High Frequency of Cytomegalovirus-Specific Cytotoxic T-Effector Cells in HLA-A*0201–Positive Subjects during Multiple Viral Coinfections

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How the cellular immune response copes with diverse antigenic competition is poorly understood. Responses of virus-specific cytotoxic T lymphocytes (CTL) were examined longitudinally in an individual coinfected with human immunodeficiency virus type 1 (HIV-1), Epstein-Barr virus (EBV), and cytomegalovirus (CMV). CTL responses to all 3 viruses were quantified by limiting dilution analysis and staining with HLA-A*0201 tetrameric complexes folded with HIV-1, EBV, and CMV peptides. A predominance of CMV-pp65–specific CTL was found, with a much lower frequency of CTL to HIV-1 Gag and Pol and to EBV-BMLF1 and LMP2. The high frequency of CMV-specific CTL, compared with HIV-1– and EBV-specific CTL, was confirmed in an additional 16 HLA-A*0201–positive virus–coinfected subjects. Therefore, the human immune system can mount CTL responses to multiple viral antigens simultaneously, albeit with different strengths.

Prior infection with Epstein-Barr virus (EBV) and cytomegalovirus (CMV) is common in individuals infected with human immunodeficiency virus type 1 (HIV-1), and both infections are associated with the development of a number of illnesses. EBV is thought to cause oral hairy leukoplakia [1], interstitial pneumonitis [1], central nervous system (CNS) lymphoma [1, 2], and leiomyosarcomas infection in AIDS patients [3]. CMV infection may cause retinitis, hepatitis, and CNS disorders [4, 5]. Coinfection by either EBV or CMV can affect HIV-1 virus load by several mechanisms, including transactivation of HIV-1 long terminal repeat [6, 7] and increased rate of progression to AIDS [8].

Cytotoxic T lymphocytes (CTL) are important in the control of viral infections. A strong CTL response has been implicated in protection against HIV-1 infection in humans [9–14] and simian immunodeficiency virus (SIV) infection in macaques [15–18]. An inverse correlation between virus load and HIV-1–specific CTL precursor (CTLp) frequencies in subjects with HIV-1 infection [10] and between CTL activity and provirus load in those with HIV-2 infection [19] has been observed. A selective reduction of HIV-1–specific CTLp frequency occurs in the advanced stages of HIV-1 infection [20, 21]. More recently, an inverse correlation was observed between HIV-1 RNA load and the number of effector CTL cells (CTLe cells) stained with HLA-A*0201 HIV-1 Gag and Pol tetramers [12]. Tetramer technology not only allows direct visualization of CTL effector cells by means of flow cytometry, but also can be used to quantify CTL without use of in vitro culture, which is necessary for limiting dilution assay (LDA). The existence of viral escape mutants also supports the concept of CTL control of viral replication [9, 22, 23]. In addition, CTL activity has been associated with protection against other human viral infections, including EBV [24] and CMV [25–27] infection.

$CD8^+$ CTL recognize foreign antigens in the context of major histocompatibility complex (MHC) class I molecules expressed on the surface of antigen presenting cells (APCs). The amount of antigen expressed on the surface of APCs may determine the functional status of the T cells [28, 29] and also the generation and maintenance of CTL [30–33]. Coinfection by heterologous viruses in lymphocytic choriomeningitis virus (LCMV)–infected mice resulted in a constant reduction of LCMV-specific CTLp [34]. This phenomenon is thought to be important in the maintenance of lymphoid system homeostasis.

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Informed consent was obtained from all subjects, and human experimentation guidelines of the US Department of Health and Human Services and of the institutional review board of The Rockefeller University were strictly followed in the conduct of clinical research.

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In humans, the effects of viral coinfections on HIV-1–specific CTL are not well documented. A few studies have measured EBV-specific CTL responses in HIV-1–infected individuals [20, 35, 36], but none have also examined CMV-specific CTL responses. As simultaneous infection with several viruses may present antigenic competition and reduce the HIV-1–specific response, it is important to ascertain whether EBV and CMV coinfections will influence the development of HIV-1–specific CTL.

We identified a patient (SC-15) who had been coinfected with HIV-1, EBV, and CMV and examined his clinical, virological, and immunological parameters over 20 months. In addition, we performed a longitudinal quantitative analysis of viral-specific CTL responses over 20 months by use of 2 different methods: (1) LDA for HIV-1–, EBV-, and CMV-specific CTLp and (2) tetramer staining for HLA-A*0201–restricted HIV-1–, EBV-, and CMV-specific CTLe. Dominant HLA-A*0201–restricted CMV-specific CTLe were found in patient SC-15 throughout the 20-month follow-up period, and a dominance of CMV-specific CTLe was further observed in an additional 16 patients at different stages of HIV-1 infection.

Materials and Methods

Study subjects. Subject SC-15 is a 30-year-old homosexual man. He first presented to his primary care physician with signs of primary viral infection and was determined to be HIV-1 positive by serologic analysis and Western blot testing in May 1996. A persistent lymphadenopathy invoked further laboratory examinations. CMV serologic analysis was performed by use of the Sorin Capture assay (CSL, Melbourne, Australia), yielding results consistent with either primary infection or reactivation of a recent infection. EBV serologic analysis was done by use of a component system, with antigen and control from Gull (Salt Lake City) and detection reagents from Silenus Laboratories (Melbourne) or Sigma (St. Louis). The HLA type of SC-15 is HLA-A2, A14, B44, DR07, DR12. The patient began antiretroviral therapy with stavudine, lamivudine, and saquinavir in June 1996 and continued on this therapy throughout the 2-year study period. The additional 16 patients included in this study were HLA-A*0201 positive and were chosen from various study cohorts established at the Aaron Diamond AIDS Research Center, New York. EBV and CMV infection were confirmed by serologic testing.

Measurement of HIV-1 virus load. Viral RNA copy numbers in plasma were determined either by reverse-transcriptase polymerase chain reaction on samples by use of the Amplicor HIV-1 monitor kit (Roche Molecular Systems, Branchburg, NJ), which has a detection limit of 400 copies/mL [37], or by the bDNA signal-amplification assay (Chiron, San Francisco), which has detection limit of 50 and 500 Eq/mL.

