low levels of plasma virus are a heterogeneous group, a small subgroup of patients with truly nonprogressive HIV infection and restriction of virus replication likely hold important clues to the basis of an effective immune response to HIV. However, the targets and magnitude of such responses necessary for effective restriction of virus replication remain incompletely understood.

A number of techniques have recently become available that allow the measurement of the magnitude of such responses by determining the number of Ag-specific CD8\(^+\) T cells. MHC tetramers permit the determination at the single-cell level of CD8\(^+\) T cells specific for a given peptide (13). These reagents have dramatically revised the estimates of the magnitude of the Ag-specific CD8\(^+\) T cell response during acute infections of mice and humans as much as 10- to 100-fold above that previously found by traditional limiting dilution analysis. It has been determined that 40–70% of the CD8\(^+\) T cells during an acute infection of experimental animals or humans may be Ag specific (14–17). The total numbers of Ag-specific CD8\(^+\) T cells during chronic infections of humans have not yet been well characterized. MHC tetramer analysis has recently permitted the quantification of HIV peptide-specific CD8\(^+\) T cells in the peripheral blood in some patients with progressive HIV disease (18). However, tetramer analysis alone allows the determination of the number of cells specific for a given peptide. It is likely the true number of Ag-specific CD8\(^+\) T cells is much larger if one were to examine the response to all HIV gene products in the context of each of the patient’s MHC class I alleles. Further, although MHC tetramer complexes are powerful reagents

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for examining the frequency of Ag-specific cells, such analyses provide no direct information on their functional state.

In the present study, we use a combination of assays to examine the frequency and function of Ag-specific cells in a detailed analysis of 21 HIV-infected patients. Several of these patients are part of a unique cohort characterized by infection >13 years, normal CD4+ T cell counts, HIV RNA below 50 copies/ml of plasma, and vigorous HIV-specific proliferative and direct CTL responses. These patients likely make up <0.8% of HIV-infected individuals (19–22). For comparison, we also include patients that fit the more commonly used clinical definition of nonprogressor and patients with progressive disease. We analyze the CD8+ T cell response by standard cytotoxic T cell assays, MHC tetramer analysis, and TCR repertoire analysis. In addition, we have combined techniques of flow cytometric detection of intracellular IFN-γ production with Ag presentation by HIV-vaccinia recombinant-infected autologous B cells. In this manner, we are better able to determine the global response to multiple HIV Ags and the functional state of Ag-specific cells.

Materials and Methods

Patients

Patients 1, 4–8, 19–21, 25, 27, and 29 have not received antiretrovirals during or before the study period. Patient 3 previously received IFN-α AZT (1/90 to 12/95) or IFN-α/αZTD/3D (1/96 to 12/96) as part of a National Institute of Allergy and Infectious Diseases protocol. This patient has remained off of antiretrovirals since that time. Patients 15 and 14 have not received antiretrovirals in the past 6 mo. HIV infection in study participants was documented by HIV-1/2 enzyme immunoassay. All subjects signed informed consent approved by the National Institute of Allergy and Infectious Disease investigational review board. Patients 1–6 were recently described in a separate report (23). Patients 3–8, 25, and 101–105 have also been reported separately (24). The patient numbers remain the same across studies to permit cross-reference. HLA class I and II typing was performed in all other patients in each experiment. Spontaneous release was documented by HIV-1/2 enzyme immunoassay. All subjects signed a consent statement (25) to inhibit cytokine secretion. At 6 h of incubation, the cells were washed twice and fixed in 4% paraformaldehyde (Sigma) and permeabilized or frozen for future use.

