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Virological Outcome after Structured Interruption of Antiretroviral Therapy for Human Immunodeficiency Virus Infection Is Associated with the Functional Profile of Virus-Specific CD8$^+$ T Cells$^\nabla$

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A clear understanding of the antiviral effects of CD8$^+$ T cells in the context of chronic human immunodeficiency virus (HIV) infection is critical for the development of prophylactic vaccines and therapeutics designed to support T-cell-mediated immunity. However, defining the potential correlates of effective CD8$^+$ T-cell immunity has proven difficult; notably, comprehensive analyses have demonstrated that the size and shape of the CD8$^+$ T-cell response are not necessarily indicative of efficacy determined by measures of plasma viral load. Here, we conducted a detailed quantitative and qualitative analysis of CD8$^+$ T-cell responses to autologous virus in a cohort of six HIV-infected individuals with a history of structured interruption of antiretroviral therapy (ART) (SIT). The magnitude and breadth of the HIV-specific response did not, by themselves, explain the changes observed in plasma virus levels after the cessation of ART. Furthermore, mutational escape from targeted epitopes could not account for the differential virological outcomes in this cohort. However, the functionality of HIV-specific CD8$^+$ T-cell populations upon antigen encounter, determined by the simultaneous and independent measurement of five CD8$^+$ T-cell functions (degranulation and gamma interferon, macrophage inflammatory protein 1β, tumor necrosis factor alpha, and interleukin-2 levels) reflected the emergent level of plasma virus, with multiple functions being elicited in those individuals with lower levels of viremia after SIT. These data show that the quality of the HIV-specific CD8$^+$ T-cell response, rather than the quantity, is associated with the dynamics of viral replication in the absence of ART and suggest that the effects of SIT can be assessed by measuring the functional profile of HIV-specific CD8$^+$ T cells.

The dynamics of human immunodeficiency virus type 1 (HIV-1) infection are influenced, in part, by the ability of virus-specific CD8$^+$ T-cell responses to control initial viremia following acute HIV-1 infection and suppress viral replication during the chronic phase of infection. There are several lines of evidence that support a critical role for the antiviral effects mediated by HIV-specific CD8$^+$ T cells. First, the appearance of cytotoxic CD8$^+$ T lymphocytes (CTLs) during acute infection coincides with a decrease in plasma viremia, and conversely, the experimental depletion of CD8$^+$ T cells in vivo results in a rapid increase in plasma viremia in the simian immunodeficiency virus macaque model (15, 32, 37, 53). Second, several HLA class I alleles are consistently linked to differential outcomes (17); for example, strong virus-specific CTL responses restricted by HLA-B*57 are typically associated with slower disease progression in HIV infection (34, 42, 44). Third, the selective immune pressure exerted on the viral genome by CTLs has been supported by studies demonstrating viral escape at targeted epitopes in HIV-1 and simian immunodeficiency virus infection (29, 49, 50). In addition, longitudinal studies of humans have demonstrated that mutational escape from CTL-mediated immune responses can be associated with progressive increases in HIV plasma viremia (26, 33, 48).

Despite the cumulative evidence demonstrating a crucial role for the antiviral effects of CD8$^+$ T cells, the identification of consistent quantitative and qualitative correlations remains an elusive goal. Detailed studies comparing the breadths and magnitudes of CD8$^+$ T-cell responses in infected persons have failed to show a relationship between these parameters and viral load (1, 8). Moreover, despite the initially promising preservation and enhancement of HIV-specific CD8$^+$ T-cell responses during acute infection with early initiation of antiretroviral therapy (ART) (46, 51), attempts to boost immune responses therapeutically, for example, through autovaccination in the setting of ART interruptions in chronic HIV infec-

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TABLE 1. Immunological characteristics of HIV-infected individuals during and after SIT

Patient	HLA serotypes	Baseline CD4 count prior to SIT	Nadir CD4 count[a]	Mean absolute T-cell count			
				During SIT[b]		After SIT[c]	
				CD4	CD8	CD4	CD8
101	A2, A3, B7, B15, Cw3, Cw6	415	257	367	1,010	320	925
102	A3, A29, B7, B44, Cw3, Cw7	1,419	453	1,201	804	1,098	729
103	A2, A24, B7, B40, Cw3, Cw6	581	246	559	571	462	448
114	A24, A25, B7, B18, Cw7, Cw12	1,090	426	1,031	801	824	1,022
119	A3, A24, B7, B8, Cw7	451	150	434	1,265	413	1,266
124	A1, A2, B40, B57, Cw3, Cw6	1,104	311	888	853	827	744

[a] Lowest measurable CD4 count.
[b] Mean absolute T-cell counts during SIT (seven cycles of 8 weeks on/4 weeks off ART).
[c] Mean absolute T-cell counts during final cessation of ART after SIT (study week 92).

tion, have proven disappointing (5, 22–24, 45, 47, 52). More recent studies have suggested that functional attributes of CD8+ T-cell responses in individuals with chronic HIV-1 infection might help to discriminate differential virological outcomes (7, 12, 13, 40, 41, 43), but whether there are epitope-specific differences in functional immune responses and the extent to which immune escape complicates this picture during chronic infection are not known.