Recombinant vaccinia viruses. Recombinant vaccinia expressing HIV-1 antigens were vAbT 299 (IIIBenv-gp160), vAbT 141 (IIIBgag-p55), vAbT 204 (IIIBpol), and rVV-IIIBnef, obtained from Dr. G. Mazzara (Therion Biologics, Boston) [11]. The EBV antigens expressed by recombinant vaccinia were EBNA3a, EBNA3b, EBNA3c, LMP2, BRLF1, and BMLF1, and the CMV antigens expressed by recombinant vaccinia were pp65, pp150, gB, and IE1. The control vaccinia used for all the experiments was a tk-virus in which the thymidine kinase gene was inactivated by insertion of a vector (pSC11) into the WR strain.

Establishing primary fibroblast cell lines. Primary fibroblast cell lines were established according to a procedure described elsewhere [27, 38]. Briefly, skin biopsy samples (4–5 mm^2, 0.5–1 mm depth) were taken from the inside of the upper arm under 1% lignocaine local anesthesia. The skin biopsy samples were processed further into smaller sections and cultured in petri dishes under a glass cover slip in MEM enriched with fetal calf serum. Outgrowth of fibroblasts occurred within 7–10 days, and a fibroblast line was subsequently established.

LDA. Three sets of LDA plates were set up simultaneously by use of cryopreserved peripheral blood mononuclear cells (PBMC), each with 8 input cell dilution, with a range of 0–32,000 cells/well. In addition, 2.5×10^4 gamma-irradiated allogeneic PBMC and 0.1 μg/mL anti-CD3 (clone 12F6) were added to each well. The cultures were kept growing for 14 days with twice a week medium change, using standard RPMI-1640 supplemented with 100 U/mL interleukin (IL)–2 and 15% fetal calf serum. A standard 4-h ^{51}Cr release assay was performed on days 13 and 14: each LDA plate was split 6 ways and assayed against autologous primary fibroblasts that had been infected with either control vaccinia or recombinant vaccinia expressing HIV-1, EBV, or CMV antigens. A well was regarded as positive for specific CTL activity only when the percentage of specific lysis exceeded 10% and was 10% more than that of the corresponding control target cells. The antigen-specific CTLp frequencies, after subtracting the background lysis to target cells infected with control recombinant vaccinia, were determined by use of the maximum likelihood method by means of a Microsoft Excel spreadsheet (kindly provided by Dr. S. Kalams, Massachusetts General Hospital, Boston), and the results were expressed as the number of CTLp per 10^6 input $CD8^+$ PBMC.

MHC class I tetramer staining. MHC class I tetramers were synthesized according to methods described elsewhere [12 39] and were folded with each of the following peptides: HIV-1–Gag (77-85: SLYNTVATL); Pol (476-484: ILKEPVHGV); EBV-LMP2 (426-434: CLGGLLTMV); BMLF1 (280-288: GLCTLVAML); and CMV-pp65 (495-503:NLVPMVATV). Cryopreserved PBMC from different time points were thawed and were stained with phycoerythrin-conjugated tetramers in conjunction with CD38 fluorescein isothiocyanate and CD8Tricolour (Caltag, Burlingame, CA). The triple-color stained PBMC samples were analyzed by use of a flow cytometer (Calibur; Becton Dickinson, San Jose, CA), and results were presented as percentage of $CD38^+$ and tetramer-positive cells in the $CD8^+$ cell population. The statistical differences between all groups of patients were analyzed by use of a standard single factor analysis of variance (ANOVA) test and a nonparametric Kruskal-Wallis test [40].

Results

Coinfection with HIV-1, EBV, and CMV in subject SC-15. Subject SC-15 was negative for HIV-1, EBV, and CMV by serology in February 1996. He first presented to his primary care physician with signs of primary viral infection in May 1996,

and repeated serology and Western blot testing yielded a diagnosis of HIV-1 positivity. A persistent lymphadenopathy invoked further laboratory examinations, which found him to be EBV VCA IgG equivocal and IgM positive by one test on 21 June and another test on 25 June, and subsequently, on 8 July 1996, he was found to be IgG positive and IgM negative. His CMV serology followed a similar course, with IgG positive and IgM equivocal by one test on 21 June and both IgG and IgM positive on 25 June. This CMV serological profile is suggestive, but not affirmative, of a primary infection; nonetheless, it is also consistent with reactivation of a recent infection. Subsequently he was IgG positive and IgM negative on 8 July 1996 (table 1). The patient was treated with antiretroviral therapy (stavudine, lamivudine, and saquinavir) on 25 June 1996.

Combination antiretroviral therapy controlled HIV-1 replication and resulted in rapid resolution of CD8$^+$ lymphocytosis. The virological and immunological changes in patient SC-15 were similar to those in others who have been treated successfully with combination therapy [41]. Within 3 weeks of the initiation of antiretroviral therapy with stavudine, lamivudine, and saquinavir, his plasma HIV-1 RNA levels dropped by >2 logs from 140,000 copies to <770 copies/mL. Additional therapy further reduced the HIV-1 RNA load to <400 copies (the level of detection) within 3 months (figure 1*A*). Over the same period, his CD8$^+$ cell count declined from 4080 to 920/mL, then remained at a stable level for ~12 months; meanwhile, although the absolute CD4$^+$ cell count fluctuated, his CD4 : CD8 ratio was approaching 1 : 1 (figure 1*B*). To determine the impact of antiretroviral therapy on the generation of CTL and the possible interference in the generation of HIV-1–specific CTL by other viral coinfections, we quantified his CTLp frequency specific for HIV-1, EBV, and CMV.