Cytotoxic T cell assays

PBMC were obtained by sodium diatrizoate density centrifugation (OrganonTeknika, Durham, NC) of apheresis donor packs. PBMC were cryopreserved in RPMI 1640 media with 10% FBS and 7.5% DMSO at −140°C. Standard 3H thymidine release assays were performed as previously described (27). Autologous EBV-transformed B cells were infected for 16 h at 37°C with vaccinia recombinant viruses vVK1 (containing the HIV-1HXB2 nef gene), vP1287 (HIV-1HXB2 gag-pol gene), vP1290 (HIV IIIB p17), vP1288 (HIVIIIB pol), vPE16 (HIV-1 pol env), vTFnef (HIV-1 pol nef), and the negative control virus vSC8 (Escherichia coli b-galactosidase (b-gal)3). Vaccinia recombinants were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The vP1287 (HIV-1HXB2 gag-pol), vP1289 (HIV-1HXB2 p17), and vP1288 (HIVIIIB pol) viruses were contributed to the National Institutes of Health AIDS Research and Reference Reagent Program by Virogenics (Troy, NY). The vTFnef virus was contributed by MedImmune (Gaithersburg, MD), and vPE16, vVK1, and VSC8 viruses were contributed by Dr. Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Target cells were 3Cr labeled, washed, and plated at 1 × 106 cells per well into 96-well round-bottom tissue culture plates. Fresh or cryopreserved PBMC were used as effectors. Cryopreserved PBMC were cultured overnight at 37°C before use as effectors. Preliminary experiments have yielded similar results with fresh or cryopreserved PBMC. Effector PBMC were added to each well at 100–125:1. All CTL assays were performed in triplicate. The percent specific cytotoxicity was calculated as follows: % specific cytotoxicity = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. The lysis of VSC8-infected targets was 12% for patient 5 and <5% in all other patients in each experiment. Spontaneous release was <2% in each experiment. The SE of individual triplicates was 1–20% of mean specific lysis. In preliminary experiments, significant lysis (>10%) was not observed in MHC class I-mismatched or CD8+ T cell-depleted cultures. All experiments were repeated at least once with similar results.

Flow cytometry

Four-color flow cytometry was performed according to standard protocols (28). Surface or intracellular staining was performed using the following Abs: FITC-conjugated anti-IFN-γ and anti-CD8 (PharMingen, Cupertino, CA), anti-BV8- and anti-BV5.1 (T Cell Diagnostics, Woburn MA); PE-conjugated anti-IFN-γ (PharMingen), and anti-CD69 (Becton Dickinson, San Jose, CA) and CyChrome-conjugated anti-CD8 and anti-CD3 (Coulter, Miami, FL), phycoerythrin-Texas Red-conjugated anti-CD8 and anti-CD3 (Coulter), peridinin chlorophyll protein-conjugated anti-CD3 and anti-CD8 (Becton Dickinson). Cells were analyzed within 24 h using either an EPICS XL (Beckman Coulter, Fullerton, CA) or a FACScalibur (Becton Dickson) flow cytometer. Surface staining with anti-BV5.1, anti-BV8, or tetramers was performed following stimulation but before fixation. Then, 0.5 µl of APC-conjugated HLA-A*0201 tetrameric complex was used to stain 2 × 106 PBMC in a 50-µl volume at 4°C for 30 min. MHC class I A*0201 complexed with either of the conserved gag-SLYNTVATL or pol-ILKEPVHGV peptides have been previously described to stain class I-restricted cytotoxic T cells specific for these peptides (18).

Intracellular cytokine detection

Target cells were prepared as described above for the direct CTL assay and used as the stimulus for intracellular cytokine detection. Intracellular cytokine detection was performed as previously described (29). Briefly, 4 × 105 PBMC were incubated with 400,000 uninfected, vacc-β-gal, or vacc-HIV-recombinant-infected autologous EBV-transformed B cells in a final volume of 2 ml of RPMI 1640 containing 10% FBS in 10-ml culture tubes (Sarstedt, Newton, NC). At 2 h of incubation, brefeldin-A (Sigma, St. Louis, MO) was added to the medium at a final concentration of 10 µg/ml to inhibit cytokine secretion. At 6 h of incubation, the cells were washed twice and fixed in 4% paraformaldehyde (Sigma) and permeabilized or frozen for future use.

IFN-γ-producing cells were permeabilized and blocked in a solution of PBS with 0.2% saponin (25% saponin in content; Sigma), 1 mM CaCl2, 1 mM MgSO4, 0.05% (w/v) NaF, 1% BSA, pH 7.4, with 5% nonfat dry milk overnight at 4°C. Cells were then aliquoted at 1 × 106 per tube and washed once in a solution of PBS/saponin. The pellet was resuspended in PBS/saponin/milk containing Abs for staining and incubated for 30 min at 4°C in the dark. Samples were then washed twice in PBS/saponin and resuspended at 500,000 µl PBS/saponin. One aliquot was stained on CD3+ CD8+ lymphocytes, and 15,000–200,000 events (100,000–700,000 total cells) were collected. Data were analyzed using either CellQuest (Becton Dickenson) or FlowJo software (TreeStar, Cupertino, CA). Color compensation settings were made with each round of staining using patient cells labeled singly with anti-CD3 labeled with specific fluorochromes.