In this study, we performed a detailed analysis of the quantitative and qualitative differences observed in HIV-specific CD8+ T-cell responses to autologous virus after a definitive cessation of ART in HIV-infected individuals with a history of structured interruption of therapy (SIT). Our results demonstrate substantial variations in the magnitudes and breadths of the HIV-specific T-cell responses for each individual tested; however, no correlation between viral load and either frequency or breadth of the cellular immune response following the cessation of therapy was found. Moreover, the observation of limited viral evolution within immunodominant epitopes targeted by CD8+ T cells during SIT and the post-SIT period suggests that immune escape did not directly influence either the HIV-specific T-cell response or virological outcome after SIT. However, the functional profile of HIV-specific CD8+ T cells did provide a clearer association with virological outcome, especially in the case of individuals experiencing low levels of plasma viremia for extended periods off ART.

MATERIALS AND METHODS

Patient characteristics, antiretroviral treatment regimens, and clinical assays. Enrollment criteria for the SIT protocol, as previously described (21), were documented HIV infection with a CD4+ T-cell count of >300 cells/mm^3 and plasma HIV RNA levels of <50 copies/ml during screening and <500 copies/ml for >6 months on a potent ART regimen comprised of a protease inhibitor (indinavir [IDV] or nelfinivir) combined with a nonnucleoside reverse transcriptase inhibitor (nevirapine [NVP] or efavirenz) and/or a nucleoside reverse transcriptase inhibitor (NRTI) (stavudine [d4T], didanosine, or lamivudine [3TC]). The study was designed for 90 patients randomly assigned to continue ART or receive seven cycles of 4 weeks without ART followed by 8 weeks with ART. However, the study was prematurely terminated due to a high failure rate in the SIT arm after 26 patients in each arm were enrolled (21). Six of the 26 individuals from the treatment interruption arm voluntarily underwent a final cessation of ART after 92 weeks of SIT. Four of these individuals received a three-drug ART regimen during SIT consisting of a protease inhibitor (IDV or NVP) and two NRTIs (3TC and d4T); one individual received a combination of 3TC, d4T, and NVP; and one individual received IDV, zidovudine-3TC (Combivir), and efavirenz. All patients were monitored every 4 weeks for changes in plasma HIV RNA load as determined by branched DNA (limit of detection, 50 copies/ml) (Quantiplex bDNA assay; Bayer, Norwood, MA) and lymphocyte subsets, including activation status, by standard flow cytometric techniques. Immunologic characteristics of the participants are provided in Table 1. Patients with a viral load of >50,000 copies/ml on two consecutive tests or with mean CD4+ T-cell counts 25% lower than the mean of enrollment values on two consecutive determinations returned to continuous ART.

Peptides. Peptide pools consisted of individual 15-mer peptides overlapping by 11 amino acids and were based on sequences derived from clade B chimeric HIV strain HXBc2/Bal R5 (Gag, Pol, Env, or Nef) and HIV strain SF2 (Tat, Rev, Vif, Vpr, and Vpu) (NIH AIDS Research and Reference Program). Total HIV-specific T-cell responses were determined using these pools at a concentration of 2 μg/ml with respect to each individual peptide. Overlapping 15-mer peptides were also grouped together in pools according to antigen; a total of 122 peptides in 23 pools for Gag and 49 peptides in 14 pools for Nef were used in a matrix format at the above-mentioned concentration per peptide to identify and quantify CD8+ T-cell responses specific for individual epitopes. Subsequent optimal peptide-mapping experiments were performed using a panel of 11-mer peptides overlapping by 10 amino acid residues. Peptides were synthesized as free acids (Mimetopes PTY, Inc., NC) at a purity of >95% and reconstituted in 100% high-performance liquid chromatography-grade dimethyl sulfoxide at a concentration of 100 mg/ml.