Broadly reactive CTL were generated during primary HIV-1 infection. To determine the impact of EBV and CMV coinfections on the normal development of the HIV-1–specific CTL response during primary infection, we did a longitudinal quantitative study of the HIV-1–specific CTLp, using the LDA. Moderate levels of CTL (CTLp from 38 ± 7 per 10^6 CD8$^+$ PBMC) specific for HIV-1 Env, Gag, and Pol antigens were

Table 1. Clinical diagnosis of coinfections in subject SC-15.

Virus	Laboratory tests (result)	Date
HIV	Negative	2/1/96
	Ab (+), Ag (+), WB (+)	5/22/96
	Ab (+), Ag (+), WB (+)	5/29/96
	Viral culture (+)	6/25/96
EBV	Negative	2/1/96
	IgM (+), IgG (?)	6/21/96
	IgM (+), IgG (?)	6/25/96
	IgM (−), IgG (+)	7/8/96
CMV	Negative	2/1/96
	IgM (?), IgG (+)	6/21/96
	IgM (+), IgG (+)	6/25/96
	IgM (−), IgG (+)	7/8/96

NOTE. CMV, cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; +, test positive; −, test negative; ?, test equivocal.

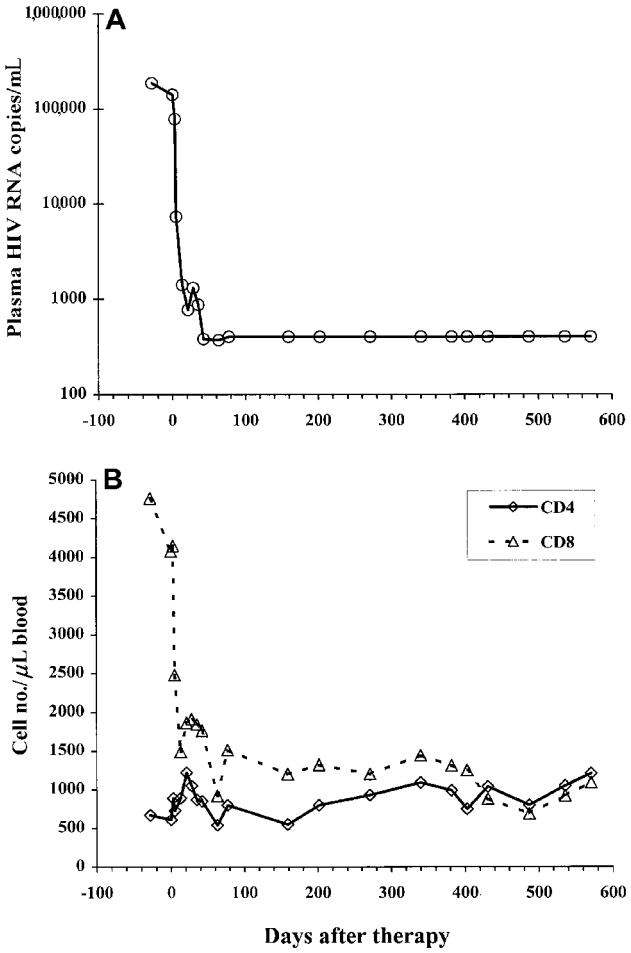

Figure 1. Clinical information over time. *A,* Plasma human immunodeficiency virus (HIV) viral load measurement was performed by use of a commercial reverse transcriptase–polymerase chain reaction method; *B,* CD4$^+$ and CD8$^+$ cell counts were determined by use of a routine clinical method, as described in Materials and Methods.

detected on the day of initiation of combination antiretroviral therapy. The number of HIV-1–specific CTL declined rapidly over the first few weeks, concomitant with the precipitous drop in virus load during the same period. However, the magnitude of these CTLp frequencies rebounded back to 50% of the starting levels soon afterward and remained at relatively stable levels for the next 20 months (figure 2 *A, B, C*). The time of rebound overlaps with the time of a transient virus load increase at week 4 to 5, so a boosting of the preexisting memory CTL might have occurred. Nef-specific CTLp were also detected at lower levels (figure 2*D*). Thus, simultaneous coinfection by 2 other herpesviruses did not have significant interference with the normal course of development of the HIV-1–specific CTL in this patient.

Sequential induction of the EBV-specific CTLp to various viral antigens. In contrast to the broadly reactive HIV-1–specific

