JOURNAL OF VIROLOGY, July 2005, p. 8171–8181 Vol. 79, No. 13
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.13.8171–8181.2005


# Limited Sequence Evolution within Persistently Targeted CD8 Epitopes in Chronic Human Immunodeficiency Virus Type 1 Infection

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Received 22 October 2004/Accepted 6 March 2005

**Studies in acute human immunodeficiency virus type 1 (HIV-1) infection indicate viral evolution under CD8 T-cell immune selection pressure, but the effects of ongoing immune pressure on epitope evolution during chronic infection are not well described. In this study, we performed a detailed longitudinal analysis of viral sequence variation within persistently targeted cytotoxic T-lymphocyte (CTL) epitopes in two HIV-1-infected persons during 6 years of persistent viremia. Responses were quantitated using freshly isolated peripheral blood lymphocytes in direct lytic assays as well as by gamma interferon (IFN-γ) Elispot assays on cryopreserved cells. Seven targeted epitopes were identified in each person. In the majority of cases, the dominant epitope sequence did not change over time, even in the presence of responses of sufficient magnitude that they were detectable using fresh peripheral blood mononuclear cells in direct lytic assays. Only 4 of the 14 autologous epitopes tested represented potential CTL escape variants; however, in most cases strong responses to these epitopes persisted for the 6 years of study. Although persistent IFN-γ responses were detected to all epitopes, direct lytic assays demonstrated declining responses to some epitopes despite the persistence of the targeted sequence in vivo. These data indicate limited viral evolution within persistently targeted CD8 T-cell epitopes during the chronic phase of infection and suggest that these regions of the virus are either refractory to sequence change or that persistently activated CD8 T-cell responses in chronic infection exert little functional selection pressure.**

Increasing evidence indicates that human immunodeficiency virus (HIV)-specific CD8 T cells play a critical role in controlling AIDS virus infections. The early decline in viremia in acute infection is associated with the emergence of a virus-specific cytotoxic T-lymphocyte (CTL) response (7, 26, 33, 40), and experimental depletion of CD8 T cells in animal models of AIDS virus infection results in dramatic increases in plasma viremia that are again brought under control once CD8 T cells return (18, 41). Moreover, in studies of acute HIV type 1 (HIV-1) infection there is strong evidence of selection for immune escape within targeted epitopes (8, 38). More recent population studies have shown HLA class I allele-specific mutations in persons with established infection (32, 47), suggesting viral imprinting by the CD8 T-cell response, although the relationship between these changes and the immune responses present in vivo were not examined. Evidence of immune escape within targeted epitopes has been documented in the chronic phase of infection (14, 15, 17, 24), although most studies have been limited to the analysis of single epitopes and few studies have simultaneously examined responses to multiple persistently targeted epitopes in longitudinal fashion.

The above data suggest that CD8 T cells apply significant immune selection pressure, but important questions remain. Although initial studies using HLA class I peptide tetramers suggested an inverse relationship between CD8 T-cell response and viral load (35), detailed studies comparing the breadth and magnitude of CD8 T-cell responses in persons with low and high viral loads have failed to show a relationship between these parameters (1, 6). More recent studies suggest functional defects in CD8 T-cell responses in persons with chronic HIV-1 infection (5, 29, 31, 42, 48), but whether there are epitope-specific differences in functional immune responses, and the extent to which immune escape is associated with persistent immune responses in the chronic phase of infection, is not known. Most studies of epitope-specific CD8 T-cell responses have relied on gamma interferon (IFN-γ) Elispot as a means to quantitate the magnitude and breadth of responses, but there is debate as to the in vivo lytic capacity of these cells. In particular, no studies have examined the impact on viral evolution within epitopes targeted by CD8 responses that are of sufficient magnitude that they can be detected by direct lysis of target cells using freshly isolated peripheral blood cells (43, 45).

In this study, we examined the ability of persistent in vivo-

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activated CD8 T-cell responses to drive viral evolution within targeted epitopes in individuals continuously viremic and maintaining strong immune responses against defined CD8 epitopes over several years. This study represents extension of a previous study in which persistent IFN-γ CD8 T-cell responses in persons with late-stage HIV-1 infection were shown to recognize the viral sequences present in vivo, suggesting a lack of selection pressure in late-stage infection (11). In this study, we performed a longitudinal analysis of two persons selected for examination because of the presence of epitopes targeted by in vivo-activated CTL responses, indicating a high level of immune activation. We included direct lytic assays, which allowed for examination of the effects of chronic-stage in vivo-activated CTL pressure on viral evolution, as well as IFN-γ Elispot assays, which allowed for simultaneous examination of a broader range of epitope-specific responses. Our data indicate that, despite persistence of strong in vivo-activated CTL responses, sequence evolution within CTL epitopes in the chronic phase of HIV-1 infection is uncommon. Moreover, they show that less-well-recognized variant sequences can continue to drive in vivo-activated CTL responses despite poor recognition of the in vivo variant.

## MATERIALS AND METHODS

**Subjects.** Two HIV-1-infected subjects who showed strong CD8 T-cell-mediated immune responses at the beginning of the study, 010-35i and 010-115i, were selected for longitudinal evaluation. HLA types for these two subjects are as follows: 35i, A*01, A*11, B*08, B*15, C*04, C*07; 115i, A*02, A*68, B*14, B*52, C*08, C*12. Several CTL epitopes recognized by these subjects were previously identified using both bulk CTL assays and CTL cloning (19, 20, 23, 46). Subject 35i has been infected at least since 1989 and was started on highly active antiretroviral therapy (HAART) in 1996. Subject 115i has been infected at least since 1987 and was started on HAART in 1996. Both subjects gave written informed consent, and the study was approved by the Massachusetts General Hospital Human Studies Committee.

**Cell lines.** Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (43) in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% or 20% (vol/vol) heat-inactivated fetal bovine serum (Sigma). RPMI 1640 medium used for all cell lines was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and HEPES (10 mM). HLA-typed reference B-LCL were also obtained from the American Society for Histocompatibility and Immunogenesis B-cell line repository.