Antibodies. Directly conjugated antibodies for flow cytometric analysis were used in the following fluorochrome combinations available from commercial sources: (i) IL-2 (interleukin-2)–allophycocyanin (APC), IL-2–phycoerythrin (PE), CD3-Cy7-APC, CD4-peridinin chlorophyll protein, gamma interferon (IFN-γ)-fluorescein isothiocyanate (FITC), IFN-γ–PE, tumor necrosis factor alpha (TNF-α)–PE, macrophage inflammatory protein 1β (MIP1β)-PE, CD14-Cy5-PE, CD19-Cy5-PE, and TNF-α–Cy7–PE (BD Biosciences, CA); (ii) CD4-Cy5.5-PE (Caltag, CA); and (iii) CD45RO-Texas Red-PE (Beckman Coulter, CA). The following antibodies were conjugated in our laboratory according to standard protocols: CD8-Qdot 705, CD27-Cascade Blue, CD57-Qdot 565, and CD107a-Alexa 680. The corresponding unconjugated monoclonal antibodies were obtained from BD Biosciences. Cascade Blue and Alexa 680 were obtained from Molecular Probes (Eugene, OR); quantum dots were obtained from the Quantum Dot Corporation (Hayward, CA).

Functional quantification of HIV-specific T cells. Frozen peripheral blood mononuclear cells (PBMCs) were thawed, resuspended at 5×10^6 cells/ml in complete medium (RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1.7 mM sodium glutamate), added to 96-well V-bottom plates (Costar Inc., NY) in 200-μl aliquots (1×10^6 cells), and rested overnight at 37°C in 5% CO_2. For individual peptide or peptide pool stimulations, costimulatory antibodies (anti-CD28 and anti-CD49d at a final concentration of 1 μg/ml; BD Biosciences, CA) and brefeldin A (final concentration of 10 μg/ml; Sigma Aldrich, MO) were added to the cells; HIV peptides were added at a concentration of 2 μg/ml, and the cells were incubated for 6 h. A negative control (costimulation alone) and a positive control (*Staphylococcus* enterotoxin B at a final concentration of 1 μg/ml; Sigma Aldrich, MO) were included in each assay. After peptide stimulation, PBMCs were washed once with fluorescence-activated cell sorter buffer (1% fetal bovine serum albumin, 0.1% sodium azide) and stained with anti-CD3-APC, anti-CD4-peridinin chlorophyll protein, and anti-CD8-FITC. The cells were then washed again, fixed and permeabilized using 200 μl of 2× fixation-permeabilization solution (Becton-Dickinson Immunocytometry Systems, San Jose, CA), washed two further times, and then stained with antibodies to the cytokines IFN-γ, TNF-α, and IL-2 (all directly conjugated to PE). After staining,

the cells were washed a final time and resuspended in 1% paraformaldehyde (Electron Microscopy Systems, Fort Washington, PA) in phosphate-buffered saline. Six-parameter flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Between 150,000 and 200,000 events were collected for each sample. Cytokine expression was calculated following subtraction of background (anti-CD28/49d alone).

Functional profiling of HIV-specific T cells by polychromatic flow cytometry. Purified PBMCs were thawed, resuspended at 2×10^6 cells/ml in complete medium, and rested overnight at 37°C with DNase I (10 U/ml; Roche Diagnostics, Indianapolis, IN). The following day, PBMCs were examined for viability and adjusted to 1×10^6 cells/ml. Costimulatory molecules (anti-CD28 and anti-CD49d), monensin (0.7 μl/ml; BD Biosciences), brefeldin A, and anti-CD107a-Alexa 680 (pretitered volume) were added; cells were then aliquoted at 1 ml to each tube containing 5 μl of peptide mixes (2-μg/ml final concentration for each individual constituent peptide) representing the HIV Gag and Nef protein sequences. An unstimulated control and a positive control (*Staphylococcus* enterotoxin B) were included in each assay. Cells were incubated for 6 h at 37°C. Following incubation, the cells were washed with fluorescence-activated cell sorter buffer and stained with the following surface antibodies: CD3-Cy7-APC, CD4-Cy5.5-PE, CD8-Qdot 705, CD27-Cascade Blue, and CD45RO-Texas Red-PE. The cells were then washed and fixed. Following fixation, the cells were washed twice in perm buffer and stained with the following intracellular antibodies: IL-2–APC, TNF-α–Cy7–PE, MIP1β-PE, and IFN-γ–FITC (BD Biosciences). After a further wash, cells were fixed, and data were acquired immediately. Polychromatic flow cytometric analysis was performed using an LSRII flow cytometer (Becton Dickinson, San Jose, CA). At least 300,000 live lymphocytes were collected, and list-mode data files were analyzed using FlowJo, version 6.3.1 (Tree Star Inc., Ashland, OR). The gating strategy was initially set to eliminate cell doublets according to forward-scatter area-versus-height properties. Subsequently, dead cells (Cascade Blue bright) and $CD14^+/CD19^+$ cells were gated out against side scatter, and lymphocytes were selected according to standard light scatter properties after confirming that any functional response originated from this population. Functional capacity was determined after Boolean gating, and subsequent analysis was performed using Simplified Presentation of Incredibly Complex Evaluations (version 2.9; Mario Roederer, VRC, NIAID, NIH) (13). All values used for analyzing a proportionate representation of responses were background subtracted. Nonspecific background was extremely low when combinations of functions were examined. With single functions, especially MIP1β, backgrounds were occasionally higher; stringent gating strategies were therefore used to ensure that only MIP1β-bright T cells were included in the analysis (13).