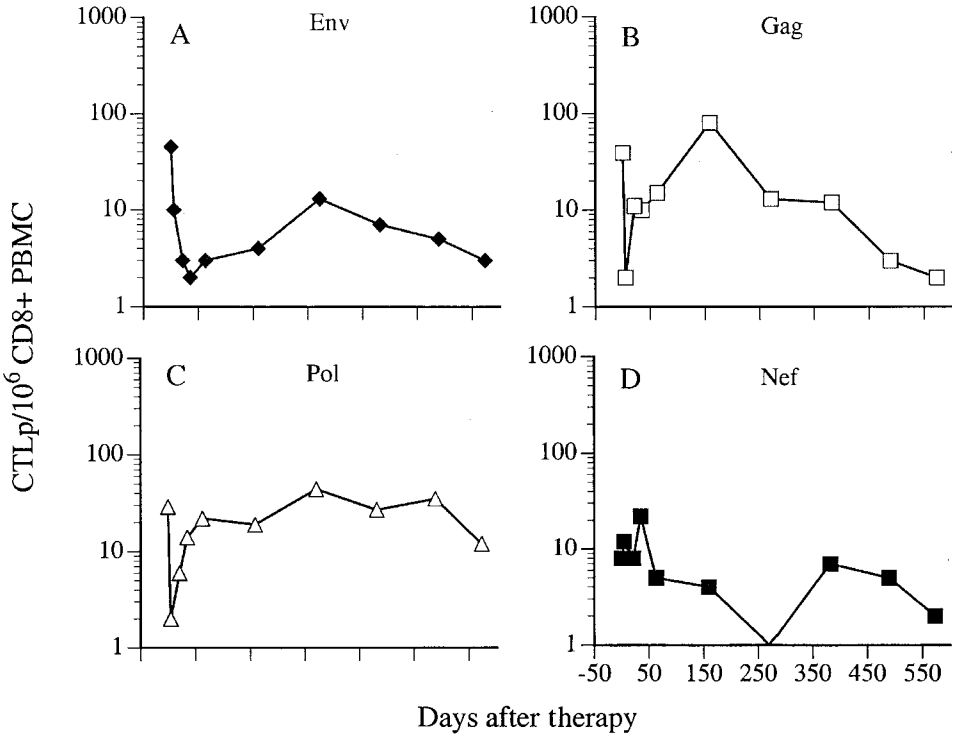

Figure 2. Detection of human immunodeficiency virus type 1 (HIV-1)–specific cytotoxic T lymphocytes (CTL). CTL precursor (CTLp) frequency was determined by use of limiting dilution assay (LDA). Effector cells were peripheral blood mononuclear cells (PBMC) cultured under LDA conditions; target cells were autologous primary fibroblasts that had been infected with either control vaccinia or recombinant vaccinia expressing HIV-1 antigens. *A,* IIIBenv-gp160; *B,* IIIBgag-p55; *C,* IIIBpol; and *D,* IIIBnef. All CTLp frequencies were presented as per 10^6 $CD8^+$ T cells.

CTL generated during the first 2–3 weeks, the EBV-specific CTL activity that developed was focused on the early viral antigen, primarily to BRLF1 (figure 3*A*). Over time, however, their dominance gave way to CTL reactive to other EBV antigens, LMP2 and EBNA3b/3c (figure 3*B*); in addition, EBNA3b seems to contain the dominant CTL epitopes among these antigens (figure 3*B*).

CTL to IE1 epitopes were detected during coinfection. CMV-specific CTL activity during coinfection with EBV and HIV-1 has not been described elsewhere; therefore, this patient provided a unique opportunity for examining the kinetics of both the induction of CMV-specific CTL and the impact of antiretroviral therapy on the development of CMV-specific CTL during coinfection. As shown in figure 4, CTL specific for the IE1 antigen were generated during the first week of therapy and remained at appreciable levels throughout the 20-month study period. In contrast, there was a delay of 1–2 weeks for CTL to other structural components, namely, gB, pp65, and pp150, to reach peak levels. In addition, the absolute numbers of CTLp to these structural proteins were less than for IE1.

The most abundant CTLe during the triple coinfections were CMV specific. Although the LDA has been widely used for quantifying memory CTL, a new technology, staining antigen-specific $CD8^+$ T cells with MHC tetramers, has proved to be a powerful alternative for quantifying CTLe [12, 39]. We therefore decided to examine the correlation between the number of CTL determined by these 2 methods. PBMC samples were stained with 5 HLA-A*0201 tetramers folded with different immunodominant peptides: (1) HIV-1-Gag (77-85: SLYNTVATL); (2) HIV-1-Pol (476-484: ILKEPVHGV); (3) EBV immediate early antigen BMLF1 (280-288: GLCTLVAML); (4) EBV latent antigen LMP2 (426-434: CLGGLLTMV); and (5) CMV structure protein antigen pp65 (495-503: NLVPMVATV). There was a dominance of HLA-A*0201–restricted CMV-specific CTLe, as high as 4.4 % of total $CD8^+$ cells, whereas the frequencies of CTLe specific for HIV-1 and EBV were much lower (figure 5). To test whether CTLp recognized the same peptides used in the CTLe tetramer assay, LDAs were set up by using conventional polyclonal stimulation with anti-CD3 and then assayed against peptide-pulsed target cells. The results demonstrated a similar CTLp frequency to all the peptide antigens (data not shown).

Because the tetramer staining for the HLA-A*0201 CMV-specific CTLe was the most prominent among the 5 tetramers used, we further followed the quantity of these cells with the CMV tetramer. As shown in figure 6, there was a rapid decline in the number of tetramer-positive cells during the first few