**Isolation of CTL clones.** Isolation and initial characterization of HIV-1-specific CTL clones have been described in detail previously (20, 21, 44). Briefly, peripheral blood mononuclear cell (PBMC) lymphocytes were cloned at limiting dilution in the presence of phytohemagglutinin or the CD3-specifc monoclonal antibody 12F6 at 0.1 μg/ml, recombinant interleukin-2, and irradiated allogeneic PMBCs. These clones were subsequently screened for HIV-1-specific CTL activity in $^{51}$Cr release assays and maintained by periodic restimulation with phytohemagglutinin or CD3-specific monoclonal antibody and feeder cells in the presence of recombinant interleukin-2.

**Recombinant vaccinia viruses.** Recombinant vaccinia viruses expressing the Gag, reverse transcriptase (RT), and Env genes from molecular clones (BH8, BH10, and PV22) derived from the HIV-1 strain IIIB were used to express viral antigens on target cells (45). Recombinant vaccinia viruses expressing serial truncations of the HIV-1 envelope glycoprotein (vPE16, -17, -18, -20, -21, and -22) were constructed from the BH8 molecular clone of HIV-1 (12, 39). The HIV-1 BH10 clone was also used to generate recombinant vaccinia viruses expressing the full-length p55 Gag protein (vAbt 141) and the p17 (vAbt 228) and p24 (vAbt 286) Gag subunits (27), while the PV22 molecular clone was used to construct vaccinia virus vectors expressing the full-length (VCF21) and serial truncations (VCF32 to -37) of the HIV-1 RT protein. The recombinant vaccinia viruses New York City Broad of Health (NYCBH), not expressing retroviral genes, and vSC8 expressing the *Escherichia coli* β-galactosidase gene (*lacZ*) were used as negative controls (10).

**Synthetic HIV-1 peptides.** Synthetic peptides corresponding to HIV-1 envelope PV22 sequence were synthesized as previously described (20). All amino acids are numbered as indicated for the HXB2 sequence, and references to other peptides and epitopes have been modified accordingly. Additional smaller peptides (8 to 20 amino acids) used for the fine mapping of epitopes were obtained commercially (Research Genetics, Alabama; Bio-Synthesis, Lewisville, Texas; Quality Controlled Biochemicals, Hopkinton, MA; and Chiron Mimotopes, Clayton, Australia) or were synthesized by standard f-Boc chemistry on a peptide synthesizer, model 432A, obtained from Applied Biosystems, Inc. (Foster City, CA). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethyl sulfoxide (Sigma) with or without 1 mM dithiothreitol (Sigma).

**Cytotoxicity assay.** Target cells consisted of B-LCL preincubated with synthetic HIV-1 peptides. Cells were then labeled with 100 μCi of $Na_2$ ($^{51}CrO_4$; 1 Ci [37 GBq]; New England Nuclear, N. Billerica, MA) for 45 to 60 min and washed three times with RPMI 1640 medium supplemented with 10% fetal calf serum. Cytolytic activity was determined in a standard $^{51}$Cr release assay (43) using U-bottom microtiter plates containing $10^4$ targets per well and the designated number of PBMCs, cell lines, or clones as effector cells. Plates were incubated in a humidified incubator at 37°C for 4 h. All assays were performed in duplicate or triplicate. Supernatants were then harvested and counted on a Cobra gamma counter (Packard Instrument Company, Meriden, CT), and percent lysis was determined from the formula 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of targets in detergent (1% Triton X-100; Sigma). Spontaneous release was less than 30% of maximal release for all reported assays.

**Elispot assay.** HIV-1-specific CD8 T-cell responses were also quantified by IFN-γ Elispot assay by using frozen PBMCs as described previously (4). Specific IFN-γ-secreting T cells were enumerated by an AID Elispot reader (AID GbmH, Strassberg, Germany), with values calculated by subtracting the negative control value and expressed as spot-forming cells (SFC) per $10^6$ input cells. A response was considered positive if there were ≥50 SFC/$10^6$ PBMCs and it was at least three times greater than the background level. Negative controls were always <30 SFC/$10^6$ PBMCs.

**Sequencing of autologous virus with viral RNA or proviral DNA.** Viral RNA was extracted from cryopreserved plasma using the QIAmp RNA Viral Mini kit (QIAGEN, Valencia, Calif.), or proviral DNA was extracted from PBMCs using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). Four microliters of plasma viral RNA or cDNA was subjected to nested PCR using 35 cycles of 30 s at 94°C, 30 s at 60°C, 2 min at 72°C, and a final extension for 20 min at 68°C. RT-PCR cycling conditions were as follows: 50°C for 60 min, 95°C for 15 min, and cycling conditions as noted above. For the second round of nested PCR, the extension time was 1 min. For each epitope a different primer set of first- and second-round primers was used (Table 1).

PCR fragments were population sequenced to identify regions of sequence variation and where necessary were additionally cloned (TOPO TA; Invitrogen, Carlsbad, Calif.). All fragments were sequenced bidirectionally on an ABI 3100 Prism automated sequencer. Sequencers (Gene Codes Corp., Ann Arbor, Mich.) and MacVector 4.1 (Oxford Molecular) were used to edit and align sequences. If the height of the secondary peak at a given residue in the chromatogram was reproducibly more than 25% of the dominant peak, a mixed base was considered present at that position.

**Flow cytometric analysis of tetramer-specific CD8 T cells.** To detect CD8 T cells binding to HIV-1-specific tetramers refolded with epitopic HIV-1 peptides, thawed PBMCs were mixed with phycoerythrin-conjugated CD8 monoclonal antibodies (BD Biosciences, San Jose, CA) and allophycocyanin-conjugated A2-SL9-specific tetramers (Beckman Coulter). Following 30 min of incubation at room temperature, cells were washed twice, fixed in 1% paraformaldehyde, and acquired on a flow cytometer (FACSCalibur; BD Biosciences) using the CellQuest software. Data analysis was performed with the FlowJo software package (Treestar, Ashland, OR).

**Statistics.** Linear correlations were calculated by use of Spearman's rank coefficient. Data analysis and graphical presentation were performed using the Prism software package.