Repertoire analysis

CD4+ T cells were isolated by depleting monocytes and CD8+ T cells using anti-CD14- and anti-CD8-coated magnetic beads (Dynal, Lake Success, NY). CD4+ T cells were then positively selected with CD4-specific beads. CD8+ T cells were positively selected directly from PBMC with CD8-specific beads. CD4+ T cells were positively selected directly from PBMC with CD8-specific beads. CD4+ or CD8+ T cell purity was documented by flow cytometry to be 95–99%. Total RNA from 106 CD4+ or CD8+ T cells was isolated using Trizol LS (Life Technologies, Grand Island, NY) and precipitated with isopropanol in the presence of Microcarrier (Molecular Re- sources Center, Cincinnati, OH). Reverse transcription was performed using Superscript II (Life Technologies) and oligo(dT) according to manufacturer protocols. Then, 5 µg of total RNA was used for analysis of TCRBV subfamily size patterns, and 1.5 µg of total RNA was used for semiquantitative analysis (30).

To analyze TCRBV transcript size patterns, 24 aliquots of the cDNA were amplified for 40 cycles in 50-µl reactions. A primer specific for each of the 22 functional TCRBV subfamilies (BV1-9, BV11-18, BV20-24) (31, 32) and an unlabeled primer specific for the TCR β-chain constant region (TCRBC) was included. The cDNAs were amplified in a Perkin-Elmer (Foster City, CA) 9600 thermocycler for 40 cycles (denaturation 25 s at 94°C, annealing 45 s at 60°C, extension 45 s at 72°C). Aliquots of the 24 PCR products were then labeled by five cycles of elongation in a “runoff” reaction with a fluorescent dye (6-carboxylfluorescein-TCRB) (30, 33). Products of these reactions were electrophoresed on 24 cm 6% acrylamide gels on a 373 DNA sequencer and then analyzed using Genescan software (Perkin-Elmer), as previously described (30).
between 690 and 1200 cells/mm$^3$ and typically maintain plasma virus levels below 50 copies/ml measured by branched chain DNA (bDNA). Patient 8 has not had viremia detected by bDNA since 1990, 11, 37). In all other patients in the present study, the proliferative response to p24 was $<1000 \Delta\text{cpm}$, which was equivalent to uninfected controls. Patients within group B meet more commonly used clinical criteria of nonprogressive disease (infection $>7$ years, peripheral CD4$^+$ T cell count $>500$ cells/$\mu l$ without antiretroviral use) (38, 39). Group C contains patients with progressive disease not receiving antiretrovirals. Because of the lack of availability of patients with progressive disease not receiving antiretroviral therapy, six patients (group D) receiving therapy but with plasma virus RNA levels $>1000$ copies/ml were included for comparison. The MHC class I and class II haplotypes of the patients are shown in Table II. HLA B*57 was overrepresented in group A patients that are part of a larger cohort of such patients examined in further detail in another report (24). Only patient 25 was heterozygous for the 32-bp deletion within the HIV coreceptor CCR5.

### Results

#### Patient characteristics

The clinical characteristics of the study patients are shown on Table I. For the purpose of discussion patients were divided into four groups. Patients within group A have been infected with HIV for at least 13 years, with stable peripheral blood CD4$^+$ T cell counts between 690 and 1200 cells/mm$^3$ and typically maintain plasma virus levels below 50 copies/ml measured by branched chain DNA (bDNA). Patient 8 has not had viremia detected by bDNA since diagnosis until a recent increase to 324 copies/ml associated with a febrile illness. Patients 3–6 have shown strong proliferative responses to p24 Ag ($\Delta\text{cpm} \geq 3000$) when stimulated with 8 $\mu$g/ml of p24 Ag (A. McNeil, unpublished observations). These responses are similar to those recently described in two patients with plasma viral RNA below 50 copies/ml plasma (11, 37). In all other patients in the present study, the proliferative response to p24 was $<1000 \Delta\text{cpm}$, which was equivalent to uninfected controls. Patients within group B meet more commonly used clinical criteria of nonprogressive disease (infection $>7$ years, peripheral CD4$^+$ T cell count $>500$ cells/$\mu l$ without antiretroviral use) (38, 39). Group C contains patients with progressive disease not receiving antiretrovirals. Because of the lack of availability of patients with progressive disease not receiving antiretroviral therapy, six patients (group D) receiving therapy but with plasma virus RNA levels $>1000$ copies/ml were included for comparison. The MHC class I and class II haplotypes of the patients are shown in Table II. HLA B*57 was overrepresented in group A patients that are part of a larger cohort of such patients examined in further detail in another report (24). Only patient 25 was heterozygous for the 32-bp deletion within the HIV coreceptor CCR5.