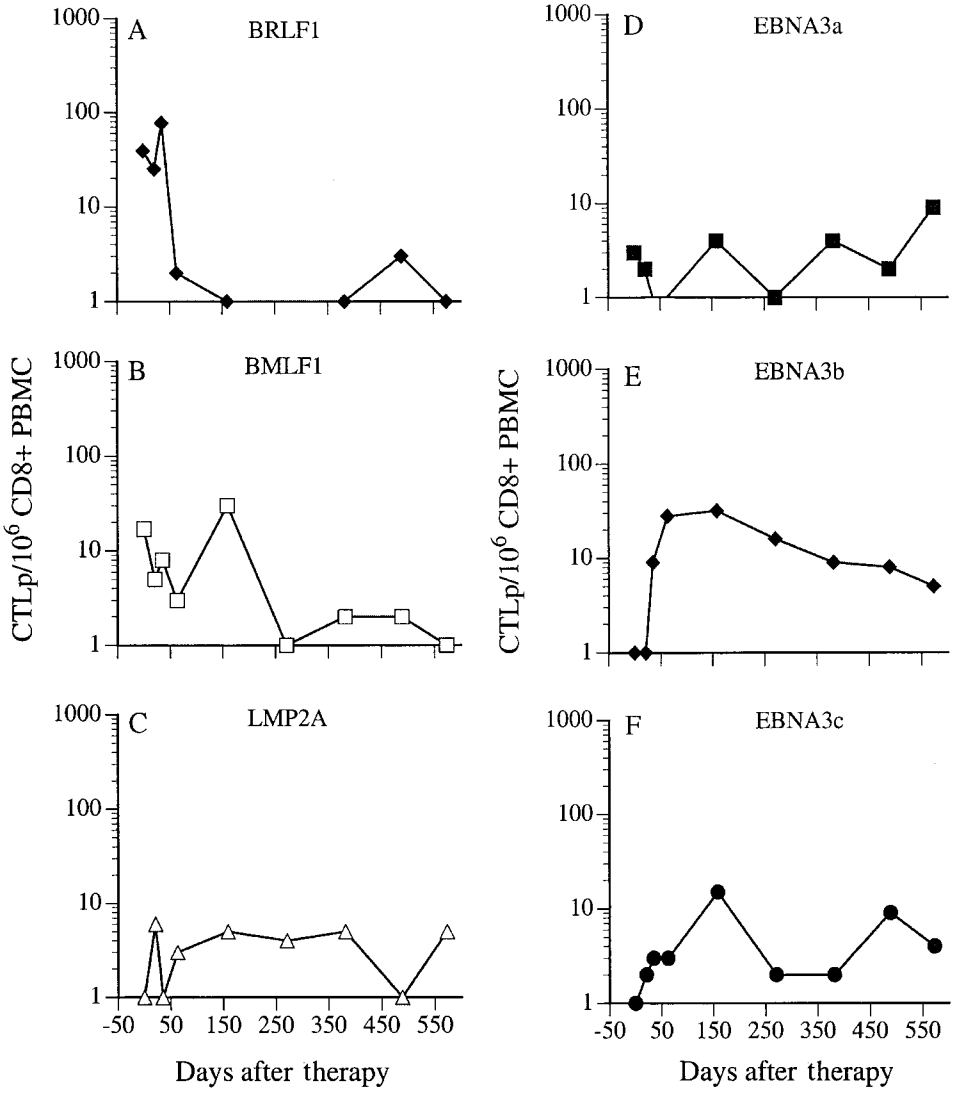

Figure 3. Detection of Epstein-Barr virus (EBV)–specific cytotoxic T lymphocytes (CTL). The following EBV antigens were included: *A,* BRLF1; *B,* BMLF1; *C,* LMP2; *D,* EBNA3a; *E,* EBNA3b; and *F,* EBNA3c. CTLp, CTL precursor; PBMC, peripheral blood mononuclear cells.

days of antiretroviral therapy. Their number was then stabilized at ~25%–50 % of the peak level for the next 20 months.

Dominance of CMV-specific CTLe in HIV-1, EBV, and CMV coinfections. Having shown the dominance of CMV-specific CTLe in 1 patient, we hypothesized that this might be a more generalized phenomenon. Because prior EBV and CMV infections are common in the general population [42–44], the majority of HIV-1–infected individuals are likely to harbor EBV and CMV viruses. We therefore selected 16 HIV-1–infected HLA-A*0201 individuals for a cross-sectional study of CTLe frequency using tetramers to HIV-1, EBV, and CMV. The patients selected were a mixture of acute seroconverters and chronically infected individuals. Some of the samples were obtained before the initiation of antiretroviral therapy, others after (table 2). Those patients with CTLe for all 3 viruses are presented in figure 7. Pre- and posttreatment data were analyzed by use of the same statistical methods. A standard single factor ANOVA showed a significant difference between the pretreatment data on the mean percentage of $CD8^+$ cells stained with tetramers for A*0201/Gag, A*0201/Pol, A*0201/BMLF1, A*0201/LMP2, and A*0201/CMV ($P < .01$). Pairwise multiple comparison (Dunnett's method) of the A*0201/CMV group mean and all other means showed that the A*0201/CMV mean is significantly different from the other means, at $P < .001$. A nonparametric Kruskal-Wallis test of the 5 groups was significant at $P < .05$. For the posttreatment data on the mean percentage of $CD8^+$ cells stained with tetramers for A*0201/Gag, A*0201/Pol, A*0201/BMLF1, A*0201/LMP2, and A*0201/CMV, a

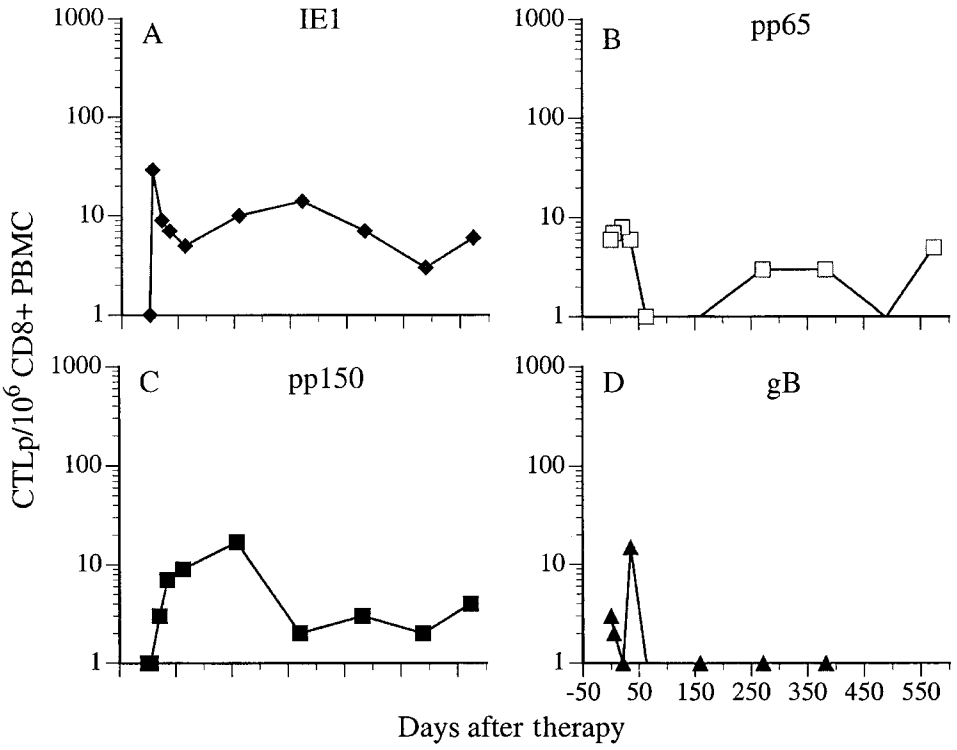

Figure 4. Detection of cytomegalovirus (CMV)–specific cytotoxic T lymphocytes (CTL). The following CMV antigens were included: *A,* IE1; *B,* pp65; *C,* pp150; and *D,* gB. CTLp, CTL precursor; PBMC, peripheral blood mononuclear cells.

standard single factor ANOVA revealed a significant difference at $P < .025$. Pairwise multiple comparison showed that the A*0201/CMV group mean was significantly different from the other means at $P < .001$. A nonparametric test could not be performed, because of the low number of data points per group.