## RESULTS

**Characterization of CTL epitopes targeted by in vivo-activated HIV-1-specific CTL.** In order to examine the effect of CTL responses on viral evolution over the chronic phase of HIV-1 infection, we screened persons with established HIV-1

TABLE 1. HIV sequencing primers

| Epitope(s) | Fragment name | 1st PCR primer | | 2nd PCR primer | |
|---|---|---|---|---|---|
| | | Location | Sequence | Location | Sequence |
| B62-RY10, A2-SL9 | 1b | 623-F | AAATCTCTAGCAGTGGCGCCCGAACAG | 692-F | CAGGACTCGGCTTGCTGAAGC |
| | | 2827-R | TAACCCTGCGGGATGTGGTATTCC | 1276-R | GGTATTACTTCTGGGCTRAAAGC |
| Cw8-TL9 | 1c | 623-F | AAATCTCTAGCAGTGGCGCCCGAACAG | 989-F | CCCTTCAGACAGGATCAGAAG |
| | | 2827-R | TAACCCTGCGGGATGTGGTATTCC | 1485-R | GTTCCTGCTATGTCACTTCCC |
| B62-GY9, B8-EI8 | 1d | 623-F | AAATCTCTAGCAGTGGCGCCCGAACAG | 1266-F | AGAAGARAAGGCTTTYAGCCC |
| | | 2827-R | TAACCCTGCGGGATGTGGTATTCC | 1755-R | TCTGGGTTCGMATTTTGGACC |
| B14-DA9, B52-RI8 | 1e | 623-F | AAATCTCTAGCAGTGGCGCCCGAACAG | 1545-F | TAATCCACCTATCCCAGTAGG |
| Cw8-RV9 | | 2827-R | TAACCCTGCGGGATGTGGTATTCC | 2094-R | TGTRGGAAGGCCAGATCTTCC |
| B8-GL9 | 2c | 1856-F | AGTGGGRGGACCCRGCCATAARGC | 2141-F | AGCAGACCAGAGCCAACAGCC |
| | | 3008-R | CTGGTGATCCTTTCCATCCCTGTGG | 2827-R | TAACCCTGCGGGATGTGGTATTCC |
| A11-AK9 | 3b | 2711-F | GGGCCTGAAAATCCATACAATACTCC | 2711-F | GGGCCTGAAAATCCATACAATACTCC |
| | | 4306-R | GGTGGCAGGTTAAAATCACYAGCC | 3276-R | GGCAGCACTATAGGCTGTACTGTCC |
| B62-LY12 | 3c | 2711-F | GGGCCTGAAAATCCATACAATACTCC | 3006-F | TTCCACAGGGATGGAAAGGATCACC |
| | | 4306-R | GGTGGCAGGTTAAAATCACYAGCC | 3623-R | TAGTGTGGGCACCCCTCRTTCTTGC |
| B14-VI10 | 3f | 2711-F | GGGCCTGAAAATCCATACAATACTCC | 3876-F | CTTTCTATGTAGATGGGGCAGC |
| | | 4306-R | GGTGGCAGGTTAAAATCACYAGCC | 4306-R | GGTGGCAGGTTAAAATCACYAGCC |
| B8-YL8, B14-EL9 | 7c | 7801-F | GGAGCAGCAGGAAGCACTATGG | 7831-F | TCAATGACGCTGACGGTACAGG |
| | | 8130-R | CTTTTTCCCACTCCCTCCAGGTCG | 8070-R | AACTAGCATTCCAAGGCACAGC |

infection for detection of cytolytic responses. Chromium release assays were performed using bulk, unstimulated PBMCs and autologous B cells infected with recombinant vaccinia viruses expressing HIV-1 proteins. Two individuals exhibiting strong ex vivo responses were selected for detailed longitudinal follow-up to address the impact of these responses on viral evolution. Kinetics of viral load and CD4 T-cell changes over time for these two subjects are shown in Fig. 1A and B, and longitudinal cytolytic responses against HIV-1 proteins using autologous B cells infected with recombinant vaccinia virus–HIV-1 vectors and freshly isolated PBMCs are shown in Fig. 1C and D. Both individuals exhibited strong CTL responses to

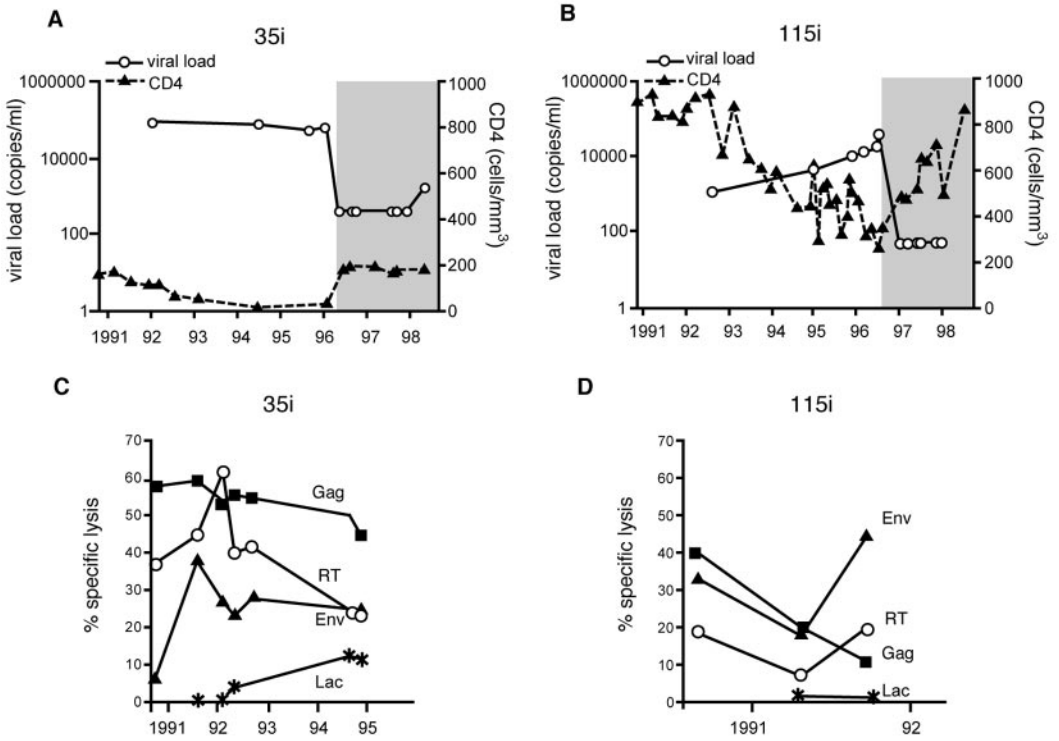


FIG. 1. (A and B) Viral load and CD4 T-cell counts over time for subjects 35i (A) and 115i (B). (C and D) Longitudinal cytolytic responses using autologous B cells infected with recombinant vaccinia virus-HIV vectors, together with freshly isolated PBMCs, in subjects 35i (C) and 115i (D). Results are shown at an effector-to-target ratio of 50:1 in a 4-hour chromium release assay at the designated times. Recombinant vaccinia virus vectors expressing Gag, RT, or Env or a control expressing the β-galactosidase gene product (Lac) were used as target cells.