#### Direct CTL activity

The magnitude of HIV-specific CD8$^+$ T cell responses detected by direct CTL assays in a subset of patients was determined. The results are shown in Fig. 1. In preliminary experiments, specific lysis above 10% was not reproducibly observed in uninfected individuals. Positive CTL activity was considered to be $>10%$ specific lysis. In preliminary experiments, differences in results obtained with fresh or cryopreserved samples varied $<10%$ at a given E:T ratio, which is within the variability of this assay. These assays were performed without restimulation of effectors and in the absence of exogenous IL-2. The predominant activity was against gag-pol (10–30% sp. act.), followed by nef and by env. The level of this activity is consistent with that previously published for
patients with progressive or nonprogressive disease (Ref. 40; reviewed in Ref. 41).

**HIV Ag-specific IFN-γ secretion**

To further characterize the CD8\(^+\) T cell response to HIV Ags by a more quantitative method, we adapted a method of intracellular cytokine staining detected by flow cytometry to enumerate Ag-specific CD8\(^+\) T cells at the single cell level. HIV-vaccinia recombinant-infected EBV-transformed autologous B cells were used as stimuli. Similar assays have been used with peptide-pulsed target cells to measure responses to CMV in one report in humans and in virus infections in mice (16, 17, 42). Such assays have the advantage of not requiring in vitro proliferation of effector CD8\(^+\) T cells, which likely results in the low and variable numbers of these cells detected by traditional limiting dilution analysis. In addition, the determination of the response to the products of whole genes in the present study is not limited to a single peptide and permits a determination of the global response to HIV Ags.

An example of intracellular staining of stimulated cells from patient 8 is shown in Fig. 2A. Gating on CD3\(^+\) CD8\(^+\) lymphocytes, the percent of cells that were CD69\(^+\) IFN-γ\(^+\) in response to a given stimulus was determined. No CD3\(^+\) CD8\(^+\) cells were shown to synthesize IFN-γ after a 6-h incubation in the absence of stimulation. When PBMC were incubated with uninfected or vaccinia-infected EBV-transformed autologous B cells, the background percent positive cells was 6.87 for patient 5 and more typically between 0.04 and 2 for the remaining patients. As the percents were equivalent under these two conditions, it is likely

![Graph](https://example.com/graph.png)

**FIGURE 1.** Direct cytolysis of autologous HIV-vaccinia-recombinant-infected autologous EBV-transformed B cells. Specific lysis shown is after subtraction of lysis of vscc8(β-gal)-infected cells.
these cells were activated by either the B cells alone or EBV Ags (43) and are not vaccinia specific. The response to individual gene products is shown in Fig. 2B. The sum of the responses to gag and pol is equivalent to the response to the gag-pol construct gene products. Similarly, the sum of responses to p17 and p24 is equivalent to the gag response, indicating this method is highly quantitative. Similarly, the percent of CD8\(^+\) T cells that produce IFN-\(\gamma\) in response to peptide-pulsed B cells closely correlates with the percent obtained by the corresponding peptide MHC tetramers (not shown), consistent with recent results obtained by an IFN-\(\gamma\) enzyme-linked immunospot assay (44). The observed frequencies of Ag-specific cells were also highly reproducible by this method in repeated experiments in each of the patients studied.