Discussion

Although EBV and CMV coinfections are common in individuals with HIV-1 infection, it is extremely rare to see patients with primary HIV-1, EBV, and CMV coinfections. In

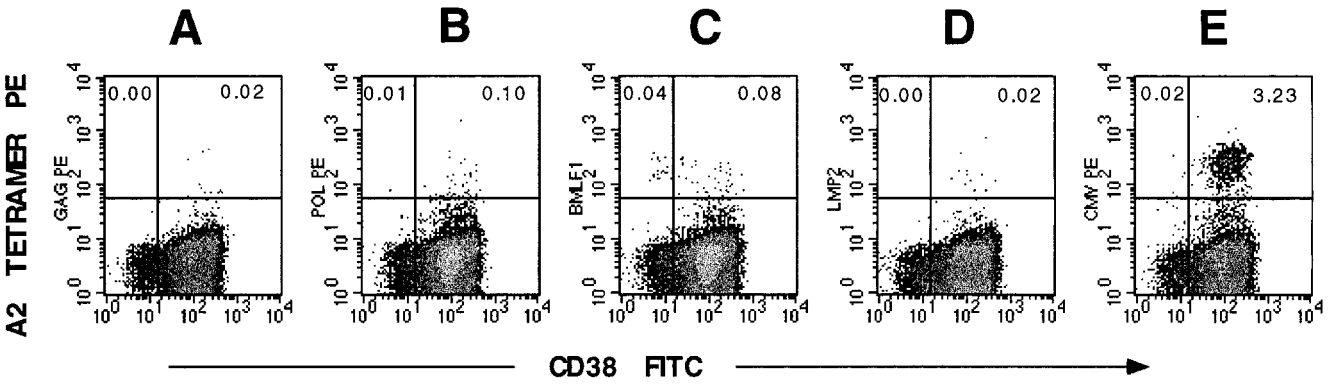

Figure 5. Cytomegalovirus (CMV)–specific effector cytotoxic T lymphocytes (CTLe) were the dominant effector cells during coinfections in patient SC-15. Peripheral blood mononuclear cells (PBMC) during the first week of therapy were stained with the following: *A,* human immunodeficiency virus type 1 (HIV-1)–Gag; *B,* HIV-1–Pol; *C,* Epstein-Barr virus (EBV)–BMLF1; *D,* EBV-LMP2; and *E,* CMV-pp65 tetramers, as described in Materials and Methods. Data presented are percentage of tetramer-positive $CD8^+$ T cells. FITC, fluorescein isothiocyanate.

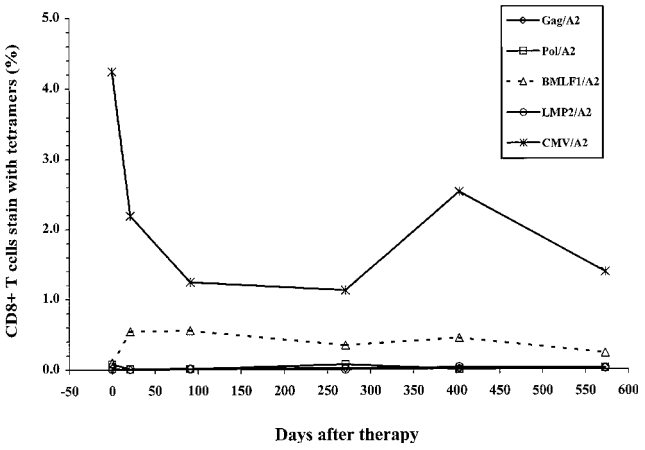

Figure 6. Cytomegalovirus (CMV)–specific effector cytotoxic T lymphocytes (CTLe) persisted at high levels for 20 months in patient SC-15. Sequential samples were stained with human immunodeficiency virus type 1 (HIV-1)–Gag (◇), HIV-1–Pol (□), Epstein-Barr virus (EBV)–BMLF1 (△), EBV-LMP2 tetramers (○), and CMV-pp65 (●), as described in figure 5 legend. Results presented are percentage of tetramer-positive $CD8^+$ T cells.

mice it was demonstrated that immunodominance of 1 CTL epitope over the others was established during primary lymphocytic choriomeningitis virus (LCMV) infection [45]. However, subsequent infection by heterologous viruses perturbs the existing memory CTL response to the initial inoculating LCMV [34]. This case offered a unique opportunity to examine CTL responses to multiple viral coinfections. We observed that CTL responses to HIV-1, EBV, and CMV developed simultaneously. Therefore, the host immune system has the potential of coping with a diversity of viral infections simultaneously during primary infection. The kinetics of these CTL responses, however, differed from each other. In addition, by studying the prevalence of CTLe to various viral antigens in an additional 16 HLA-A*0201–individuals with HIV-1, EBV, and CMV coinfections, we demonstrated that CMV-pp65–specific CTL were the most prevalent effector cells among all effector cell populations.

The CTL response to multiple HIV-1 antigens, including Env, Gag, Pol, and Nef, developed early in subject SC-15. These observations are in agreement with published data [9, 11]. Our study, however, further illustrated the kinetics of HIV-1–specific CTL during antiretroviral therapy. After the initial appearance of a broadly reactive CTL response, the level of these CTLp declined rapidly over the first few weeks after the onset of combination therapy, concomitant with the precipitous drop of virus load during the same period. The magnitude of these CTLp rebounded to the starting levels soon afterward and remained relative stable for the next 18 months. The initiation of the CTLp rebound was associated closely with a transient virus load increase, suggesting that this may have boosted the preexisting memory CTL. Another possibility is that coinfection by EBV and CMV leads to more T-cell activation with increased production of cytokines, thereby nonspecifically activating the HIV-1–specific memory CTL. The persistence of these CTL may indicate there are still low levels of viral replication in either the blood or the lymph nodes.