TABLE 2. HLA class I-restricted T-cell epitopes recognized by subjects 35i and 115i

| Subject (HLA type) | Protein | HLA restriction | Peptide name | Amino acids | Epitope sequence | Direct lysis (%)[b] |
|---|---|---|---|---|---|---|
| 35i (A*01, A*11, B*08, B*15, C*04, C*07)[a] | Gag | B62 | RY10 | 20–29 | RLRPGGKKKY | 19[1] |
| | Gag | B8 | EI8 | 260–267 | EIYKRWII | 32[1] |
| | Gag | B62 | GY9 | 269–277 | GLNKIVRMY | 61[1] |
| | Pol | B8 | GL9 | 173–181 | GPKVKQWPL | 8[1] |
| | Pol | A11 | AK9 | 313–321 | AIFQSSMTK | 22[1] |
| | Pol | B62 | LY12 | 415–426 | LVGKLNWASQIY | 39[1] |
| | Env | B8 | YL8 | 586–593 | YLKDQQLL | 27[1] |
| 115i (A*02, A*68, B*14, B*52, C*08, C*12) | Gag | A2 | SL9 | 77–85 | SLYNTVATL | 14[2] |
| | Gag | Cw8 | TL9 | 180–188 | TPQDLNTML | 20[2] |
| | Gag | B52 | RI8 | 275–282 | RMYSPTSI | ND |
| | Gag | B14 | DA9 | 298–306 | DRFYKTLRA | ND |
| | Gag | Cw8 | RV9 | 305–313 | RAEQASQEV | 17[2] |
| | Pol | B14 | VI10 | 651–660 | VTDSQYALGI | 21[2] |
| | Env | B14 | EL9 | 584–592 | ERYLKDQQL | 25[3] |

[a] HLA; B15 represents a member of the B62 superfamily (30).
[b] Date of assay was as follows: 1, 3/1991; 2, 8/1991; 3, 4/1992. ND, not done. Specific lysis of target cells expressing the control lacZ protein ranged from 0 to 1% for these assays.

multiple HIV-1 proteins, as evidenced by direct lysis of target cells expressing processed viral antigens in standard 4-h chromium release assays.

In order to further define the targets of these immune responses, overlapping peptides were used to define the optimal epitopes targeted, screening by both cytolytic assays using autologous chromium-labeled B cells sensitized with peptide, as well as IFN-γ Elispot assays. For each epitope, the optimal epitope was defined by determining the shortest peptide that sensitized target cells for lysis at the lowest concentration (data not shown) (20). We focused our analysis on seven optimal epitopes identified in each subject that were found to be recognized using either bulk CTL or Elispot assays. At the time of initial screening, we were able to map five epitopes in subject 115i and six in subject 35i directly by using fresh PBMCs, demonstrating that these responses were highly activated in vivo. We later identified an additional two epitopes for subject 115i and one in 35i by comprehensive IFN-γ Elispot assay from frozen cells (Table 2). Since direct lytic assays can only be performed using freshly isolated PBMCs, we were not able to test whether these epitopes were also targeted by freshly isolated PBMCs. Together, these data indicate that multiple epitopes can be targeted by circulating CD8 T cells that exhibit immediate cytolytic activity in the chronic phase of infection and that these same responses can be confirmed by IFN-γ Elispot assay.

**Evolution of CTL responses during chronic HIV-1 infection.** Previous studies in persons with late-stage disease have shown a lack of immune escape within dominant epitopes defined by IFN-γ Elispot analysis (11), but longitudinal assays examining multiple epitopes shown to be simultaneously highly targeted in vivo, and including comparison of direct lytic assays to IFN-γ Elispot, have not been performed. To address this, we performed a longitudinal analysis of these responses over time in each subject. We evaluated CTL responses using IFN-γ Elispot assay and $^{51}$Cr assay at multiple time points during a 5-year period for both subjects (Fig. 2) and extended these analyses using the Elispot assay alone for an additional 1 year.

In subject 35i, the IFN-γ Elispot response remained relatively stable for all seven epitopes except B8-GL9 during the course of study, and over this time period there was a steadily declining CD4 count but relatively stable plasma viral load between 55,000 and 95,000 RNA copies/ml plasma. In contrast, direct lytic assays showed progressive decline for most targeted epitopes after 2 years of follow-up, except for a single epitope in Gag (B62-GY9). In subject 115i, direct lytic assays and Elispot responses both remained relatively robust over the course of study, despite the fact that viral load was gradually increasing and CD4 counts were gradually decreasing, although never to the low-level CD4 counts observed in subject 35i. Moreover, in study subject 115i, CD8 T-cell responses against the A2-SL9 epitope were also longitudinally assessed using major histocompatibility complex class I tetramers refolded with SL9 epitopic peptide. The magnitude of A2-SL9 tetramer-specific CD8 T cells was highest in the early years of the analysis (1.04% in 1993) and subsequently declined (0.14% in 1996), consistent with the results obtained using IFN-γ Elispot analysis of the A2-SL9-specific CD8 T-cell response.

Together, these data indicate the persistence of IFN-γ Elispot assay positivity for most epitopes in both subjects but the variable persistence of CD8 T cells with direct cytolytic function. Furthermore, we observed a positive correlation between the magnitude of IFN-γ-secreting epitope-specific CD8 T cells, as determined by Elispot assays, and the corresponding antigen-specific cytotoxicity of these cells. However, this correlation reached the level of statistical significance only in study subject 35i (Fig. 3).