The summary data of the percent of CD3\(^+\)CD8\(^+\) lymphocytes which were CD69\(^+\) IFN-\(\gamma\)^+ in response to a given HIV gene product are shown in Fig. 3. By this method, the fraction of cells that were HIV specific varied from 0.8 to 18.0%. Preliminary experiments over a broad range of E:T ratios showed no higher response to increased target numbers, only higher background activity to the vac-\(\beta\)-gal control. Similar to the direct CTL data, the predominant activity was again directed against gag-pol with a frequency of CD69\(^+\) IFN-\(\gamma\)^+ cells between 0.43 and 17.02%. The response in patient 8 was observed in multiple samples taken over several months and is dramatically higher than that observed in other patients. It is also higher than one would predict based upon direct CTL. It is possible this response is related to a recent increase in plasma viremia associated with a febrile illness and may diminish with time. With the exception of the response in patient 8, there was a good correlation between the percent of CD8\(^+\) T cells reacting to gag-pol and direct cytolytic activity to this target \((R^2 = 0.796, p = 0.007)\). If the response of patient 8 is included in this correlation, it is no longer significant \((R^2 = 0.3, p = 0.15)\). The
lack of a single-cell assay for cytolytic activity does not permit the effector CTL function of IFN-γ-producing cells to be directly confirmed. It has recently been shown in humans and in mouse models of viral infection that even memory CD8 T cells will rapidly secrete IFN-γ upon restimulation and may remain cytolytically active (16, 45, 46). Thus, the cells detected in the current study by intracellular cytokine staining are likely a composite of Ag-experienced cells with either memory or effector function.

High numbers of Ag-specific CD8 T cells were not unique to long-term nonprogressor (LTNP) patients with low levels of plasma viral RNA and strong proliferative responses. In some cases, the lowest frequencies were observed in LTNP (patients such as 5 and 6) with <50 copies of viral RNA/ml plasma. No significant difference in the number of HIV-specific CD8 T cells was observed in group A (mean 7.7 ± 2.1) compared with all other patients not receiving antiretrovirals (mean 7.5 ± 2.1, p = 0.5), even those with progressive disease and high-level viremia. No higher frequency of gag-specific CD8 T cells was found in group A when compared with other patients not receiving antiretrovirals (p = 0.3). No significant correlation between the number of HIV-specific CD8 T cells and the level of plasma viral RNA was found (p = 0.3). Because of the lack of availability of patients with progressive disease not receiving antiretroviral therapy, six patients with progressive disease and high-level viremia were included (group D). Again, large numbers of HIV-specific cells were observed, and no significant difference was found when compared with group A (mean 4.4 ± 1.2, p = 0.5). Given that antiretroviral therapy decreases the number of HIV-specific CD8 T cells (47), these results likely underestimate the total number of Ag-specific cells that would be observed in these patients if therapy was removed. These results do indicate that the number of HIV-specific CD8 T cells is no lower in patients with progressive disease when compared with nonprogressors with high-level restriction of plasma viremia.

In some virus infections in experimental animals, the number of IFN-γ-secreting CD8 T cells specific for a given peptide has been found to be similar to that detected by MHC tetramers (16, 17). Given that such large numbers of Ag-specific cells were found in HIV-infected patients, one concern is that the number of activated cells detected may be increased through bystander effects of cytokine secretion in vitro. However, the duration (6 h) of the assay and addition of brefeldin-A to stop cytokine secretion makes this possibility unlikely. To address these issues, we used two well-characterized HLA-A*0201 tetramer complexes to detect Ag-specific cells and determine the fraction able to secrete IFN-γ under the current experimental conditions (18).

Four of 11 patients tested were MHC class I A*0201 positive. Of these patients, only two had >0.1% of CD8 T cells specific for the conserved gag (SLYNTVATL) or pol (ILKEPVHGV) peptide. The tetramer staining of PBMC of patients 3 and 19 is shown in Fig. 4A. No bystander activation of these cells was observed when cells were stimulated with any recombinant encoding non-p17 gene products. Although no bystander activation was observed, many MHC tetramer T cells did not produce IFN-γ.
the experimental conditions in Fig. 4A, almost one-half of MHC tetramer+ cells did not produce IFN-γ upon stimulation. Because the frequency of SLYNTVATL-specific cells is lower than the total responsive to the entire gag-pol gene product (Fig. 3), the number of target cells of patient 3 was increased to a 1:1 ratio to engage all Ag-specific cells (Fig. 4B). This increases the numbers of cells responding but also dramatically increases the nonspecific activity to other Ags such as to β-gal. Under these conditions, 73% of MHC tetramer+ cells could be induced to make IFN-γ. Higher numbers of responding MHC tetramer+ cells were not observed with higher numbers of target cells or longer stimulation at 12 or 24 h. Thus, of all MHC-gag tetramer+ cells only a subset produced IFN-γ when stimulated with the gag or gag-pol gene product.