Sequencing of autologous virus. HIV virions were concentrated from 1 ml of plasma by centrifugation at $17,530 \times g$ for 75 min at 4°C. The supernatant was removed, and the pellet was resuspended in 1 ml of Trizol-LQ (Invitrogen). Viral RNA was extracted according to standard protocols, and cDNA was prepared using the Superscript first-strand synthesis system with a mixture of random hexamers and oligo(dT). A two-step nested PCR amplification of the viral cDNA was performed using Platinum *Taq* polymerase (Invitrogen); outer and inner primer sets for HIV *gag*, *pol*, *env*, and *nef* were designed and used as previously described (4). Amplification products were gel purified and cloned using the TOPO TA cloning kit (Invitrogen). Plasmids were transformed into competent Top10 F′ *Escherichia coli* cells (Invitrogen). Individual clones were screened using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) complementation, and colony DNA was prepared using Qiagen Miniprep kits. Clones were sequenced bidirectionally on an ABI 3100 Prism automated sequencer and assembled using Sequencher, version 4.5 (GeneCodes Inc., Ann Arbor, MI). At least 10 clones were sequenced for each PCR product, and the autologous viral sequence was compared to the consensus HxB2 sequence.

RESULTS

Virological and immunological characteristics of SIT patients following cessation of ART. Six of 26 chronically infected patients who received SIT comprising at least seven cycles of 4 weeks off ART followed by 8 weeks on ART underwent a definitive interruption of therapy at study week 92 (end of the last cycle on therapy) (21). Plasma viremia and T-cell subset counts were monitored during the course of SIT and the drug cessation period (Fig. 1). Individuals resumed ART if their HIV plasma loads exceeded the clinical failure criteria of >50,000 copies/ml on two consecutive tests or >10,000 copies/ml on three consecutive tests or if their mean $CD4^+$ T-cell count on two consecutive determinations was 25% lower than the mean of the screening and enrollment values. Relatively stable $CD4^+$ and $CD8^+$ T-cell counts were observed for each individual during SIT and the post-SIT period off ART (Table 1).

All six individuals experienced a rebound in plasma viremia after the cessation of ART and maintained various levels of detectable plasma HIV RNA levels throughout the final drug interruption period (Fig. 1). The virological characteristics are summarized in Table 2. To test for predictors of the virological response after cessation of ART, the mean plasma viral load (pVL) off therapy (after SIT) was compared to the pVL at SIT cycle 1, at week 4 off ART (Fig. 1). Previous findings from SIT pilot studies suggested that rebounding virus during the first off-drug cycle is a reasonable predictor of pretreatment viral set point (45, 47). The duration off ART was used as a secondary predictor because longer periods off therapy suggest a more favorable virological response. For each patient, the plateau pVL was defined as the mean (geometric) pVL during the post-SIT period (median number of measurements, 8; range, 2 to 13). The mean time off therapy was 26 weeks (median, 33 weeks; range, 8 to 52 weeks). All six individuals experienced pVLs off ART that were lower than the estimated viral set point determined from cycle 1, at week 4 off ART. Two individuals (patients 101 and 114) developed especially favorable virological responses during the post-SIT period, while one individual (patient 124) experienced a more modest response (Table 2). The least favorable virological outcomes were observed for patients 102, 103, and 119. Patient 119 experienced a minimal reduction in mean pVL off ART (6.11-fold) and returned to therapy after 12 weeks off ART due to three consecutive viral load measurements of >10,000 copies/ml. In contrast, patient 102 remained off ART for the longest period of time (52 weeks), despite having the smallest reduction between levels of viremia post-SIT and the estimated viral set point (Table 2). Patient 103 was off ART for 8 weeks and recommenced continuous therapy after two consecutive pVL measurements of >50,000 HIV RNA copies/ml. It should be noted that this individual entered the SIT study with genotypic resistance to efavirenz (K170R). The mutation was not detected until week 60 of SIT. At this time, the ARV regimen was changed to zidovudine-3TC-IDV, and the individual continued to the end of the SIT period and elected to halt ART. At week 96, the additional resistance mutations D76N (NRTI) and K70R (NRTI) were detected.