**Longitudinal assessment of immune selection pressure during chronic HIV-1 infection.** The above data indicate an overall decline in circulating CD8 cells with direct lytic potential in subject 35i and overall persistence of these responses in subject 115i. We next assessed the extent to which immune escape might be related to the variable evolution of immune responses to these epitopes. We therefore bulk sequenced plasma viral RNA and PBMC DNA from four to six different time points in each subject. In order to assess the mixture of variants present in vivo, we additionally sequenced at least six clones on at least two (first and last) time points. In addition, we assessed the ability of PBMCs from each subject to recognize the in vivo viruses detected, using standard peptide titration assays (Fig. 4

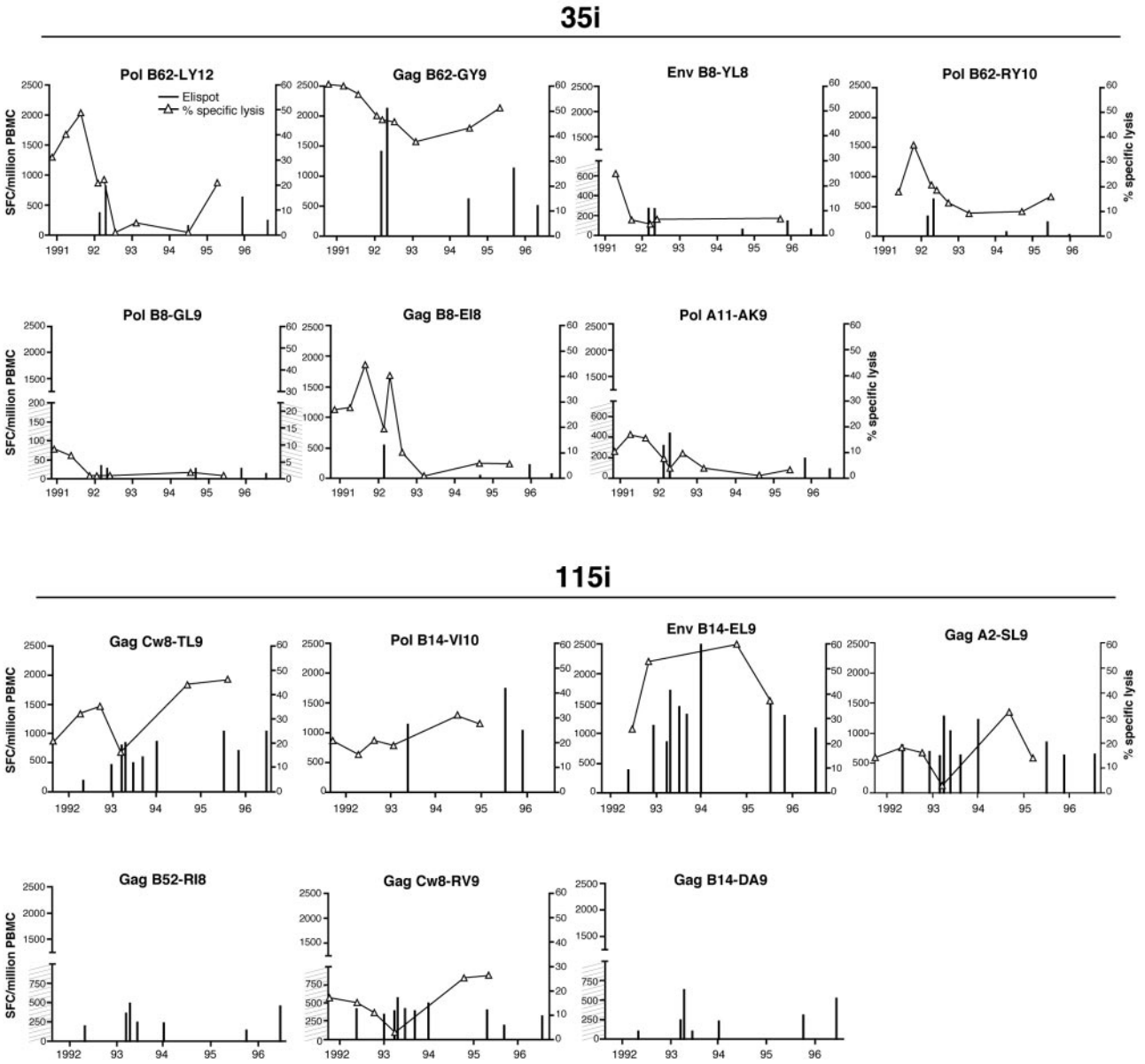


FIG. 2. Longitudinal IFN-γ Elispot and cytolytic responses for each epitope in subjects 35i and 115i. The cumulative magnitude of Elispot responses in spot-forming cells/$10^6$ PBMCs (black bars) is indicated on the left *y* axis. The magnitude of specific lysis (open triangles) is indicated in percentages on the right *y* axis. Discontinuity of the *y* axis scaling is highlighted by gray hash marks.

and 5). Of the seven epitopes analyzed in subject 35i (Fig. 4), three remained unchanged from the clade B consensus sequence for the entire duration of follow-up (B62-LY12, B62-GY9, and B8-YL8), yet the direct lytic PBMC response to B62-LY12 and B8-YL8 declined markedly over this time period. The remaining four epitopes in subject 35i (B62-RY10, B8-EI8, B8-GL9, and A11-AK9) were similarly not observed to evolve over time, although in these the autologous viruses exhibited a single amino acid change compared to the consensus clade B sequence. Since this difference was static, evolving epitope mutations could not account for the decline in in vivo-activated responses. This conclusion was further supported by peptide titration curves comparing autologous sequence to the consensus clade B sequence for these four epitopes. These assays revealed that one autologous variant was equally well recognized (B8-EI8), one autologous variant was slightly better recognized (B62-RY10), and one autologous variant was slightly less well recognized at low peptide concentrations but better recognized at high peptide concentrations (B8-GL9) than the consensus sequence. Only one of these autologous epitopes was not recognized at all (A11-AK9), consistent with it being a CD8 T-cell escape variant. This epitope contained a K-R substitution at position 9 from the earliest time point examined and did not vary over time. Examination of the longitudinal lytic assays and IFN-γ assays against the consensus peptide, however, indicated persistence