Nearly all MHC-gag tetramer+ cells of patients 3 or 19 were ultimately capable of producing detectable IFN-γ after stimulation with PMA/ionomycin (Fig. 3B). Surface staining was used to further characterize the population of CD3+CD8+ MHC tetramer+ cells. Of unstimulated CD3+CD8+ MHC tetramer+ cells, 46% were CD38+, 90% were CD27+, and 72% were CD45RA−. This result is consistent with the vast majority of these cells being Ag-experienced memory or effector cells (13, 18, 48).

Analysis of the CD8+ T cell receptor repertoire

Large virus-specific expansions previously have been found in some acute and chronic infections in humans (15, 43, 49–51). Given that patients in the present study had large numbers of Ag-specific cells, it was of interest to determine whether large HIV-specific expansions existed within the CD8+ T cell Ag receptor repertoire. Of the patients examined (1–6), large expansions within the CD8+ TCR repertoire were found in patients 3–6. In each case, these expansions were associated with expansion of a single-sized transcript (Fig. 5). Sequence analysis revealed that these expansions were monoclonal. In one case (patient 3), this monoclonal expansion made up ~30% of the circulating CD8+ T cells. In patients 3 and 6, these expansions were shown to persist over 4 years of study. The CD4+ T cell repertoire was also analyzed in these patients and found to be polyclonal. No subfamily was expanded to >15%, and all size pattern distributions were gaussian and indistinguishable from those of uninfected individuals.

We then determined if HIV specificity of these expanded CD8+ T cell clones could be detected (Fig. 6). BV subfamily-specific Abs were used to stain the CD8+ T cell expansions of patients 3–5. No BV6 Ab that recognized the expansion of patient 6 was found. The percent of CD8+ T cells that stained with BV-specific Ab correlated well with the percentages detected by semiquantitative PCR. No HIV specificity could be found within the BV8 expansion of patient 3 and the BV5 expansion of patient 5. Although HIV specificity was not demonstrated for most of the expansions found, the BV5.1 expansion of patient 4 was found to be nef specific. Some decrease in the intensity of BV5.1 Ab staining is observed on IFN-γ+ cells, consistent with activation-induced TCR down-regulation. Small numbers of gag-pol-specific cells were also detected in this subfamily and likely represent the lower frequency expansions.
clones detected by size pattern analysis and sequencing of BV5.1 in this patient. Although the expansion makes up the vast majority of clones in the BV5.1 subfamily, only 20% of the BV5.1 cells produced IFN-γ in response to the nef gene product. Higher numbers of IFN-γ-producing cells were not observed in response to higher numbers of infected target cells.

**Discussion**

In the present study, very high numbers of HIV-specific CD8+ T cells were detected in patients across a broad spectrum of plasma virus load. Using a method that couples the capability of studying the frequency of Ag-specific cells provided by intracellular cytokine staining with the ability to study a wide array of epitopes inherent to the HIV-vaccinia recombinant-infected B cell system, between 0.8% and 18% of the circulating CD8+ T cells were found to be specific for HIV. These numbers are ~3–15 times the number previously estimated by enzyme-linked immunospot or tetramer analysis (18, 40, 47, 52–54). In some cases, this percent approaches the total number of CD3+CD38+ T cells previously believed to be mostly due to bystander activation in infected patients (21). This study includes a unique cohort of LTNPs that has been infected >13 years, has strong proliferative responses to HIV Ags, and maintains plasma virus levels <50 copies/ml. Although these patients appear to have a potent ability to restrict virus replication, this was not associated with higher numbers of HIV-specific CD8+ T cells measured in vitro. Similar high levels of virus-specific CD8+ T cells were observed in these patients as some patients with progressive infection and 55,000 copies of viral RNA per ml of plasma or other patients with plasma viremia poorly controlled by antiretroviral therapy. It has previously been proposed that patients that maintain effective restriction of HIV replication do so because of greater HIV-specific CD8+ T cell responses. However, previous studies have not consistently demonstrated a significant correlation between levels of plasma virus and CD8+ T cell responses measured in vitro (7, 8, 10, 21, 37, 55, 56). It has been further suggested that significant correlations between plasma virus levels and measures of HIV-specific CD8+ T cell responses were not detected because the assays used