Relationship between the magnitude of the HIV-specific immune response and virological outcome in SIT-experienced patients following cessation of ART. Quantitative aspects of the HIV-specific immune response, defined by magnitude and breadth, have frequently been used in attempts to define the correlates of T-cell efficacy in relation to pVL (1, 10, 16, 47). In order to assess whether the magnitude of the total HIV-specific immune response was an immunologic predictor of plateau pVLs observed during the off-ART period following SIT, $CD4^+$ and $CD8^+$ T-cell responses to different viral proteins were quantified by flow cytometry according to direct ex vivo cytokine (IFN-γ, TNF-α, and IL-2) production upon stimulation with peptide pools spanning the consensus HIV-1 Gag,

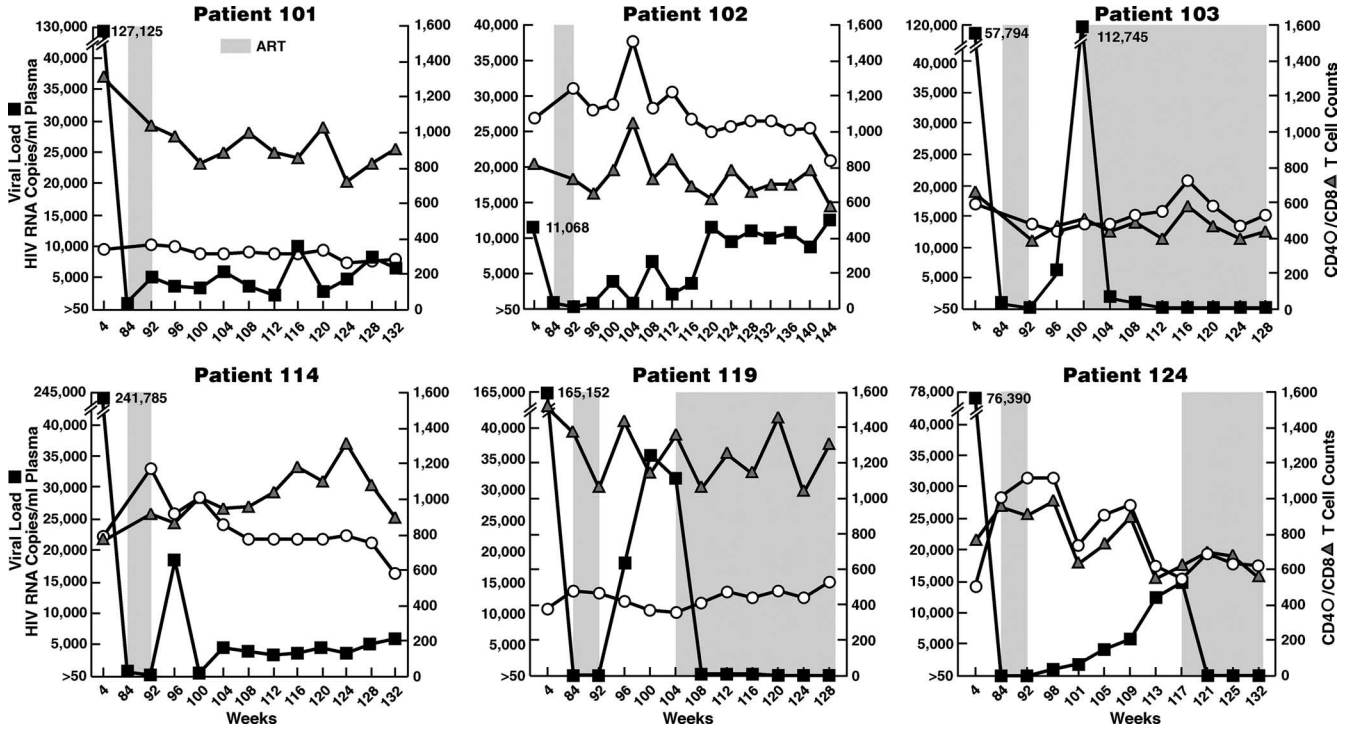

FIG. 1. Immunological and virological characteristics of HIV-infected individuals during SIT and definitive cessation of ART. A cohort of six individuals with chronic HIV infection received SIT comprised of 8 weeks on ART followed by 4 weeks off ART for a period of up to 92 weeks. At week 92 of the study, individuals voluntarily stopped ART. Viral load (HIV RNA copies/ml plasma) and absolute numbers of $CD4^+$ and $CD8^+$ T cells per mm^3 of blood were monitored throughout treatment interruption and cessation periods. Gray shading depicts periods on ART. Patients 103, 119, and 124 restarted ART at weeks 100, 104, and 117, respectively.

Pol, Nef, Env, Rev, Tat, Vpu, Vpr, and Vif proteins. HIV-specific $CD4^+$ and $CD8^+$ T-cell responses were measured at week 84 (start of final on-ART cycle) and at one or more further time points during the final cessation of therapy. A heterogeneous pattern of T-cell responses was observed with representative patient profiles shown in Fig. 2. In general, total HIV-specific $CD4^+$ T-cell responses were lower in magnitude than $CD8^+$ T-cell responses, and responses to Gag and Nef peptides were immunodominant in the $CD8^+$ T-cell populations. Patients 101 and 114, considered to be the best responders virologically, showed substantial increases in the magnitudes of their HIV-specific $CD8^+$ T-cell responses. The immunodominant $CD8^+$ T-cell target protein in