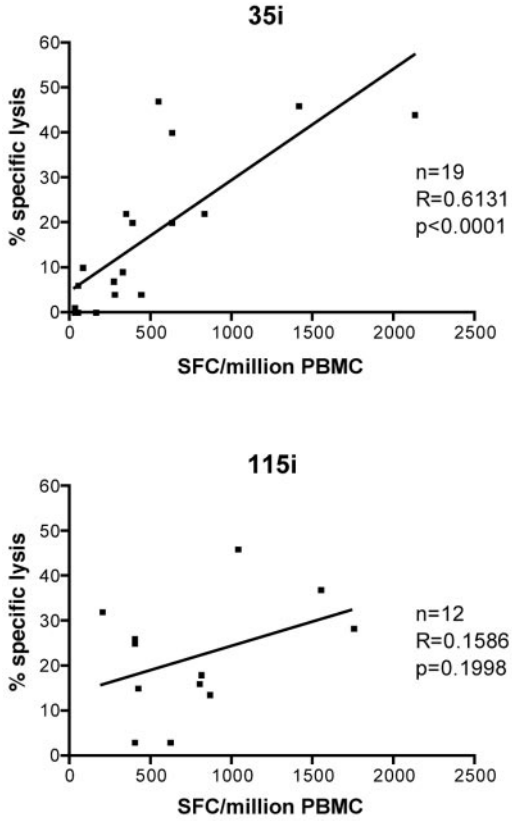


FIG. 3. Correlation between the magnitude of IFN-γ-secreting HIV-1-specific CD8 T cells and their corresponding cytotoxic activities in the two study subjects. All time points were included for which both functional assays were performed.

of the IFN-γ response and loss of the direct lytic response (Fig. 2). Thus, over the course of follow-up in this person with declining CD4 T-cell counts and stable viral load, there was little evidence of viral evolution within persistently targeted epitopes and yet marked differences in the in vivo bulk lytic activity that could not be linked to changes in viral sequence. Moreover, the lack of sequence changes in flanking epitopes (data not shown) suggested that the lack of targeting was not due to differences in antigen processing over time.

Similar detailed analyses were performed in the second subject, 115i (Fig. 5). Two epitopes remained unchanged compared to consensus sequence over time (Cw8-TL9 and B14-VI10), despite the fact that both appeared to be under strong CTL selection pressure as evidenced by persistent in vivo bulk lytic responses in $^{51}$Cr release assays. One epitope exhibited no evolution but contained a single amino acid substitution compared to consensus (B14-EL9). In peptide titration assays the variant was clearly less well recognized, with a 50% sensitizing concentration of peptide by IFN-γ assay (34) of 4.2 μM compared to 0.42 μM for the consensus peptide, consistent with previously published data from this subject using CTL clones specific for this epitope (23). Even though this variant epitope appeared to be less well recognized, a strong in vivo lytic response persisted at each time point tested. Two epitopes (B14-DA9 and B52-RI8) exhibited one or two amino acid mutations at the first time point and yet reverted largely to consensus sequence over time in the continued presence of an Elispot response to the wild-type epitope. For only two of the seven epitopes in subject 115i (A2-SL9 and Cw8-RV9) was there evidence of ongoing immune escape, as shown by lack of CD8 T-cell recognition of the dominant in vivo epitope. However, for both epitopes there persisted in vivo a strong cytolytic and IFN-γ response to the infrequently detected wild-type epitope.

Together, these data indicate that of the 14 persistently targeted epitopes evaluated longitudinally over the course of chronic infection, the sequences within the epitopes remained relatively fixed despite persistent immune recognition, which in some cases declined in direct lytic assays but always persisted by IFN-γ assay. Of the 14 epitopes, only 4 represented potential immune escape variants (1 of 7 in subject 35i, 3 of 7 in subject 115i), but in each case a response to the consensus sequence persisted.

## DISCUSSION

In this detailed longitudinal study examining multiple CD8 T-cell epitopes in two subjects, we find that the sequence evolution within persistently targeted CTL epitopes is uncommon in the chronic phase of HIV-1 infection. The majority of CD8 T-cell epitopes remained largely unchanged over time, despite the continued presence of a CD8 T-cell response, often of sufficient magnitude to be detectable in direct lytic assays using fresh PBMCs. Moreover, only 4 of 14 targeted epitopes targeted by these subjects in the chronic phase of infection were consistent with immune escape. Even in the presence of such apparent immune escape, a persistent CD8 T-cell response to the consensus B clade sequence by IFN-γ production was detected. The most striking change over time was the magnitude of the in vivo-activated CTL response in subject 35i, as measured by direct lysis of HIV-expressing target cells using freshly isolated PBMCs. However, the decline in magnitude of these responses could not be linked to immune escape, but rather suggested a progressive functional defect in this person with CD4 T-cell numbers declining from 200 cells/mm$^3$ to less than 20 cells/mm$^3$. Overall, these data provide the picture of a relatively static relationship between virus and host immune response in the chronic phase of infection, in marked contrast to what has been observed in longitudinal studies in acute infection (8, 13, 28, 34, 38).

The reasons for the lack of viral evolution in the majority of targeted CD8 epitopes over time despite the presence of strong in vivo-activated CTL responses are unclear. All epitopes studied here have been shown to tolerate sequence changes, based on sequences submitted to the Los Alamos Sequence Database (http://hiv-web.lanl.gov). However, of the five epitopes that remained homologous with consensus clade B sequence throughout the period of observation (35i, B62-LY12, B62-GY9, and B8-YL8; 115i, Cw8-TL9 and B14-VI10), all except B8-YL8 are more than 90% conserved among clade B sequences in the Los Alamos Sequence Database, and most are highly conserved across other clades, suggesting constraints on sequence evolution within these regions. The use of non-autologous viral sequences for initial epitope mapping studies in these two subjects may have resulted in a failure to detect responses against more variable regions and thus a bias towards analysis of more conserved epitopes (3). The epitope that showed the most variability over time, A2-SL9 in subject