![FIGURE 6. HIV specificity of CD8+ T cell BV subfamily expansions. See Fig. 2A. CD3+CD8+ cells that stained with the indicated BV subfamily subtype-specific Ab and produce IFN-γ in response to the indicated stimuli are shown.](https://www.jimmunol.org/doi/abs/10.4049/jimmunol.1089)
require in vitro stimulation, are poorly reproducible, or are not reliably quantitative. Subsequent more quantitative analyses using MHC tetramers dramatically increased estimates of the numbers of HIV-specific CD8\(^+\) T cells by 10- to 100-fold and demonstrated an inverse correlation with plasma viremia (18). In contrast, a similar correlation was not observed in the present study. However, these results are in agreement with one very recent report in which the numbers of CD8\(^+\) T cells specific for previously described HLA-A- and -B-restricted peptides detected by enzyme-linked immunospot assays in patients with progressive HIV infection did not correlate with plasma viral load or CD4\(^+\) T cell count (54).

There are a number of important differences that may account for the apparent inconsistencies between the results of the present study with recent observations using HIV peptide MHC tetramers (18). First, the patient population studied here is shifted toward patients with nonprogressive infection with lower plasma virus loads. Previous studies have identified patients with low levels of HIV-specific CD8\(^+\) T cell responses in end stage disease (8, 54). Conversely, low responses have also been observed by a variety of methods in some LTNPs with very low virus loads similar to patients 5 and 6 (8, 21, 40, 54) suggesting the measured CD8\(^+\) T cell responses may be dependent on virus replication in such patients. Thus, the results of such correlations might be dramatically affected by inclusion of patients at either extreme of HIV infection. Second, the number of Ag-specific cells even for conserved peptide sequences is highly variable across patients regardless of viral burden. Although four of the patients tested are A2 positive, only patients 3 and 19 stain >0.1% of CD8\(^+\) T cells with the SLYN TVATL-A2 tetramer, consistent with some previous observations (40, 53). Further, upon mapping the response to gag peptides of nine of these patients, the number of IFN-\(\gamma\)\(^-\) cells detected in response to a given peptide restricted to a single MHC allele may range from 0 to 5% with no association with viral burden (24, 54). Last, tetramer analysis alone examines the response specific for a given MHC allele. Because of the difficulties of mapping and production of tetramers of a given peptide, analysis is commonly done on only the more common MHC A and B alleles. Thus, when the global response to HIV gene products in the context of each of a given patient’s MHC alleles is measured, considerably higher frequencies of Ag-specific cells are found. Although these frequencies are quite high, it is likely they still may underestimate the true frequency of HIV-specific cells if one were to measure the total response to the patient’s virus or cells in the lymphoid tissues.

Although large percentages of the circulating cells were specific for HIV they were not typically concentrated within large monoclonal expansions but rather were scattered throughout the TCR repertoire. Overall, this is consistent with one recent report in which gag tetramer\(^+\) cells were found to be contained within some expanded BV subfamilies by flow cytometry (52). Upon examination of the CD8\(^+\) TCR repertoire of the patients in the present study, some extremely large monoclonal expansions (up to 30%) were observed. Yet in only a minority of cases were these expansions found to be specific for the HIV isolate tested. It is possible these expansions are in fact HIV specific and do not react with the isolates represented by the vaccinia recombinants used. Alternatively, they are not HIV specific and similar to those observed in HIV-uninfected individuals (57–62) or are specific for other chronic virus infections such as CMV or EBV Ags not actively expressed in transformed B cells. The lack of similar expansions within the CD4\(^+\) T cell compartment confirms the observed expansions in CD8\(^+\) T cells are not due to superantigen effects nor contamination with CD4\(^+\) T cells. An analysis of Ag-specific CD4\(^+\) T cells in some patients in the present study has shown that in these patients between 0.2% and 0.8% of circulating CD4\(^+\) T cells make IFN-\(\gamma\) in response to HIV p55 Ag (63). Taken together, these data are then consistent with those from experimental animals showing that the CD4\(^+\) T cell repertoire is much less prone to large monoclonal expansions and the numbers of virus-specific CD8\(^+\) T cells are as much as 10-fold larger than those of CD4\(^+\) T cells (49, 61, 64–66).