patient 101 was Gag, while the corresponding responses in patient 114 targeted primarily Nef. Interestingly, the lowest-magnitude T-cell responses were observed in patient 102, despite the maintenance of a mean pVL of 4,778 copies/ml for at least 52 weeks off therapy. Of note, two of the three individuals with the poorest virological response post-SIT (patients 103 and 119) also developed potent HIV-specific $CD8^+$ T-cell responses. These data suggest that the magnitude of the HIV-specific T-cell response alone is insufficient to predict virological outcome in these individuals.

TABLE 2. Virological characteristics of HIV-infected individuals during and after SIT

Patient	Viral set point (HIV copies/ml)[a]	Mean pVL after SIT[b] (HIV copies/ml)	Time off ART (wk)	Fold reduction in viral set point versus no ART
101	127,125	4,660	40	27.28
102	11,068	4,778	52	2.32
103	57,794	21,888	8	2.64
114	241,785	3,956	40	61.12
119	165,152	27,045	12	6.11
124	76,390	4,458	25	17.14

[a] Viral set point (estimate) is pVL at week 4 off ART of the first SIT cycle.
[b] Geometric mean.

Relationship between the breadth of HIV-specific T-cell responses and virological outcome. To quantify the breadth of the HIV-specific $CD8^+$ T-cell response, a peptide matrix comprising 23 pools (122 total peptides) of 15-mer peptides overlapping by 11 amino acids for Gag was used to stimulate PBMCs directly ex vivo from multiple time points during treatment interruption for all six individuals. In addition to Gag, a Nef matrix comprising 14 pools of peptides (49 total 15-mer peptides) was used to stimulate cells from patient 114. A complete summary of the matrix peptide analysis is shown in Table 3. Overall, no consistent patterns were observed to relate the breadth of the HIV-specific T-cell response with virological outcome.

Of the two individuals with the highest-magnitude HIV-specific $CD8^+$ T-cell responses, a broad response was observed in patient 101, with $CD8^+$ T cells recognizing a total of 18 peptides corresponding to Gag p17 (positions 1 to 35), p24 (positions 265 to 303), and p24 (positions 309 to 347), and a narrow response was observed in patient 114, with $CD8^+$ T cells targeting a single peptide, LKETINEEAAEWDRV, in