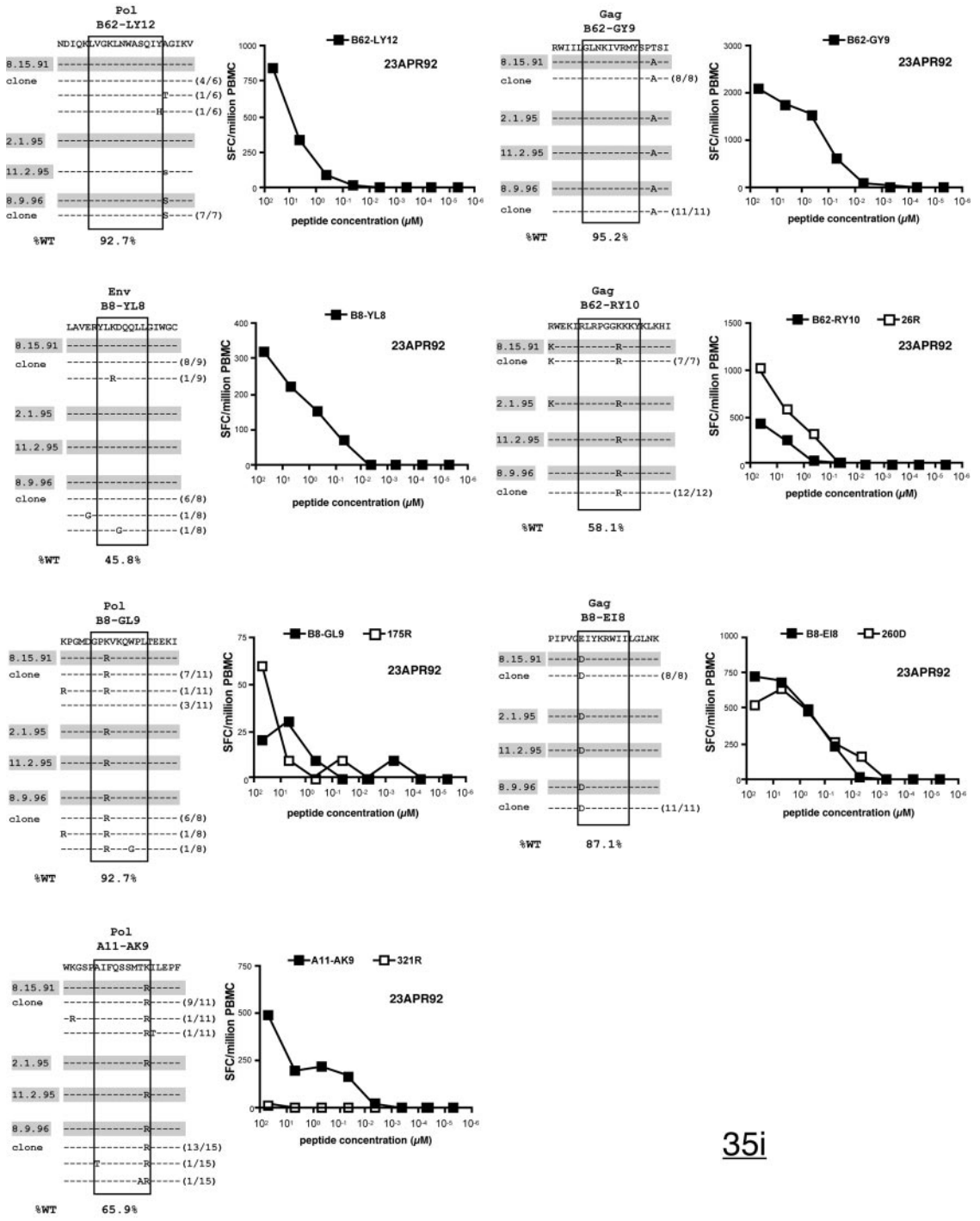


FIG. 4. Longitudinal sequence data and peptide titration curves at limiting peptide concentrations for seven epitopes in subject 35i. Shaded sequences represent bulk sequence data. The percentage of reported consensus sequences for each epitope is shown and is based on the Los Alamos Sequence Database (http://hiv-web.lanl.gov). The date of peptide titration assay is indicated.

115i, was also the most highly polymorphic among all epitopes examined in this study, with only 32% of submitted sequences corresponding to the consensus sequence. However, the likelihood that this variability is driven by immune selection pressure is unclear, since similar variability within this epitope is also seen in persons who do not express HLA-A2 (9). While suggesting that this region is tolerant of variation that may occur by nonimmunologic mechanisms, some of the variability within this epitope may be the result of immune selection pressure and subsequent transmission of epitope variants to subjects without the HLA-A2 allele. This is a mechanism whereby immune selection pressure has been described to