Although large numbers of Ag-specific cells were in some cases detected by either tetramer analysis or repertoire analysis, only a subset of these cells were able to activate, as measured by CD69 staining, or produce IFN-\(\gamma\) when stimulated through the TCR. Only approximately one-half of the MHC tetramer\(^+\) cells of patients 3 or 19 produced IFN-\(\gamma\) in response to the appropriate HIV gene product. It is possible that the MHC tetramer\(^+\) IFN-\(\gamma\)\(^-\) cells are unable to make IFN-\(\gamma\) after TCR engagement such as CCR7\(^+\) memory cells (67) or alternatively noneffector Ag-specific cells recently observed (68). However, unlike the situation in lymphocytic choriomeningitis virus-infected mice under conditions of CD4\(^+\) T cell depletion, tetramer\(^+\) IFN-\(\gamma\)\(^-\) cells in the present study were able to produce cytokine upon stimulation with PMA/ionomycin. It is also possible that these MHC tetramer\(^+\) IFN-\(\gamma\)\(^-\) cells are a subpopulation of MHC tetramer\(^+\) cells that are not Ag specific. This is a possibility given the avidity of the MHC-peptide complex was increased by producing tetramers to allow staining of Ag-specific cells. However, this is not necessarily the case given the BV 5.1 expansion of patient 5 was nef specific and monoclonal yet only 20% of these cells produced IFN-\(\gamma\) even under conditions of high numbers of infected APCs. This result suggests that the Ag-specific cells detected by MHC tetramers that do not activate or accumulate cytokine in response to HIV gene products may be Ag-specific cells of relatively low avidity and may have a more limited ability to activate in response to stimulation through the TCR.

It should be noted that conclusions regarding protective immunity based upon correlations between plasma viremia and parameters of HIV-specific immunity should be approached with some caution. Because no inverse relationship was found between plasma virus and the numbers of Ag-specific CD8\(^+\) T cells measured in vitro does not imply these cells are not important mediators of protective immunity or restriction of virus replication in some infected patients. For reasons pointed out above the relationship between the measured CD8\(^+\) T cell response and plasma viremia is likely quite complex and dependent upon virus replication and stage of disease. Further, the qualitative nature of the CD8\(^+\) T cell response may be quite different in the context of a vaccine that might induce CD4\(^+\) and CD8\(^+\) T cell responses than in HIV-infected individuals in the context of diminished CD4\(^+\) T cell help. It is now clear by more direct evidence that CD8\(^+\) T cells are important mediators of the restriction of virus replication observed in several experimental animal models of HIV infection (1–4). Similarly, the cells of patients 3–8 are able to restrict autologous and challenge virus replication when engrafted into SCID-Hu animals and restriction of challenge virus replication is abrogated by CD8\(^+\) T cell depletion (23). However, this activity was not correlated with higher CD8\(^+\) T cell responses in standard assays of suppression or cytolysis. Similarly, such patients do not appear to be distinguished by higher numbers of Ag-specific CD8\(^+\) T cells than patients with progressive disease in the present study. Although high numbers of HIV Ag-specific CD8\(^+\) T cells are maintained in infected individuals with progressive disease, these appear to have a limited capacity to restrict virus replication. The important question that remains from these results and those of others is how high-level viremia persists in many patients despite such large numbers of HIV-specific CD8\(^+\) T cells. It appears that the ability of patients within group A to restrict virus replication
may lie in not the number of virus-specific cells but likely in other, qualitative measures of their CD8+ T cell response that are not accounted for in traditional assays. It should be mentioned that in several models of anti-tumor responses or disruption of CD4+ T cell function during virus infection, tumor or infection is poorly controlled by CD8+ T cells in vivo yet may retain cytoplastic activity detected in vitro (69–74). In addition to CD8+ T cell avidity, other measures of the peptide targets, CD8+ T cell-derived suppressive factors, and MHC down-regulation, which may better model in vivo restriction of virus replication, are each being pursued as part of ongoing work. Further studies of such patients with very low levels of plasma virus and maintenance of strong proliferative responses may provide important clues to qualitative differences in the HIV-specific immune response that lead to effective restriction of virus replication.

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References


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