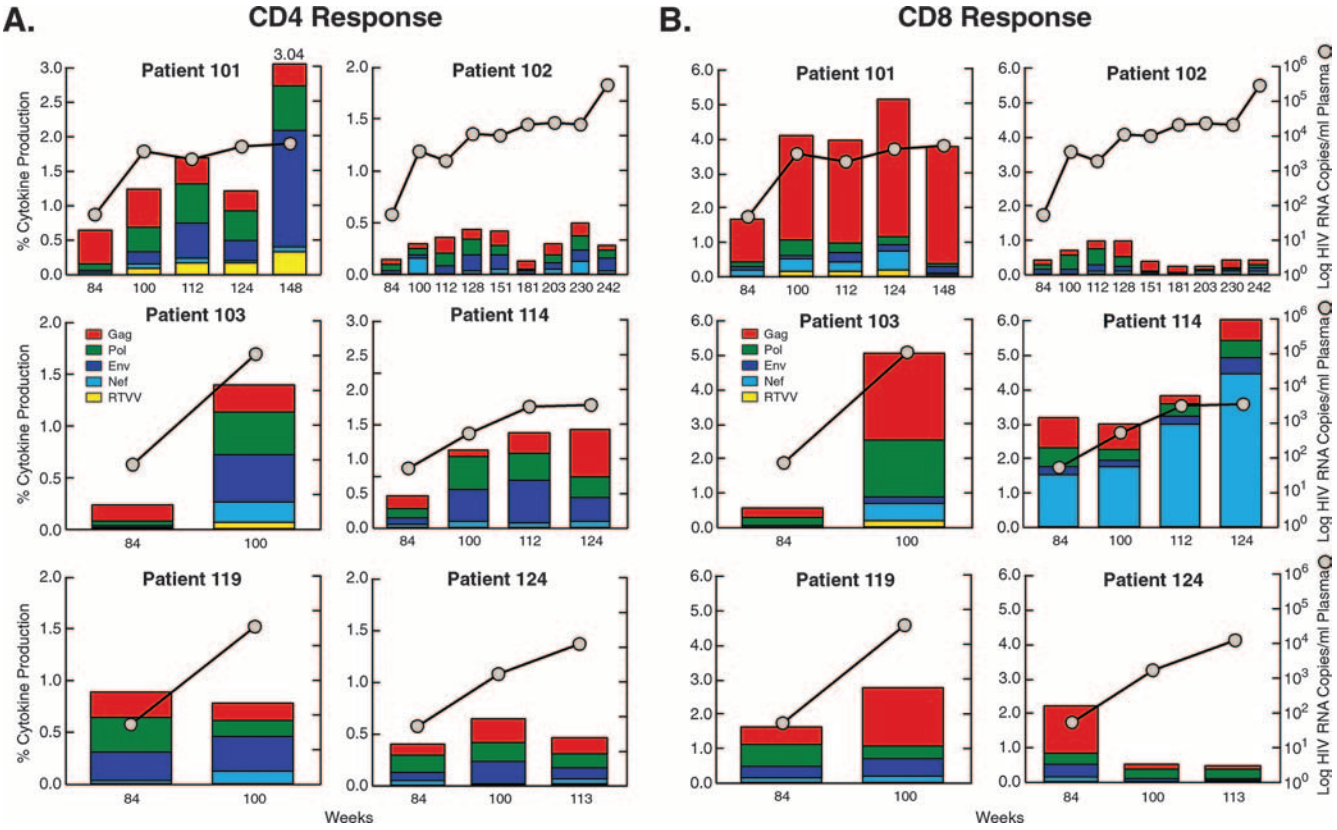

FIG. 2. Magnitude of the total HIV-specific T-cell response. The total magnitudes of the $CD4^+$ (A) and $CD8^+$ (B) T-cell responses determined using mixes of overlapping peptides specific for Gag, Pol, Env, Nef, and Rev and Tat, Vpu, Vpr, and Vif (RTVV) are shown for each subject as the percent cytokine production during the first week of the last cycle on ART (week 84) and subsequent time points after the cessation ART. Levels of pVL are shown as $\log_{10}$ HIV RNA copies/ml. Overall, the magnitude of the total HIV-specific T-cell response was not predictive of virological outcome.

Gag p24 (positions 201 to 217) and a single region in Nef. A narrow, low-frequency $CD8^+$ T-cell response to one peptide in Gag was observed for patient 124, despite a relatively favorable virological response off therapy.

The breadths of the HIV-specific immune response in individuals with poorer virological outcomes also varied. Cytokine-producing $CD8^+$ T cells from patient 119 recognized six peptides from Gag, while patient 102 exhibited a low-frequency $CD8^+$ T-cell response narrowly directed to three overlapping peptides in Gag p17 (positions 1 to 31). For patient 103, the individual with the worst virological outcome post-SIT, the $CD8^+$ T-cell response was the broadest compared to all other individuals, with a total of 24 peptides from four separate regions of Gag being recognized (Table 3). Thus, the high degree of variability in the breadth and magnitude of the HIV-specific $CD8^+$ T-cell responses observed between SIT-experienced individuals with different pVL plateaus off ART suggests that these quantitative aspects of the immune response do not correlate with virological outcome.