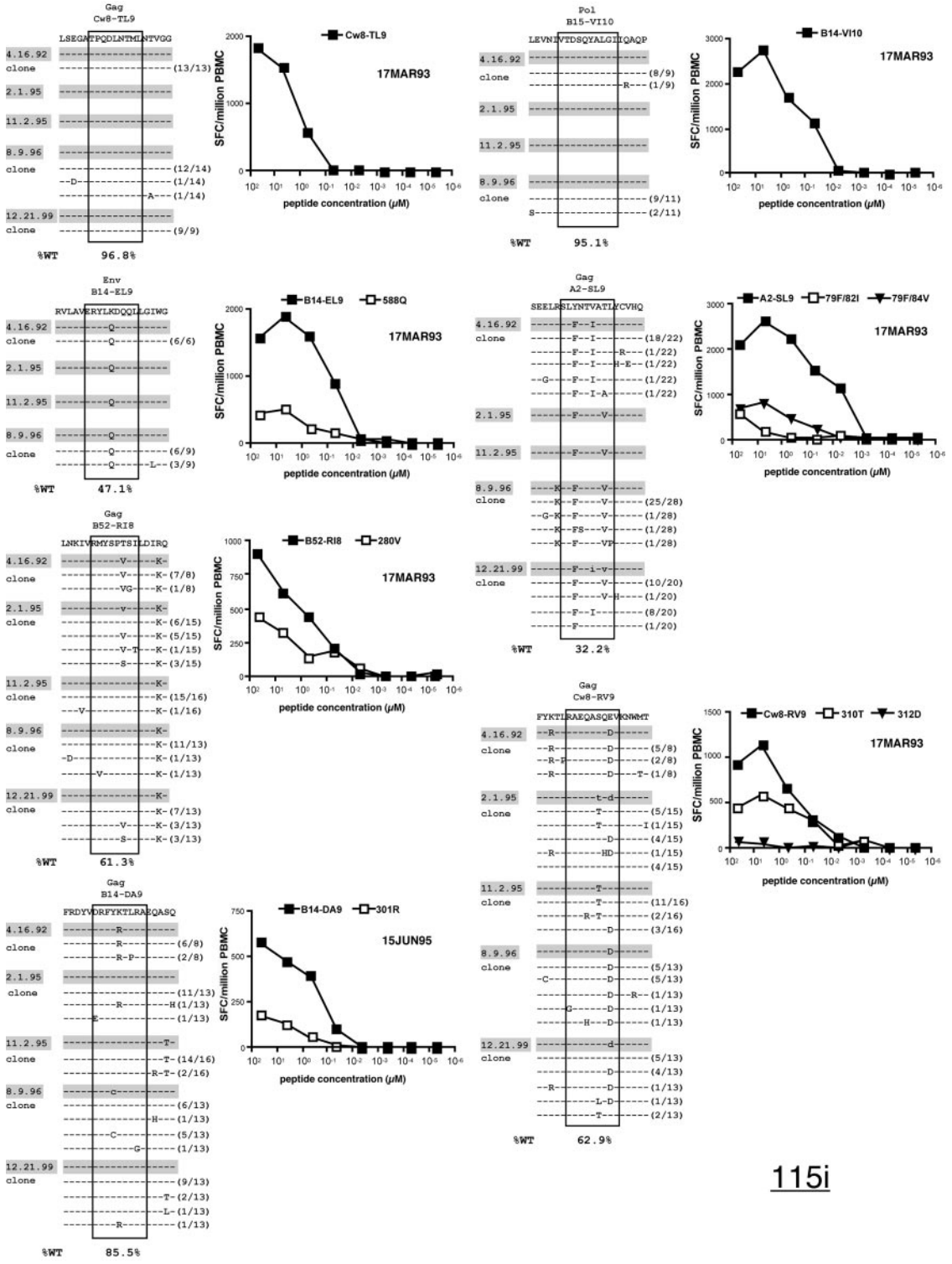


FIG. 5. Longitudinal sequence data and peptide titration curves at limiting peptide concentrations for seven epitopes in subject 115i. Shaded sequences represent bulk sequence data. The percentage of reported consensus sequences for each epitope is shown and is based on the Los Alamos Sequence Database (http://hiv-web.lanl.gov). The date of peptide titration assay is indicated.

shape viral evolution (32). However, other epitopes for which consensus sequences make up less than 50% of reported isolates, indicating tolerance for sequence variation, did not change over time despite continued presence of immune responses. Such observations suggest that the in vivo responses that persist in chronic infection may exert little selection pressure, despite being present in high numbers and clearly activated in vivo.

This study is important to view in light of other studies of viral evolution under immune selection pressure. In the acute stage of HIV-1 and simian immunodeficiency virus infection, viral evolution within targeted epitopes has been well documented (8, 13, 28, 34, 38). Some studies in chronic infection have likewise shown evolution of immune escape, focusing on limited numbers of epitopes (14, 15, 24). A study of A2-restricted Gag and RT epitopes, the former identical to the Gag epitope examined in this study, showed the development of mutations in those persons with initial CD8 T-cell responses to consensus B clade virus and subsequent predictable decline in magnitude of responses (17). In contrast, our data, examining a broader range of epitopes restricted by diverse HLA alleles show persistence of some responses, indeed, persistence of circulating cells with direct cytolytic activity, even when the in vivo virus contains an apparent escape mutation. Moreover, the longitudinal studies herein demonstrate that for the majority of persistent chronic-phase immune responses in these two individuals there is little evidence of ongoing immune-driven viral evolution, even though all of the epitopes investigated have been shown to tolerate sequence change.

Our results support an emerging view that there are functional defects in the chronic stage of HIV-1 infection. Although IFN-γ Elispot assays do not differentiate persons with progressive infection from those with nonprogressive infection (1, 6), the ability of CD8 T cells to proliferate to viral antigen seems to be reserved to those persons with nonprogressive infection (29, 31). In the present study the functional defect in responses is implied, in that despite what should be strong immune selection pressure there is little evidence of ongoing viral evolution, even though these epitopes can tolerate sequence variation. An intriguing possibility is that late-stage infection is characterized by persistence of CTL responses to epitopes that are less efficiently targeted by CD8 T cells, despite detection of high levels in the circulation. Recent studies showing loss of HIV-specific CD4 T cells during the transition to chronic infection provides one potential explanation for this observation (29). Additional evidence supporting a functional impairment of CD8 T-cell responses in vivo comes from the two epitopes in 115i (B52-RI8 and B14-DA9), both of which were consistent with immune escape variants that reverted to wild-type sequences during the course of this study, despite persistence of immune responses to the wild-type epitope.

In several instances we found persistence of Elispot responses, and in some cases strong in vivo-activated CTL responses, even though the predominant in vivo variant was clearly less well recognized than the consensus sequence, consistent with prior studies evaluating recognition of envelope epitopes by CTL clones (23). Based on the knowledge about viral escape in acute and early-stage infection, the CD8 T-cell response normally declines after escape occurs (2, 8, 15, 24), and in chronic infection HIV-1-specific responses decline after initiating HAART (16, 22, 36, 37). However, in the four epitopes (A11-AK9, B14-EL9, A2-SL9, and Cw8-RV9) that represented potential escape mutants, a response to the consensus sequence persisted. It is possible that persistence of responses to the nondetected consensus sequences may be due to episodic epitope reversion (13, 28), or to the phenomenon of original antigenic sin as applied to cellular immune responses (25). These possibilities, which have important implications for prophylactic and therapeutic vaccines, will require further investigation.

In summary, we show persistence of HIV-1-specific CD8 T-cell responses to multiple epitopes in the chronic phase of HIV-1 infection, in some cases in the presence of persistent in vivo-activated CTL; yet, despite this immune pressure, sequence evolution within CTL epitopes is uncommon. Since most of these epitopes are known to tolerate sequence variation, these results emphasize that HIV-1-specific T-cell responses in the chronic phase of infection are strikingly different from those in the acute phase of infection and provide further evidence for a functional impairment in these responses.

## ACKNOWLEDGMENTS

We thank the subjects participating in this study.

This study was supported by the National Institutes of Health (RO1 AI28568 to B.D.W. and R01 AI39966 to S.A.K.), the Japanese Foundation for AIDS Prevention (T.K.), the Doris Duke Charitable Foundation (B.D.W.), and the Howard Hughes Medical Institute (B.D.W.). S.A.K. is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation, and B.D.W. is a Doris Duke Distinguished Clinical Scientist.

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