In patient SC-15, EBV and CMV coinfections did not have adverse effects on the normal development and maintenance of HIV-1–specific CTL during primary infection. In contrast to the broadly reactive HIV-1–specific CTL during the first 2–3 weeks, the EBV-specific CTL activity was focused on the early viral antigens, namely, BRLF1 and BMLF1. Over time, however, their dominance gave way to CTL reactive to the late EBV antigens LMP2 and EBNA3b/3c, of which EBNA3b seemed to contain the most dominant CTL epitopes among late antigens. Such a highly prevalent CTL response to EBV early antigens during primary EBV infection has also been observed by others [42, 46]. The rapid expansion and contraction of the number of CTLp is consistent with an EBV-driven CTL evolution process [42, 47]. Furthermore, because the pattern of EBV-specific CTLp fluctuation was independent of changes in the HIV-1 virus load, the antiretroviral therapy did not appear to have a direct impact on the normal course of development of the EBV-specific CTL activity.

The development of CMV-specific CTL response was similar to that of EBV; that is, the initial CTL response was mostly specific for early antigens, followed by responses to antigens encoded by the later structural genes. Memory CTL specific for the IE1 antigen were detected during the first week of therapy and remained at an appreciable level throughout the 20-month study period. In contrast, there was a delay of 1–2 weeks for CTL to other structural components (gB, pp65, and pp150) to reach peak levels. Although CTL responses to both IE1 and

Table 2. Clinical characteristics of patients included in the cross-sectional tetramer staining.

	Time of blood sample			Findings		
Patient ID no.	Date	Stage of infection	Antiretroviral therapy[a]	$CD4^+$ cells/mL	$CD8^+$ cells/mL	Virus load, copies/mL
2004	1/21/97	Acute	Before	47	671	633,230
2003	12/5/96	Acute	Before	596	917	563,760
913	9/4/97	Acute	Before	697	1045	22,420
911	3/13/97	Acute	Before	685	1134	21,800
1013	9/24/96	Chronic	Before	124	1135	786,372
1010	9/11/96	Chronic	Before	284	320	248,433
33	2/9/96	Chronic	Before	557	3101	242,700
1002	9/3/96	Chronic	Before	537	1096	76,916
1004	9/3/96	Chronic	Before	402	660	27,077
1009	9/4/96	Chronic	Before	665	927	22,288
1306	12/2/97	Acute	After	720	1241	99
1309	1/16/98	Acute	After	659	288	99
1302	10/15/97	Acute	After	627	627	99
912	12/11/97	Acute	After	579	434	99
2004	1/14/98	Acute	After	308	1322	61
3	2/21/97	Acute	After	426	352	50

[a] Time of sample relative to the start of antiretroviral therapy.

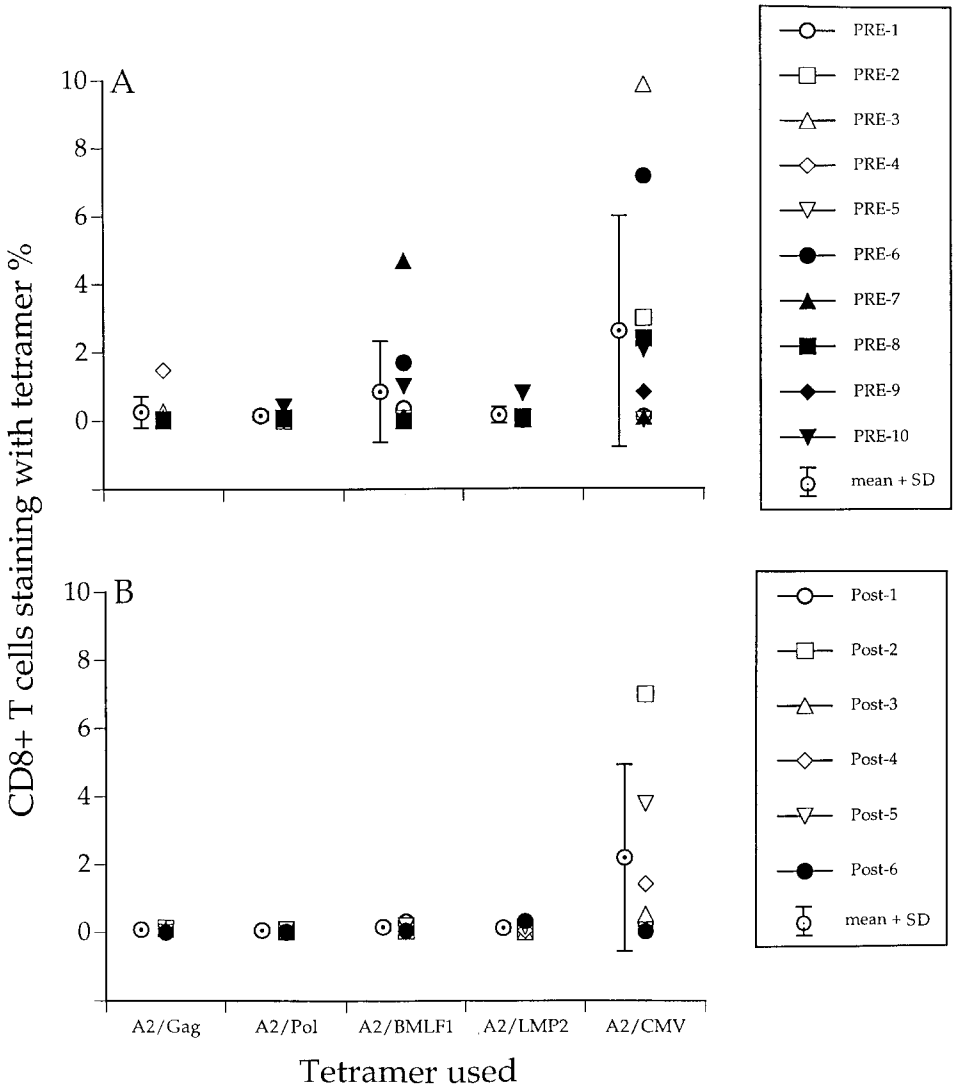

Figure 7. Cross-sectional study revealed the dominance of cytomegalovirus (CMV)–specific effector cytotoxic T lymphocytes (CTLe) in an additional 16 patients. Peripheral blood mononuclear cells (PBMC) from a single time point of each of the patients in table 1 were stained with human immunodeficiency virus type 1 (HIV-1)–Gag, HIV-1–Pol, Epstein-Barr virus (EBV)–BMLF1, EBV-LMP2, and CMV-pp65 tetramers. Results presented are percentage of tetramer-positive $CD8^+$ T cells. "Pre" and "Post" indicate pre- and posttreatment data for the designated patients.

pp65 CMV antigens have been described in healthy, asymptomatic individuals [5, 48], CTL activity during primary CMV infection has not been described previously. It is interesting to note that there were more CTL to pp65 than to IE1 antigen during chronic CMV infection [49]. This discrepancy in the dominant CTL response may be a genuine reflection of the difference in acute versus chronic CMV infection. Indeed, the absolute frequencies of IE1-specific CTL (1–30/10^6 $CD8^+$ PBMC) we observed during acute infection are in the same range as reported by others [27, 38]. The pp65-specific CTLp frequency detected was lower than reported previously [49] and much lower than we would have expected based on staining with the CMV tetramer. This lower than expected pp65-specific CTLp might be the result of the stimulation procedure used in generating memory CTL. We deliberately chose to use a polyclonal anti-CD3 monoclonal antibody to expand all the T cells, because we wanted to quantify all the CTL to all 3 viruses simultaneously. In contrast, others have used either CMV-infected fibroblasts or peptide-labeled BLCL as stimulator cells for the generation of CMV-specific CTL [27, 38, 49]. If tetramers specific for the IE1 epitopes were available, it would be useful to follow CMV-specific CTL responses with both IE1- and pp65-specific tetramers to see what the magnitude of IE1-specific CTLe is in comparison with pp65-specific CTLe. Fur-

thermore, because the kinetics of the CMV tetramer staining cells is similar to that of the total $CD8^+$ cell count (figure 1*B*) but differs from the kinetics of HIV-1–specific CTLp, this result indicates an HIV-1 antigen–independent evolution of CMV-specific CTLe.

One striking finding is the dominance of CMV-specific CTLe in those who have contracted HIV-1, EBV, and CMV triple coinfections. Although memory CTL to all these viruses exist and their frequencies are similar, each has a different rate of expansion into effector cells. One possible explanation is that different viral antigens are processed and presented with different efficiency. Among all permissive cells, the principal target cells for HIV-1 are $CD4^+$ T cells and for EBV are epithelial and B cells [48, 50], whereas for CMV, the target cells are monocytes and macrophages [51, 52]. Whereas B cells and epithelium cells can present antigen, only the macrophage is the professional antigen-presenting cell. Thus, the CMV antigens are likely to be presented to CTL preferentially, given that the other conditions are equal. Furthermore, some of the CMV proteins are associated with MHC class I down regulation [53], which could result in decreased presentation of other viral antigens, therefore resulting in an inhibition of the processing and presentation of other antigens. Because CMV has the advantage of gaining entry to professional antigen presenting cells and a subsequent advantage in antigen presentation, it is not surprising that CTL to CMV are still generated in vivo.

It is also possible that the observed differences in CTLe levels were a reflection of the variable T-helper activities to different viruses. Defects in the HIV-1–specific T-helper cells during primary infection have been well documented; therefore, stronger T-helper responses may have been induced to EBV and CMV than to HIV-1. It is also known that CMV pp65 contains a number of T-helper epitopes [25, 49]. However, whether the pp65-specific T-helper activity was stronger than that of EBV-specific T-helper activity is yet to be determined.

Although the LDA has been the standard method widely used for quantifying the precursors of memory CTL (CTLp), staining antigen-specific $CD8^+$ T cells with MHC tetramers has proved to be a powerful alternative for quantifying CTLe. The number of CTL measured by LDA is very much lower than that measured by tetramer, as is seen in our results. By using these tetramers (including HIV-1-Gag, HIV-1-Pol, EBV-BMLF1, EBV-LMP2, and CMV-pp65), we found that the most abundant CTLe during the triple coinfections were CMV specific, consisting of ~5% of total CD8 cells, whereas the other effector cells were at much lower levels. Because the tetramers define viral CTL recognizing a few dominant epitopes restricted through HLA-A*0201, it is possible that CTL in subject SC-15 recognize HIV-1 and EBV antigens that were not included in the study, or recognize similar epitopes through HLA alleles other than A*0201. HLA-A*0201 may not be a major presentation allele for EBV. It has been demonstrated, in a population where HLA-A11 is highly prevalent, that the endogenous EBV strains in those populations are mutated at A11 anchor residue [24]. HLA-A*0201 reactivity may have followed a similar pattern in the patients we have studied. Our results indicate that CMV accounts for the majority of HLA-A*0201–restricted CTL.

It is interesting to note that the variation of CMV-specific CTLe followed kinetics similar to those of the absolute $CD8^+$ cell count. Specifically, there was a high level of CMV-specific CTLe initially, which rapidly fell during the first few days of the antiretroviral therapy, then stabilized at ~10%–15% of the peak level for the next 6 months. This could be due to a reduction of CMV virus load over the same period, reflecting an antigen-driven CTL evolution, or to a nonspecific event associated with the general reduction of immune activation, as a consequence of the control of HIV-1 replication by antiretroviral therapy.

In this study, we used 2 different methods to quantify HIV-1–, EBV-, and CMV-specific CTL in a patient with triple coinfections. Each method provided complementary information on the dynamics and kinetics of CTL development in response to multiple external pathogens. LDA and the tetramer staining method may not be measuring the same population of cells. Whereas MHC tetramers are more useful in quantifying epitope-specific CTL in subjects with known HLA types, the LDA method is useful to examine the breadth of CTL to a large number of antigens and also to detect memory cells with proliferation potential. For a thorough investigation of host immune potential, both methods are needed.

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