Functionality as a qualitative measure of T-cell responses to targeted HIV proteins. Emerging evidence suggests that qualitative, rather than quantitative, aspects of the HIV-specific T-cell response may be better correlates of virological changes that occur in the absence of ART (11, 13, 18, 30). To assess whether there were differences in HIV-specific $CD8^+$ T-cell functionalities between individuals with different virological outcomes, HIV-specific $CD8^+$ T-cell populations were stimulated with the relevant targeted antigens (Gag or Nef) and analyzed by polychromatic flow cytometry to examine multiple functional readouts. Specifically, the following effector functions were assessed simultaneously and independently: $CD8^+$ T-cell degranulation, determined by CD107a mobilization; the production of the cytokines IFN-γ, TNF-α, and IL-2; and the production of the chemokine MIP1β. Following the stimulation of unfractionated PBMCs with HIV Gag or Nef peptide mixtures, the expression of each functional marker was examined after stringent gating on $CD8^+$ T cells as described previously (13). A Boolean algorithm yielding 32 unique response patterns comprising every combination of the five individual measurements (CD107a, IFN-γ, MIP1β, TNF-α, and IL-2) was applied to the gated cells. The frequency of each of these individual response patterns was then calculated, and the contribution of each combination to the total response was determined (Fig. 3).

Polyfunctionality was most apparent in the HIV-specific $CD8^+$ T-cell populations from the individuals who maintained low levels of plasma viremia during the post-SIT period. The majority of the Gag-specific $CD8^+$ T cells in patient 101 and Nef-specific $CD8^+$ T cells in patient 114 expressed a combination of "3+" and "4+" functions (Fig. 3). Furthermore, a

TABLE 3. Summary of the breadth of the CD8+ T-cell response after SIT[a]

Patient	No. of peptides[b]	Amino acid sequence	Frequency[c]	Protein	Positions
103	6	LRPGGKKKYKLKHIVWASRELERFAVNPGLL	0.658	Gag p17	21–51
	6	QPSLQTGSEELRSLYVNTAVATLYCVHQRIE	0.644	Gag p17	65–93
	6	AMQMLKETINEEAAEWDRVHPVHAGPIAPGQ	0.689	Gag p24	197–227
	6	DCKTILKALGPAATLEEMMTACQGVGGPGHK	0.741	Gag p24	329–359
101	6	MGARASVLSGGELDRWEKIRLRPGGKKKYKLKHIV	0.689	Gag p17	1–35
	6	WIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKT	0.698	Gag p24	265–303
	6	ASQEVKNWMTETLLVQNANPDCKTILKALGPAATLEEMM	0.745	Gag p24	309–347
119	3	LDRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGL	0.854	Gag p24	13–50
	2	LLVQNANPDCKTILKALGP	0.709	Gag p24	321–339
	1	EEMMTACQGVGGPGH	0.629	Gag p24	344–359
114	1	LKETINEEAAEWDRV	1.238	Gag p24	201–217
	2	IHSQRRQDILDLWIYHTQGYF	1.455	Nef	101–121
102	3	MGARASVLSGGLEDRWEKIRKLRPGGKKKYKL	0.154	Gag p17	1–31
124	1	EKAFSPEVIPMFSAL	0.188	Gag p24	161–175

[a] Patient responses are listed from broadest to narrowest.
[b] Number of peptides indicates the number of matrix peptides defining an epitope region.
[c] Frequency indicates the mean cytokine response of matrix peptide(s) per epitope region.

proportion of HIV-specific CD8+ T cells in individuals with a good virological outcome were positive for all five functions measured (Fig. 3); this high degree of functionality was observed previously in long-term nonprogressors (13). Interestingly, when the functional profiles of Gag- and Nef-specific CD8+ T-cell populations from patient 114 were compared, the Gag-specific CD8+ T-cell response was lower not only in magnitude but also in functionality than the Nef-specific response, suggesting a relationship between functionality and immunodominance (Fig. 3).

In patient 102, a polyfunctional pattern similar to those of patients 101 and 114 was observed. There was a modest reduction in plasma viremia off ART compared to the viral set point in this individual and a low-frequency Gag-specific CD8+ T-cell response; however, the length of time without therapy (52 weeks) may provide an additional virological factor that associates with the polyfunctional nature of the HIV-specific CD8+ T-cell response. For patients 119 and 124, the majority of the Gag-specific response was composed primarily of cells expressing two functions or less. The most monofunctional CD8+ T-cell response was observed for patient 124 despite a relatively good virological outcome. Further monofunctionality was observed in CD4+ T-cell populations from all six patients analyzed. However, the low frequency of the total HIV-specific response in this T-cell subset made it technically difficult to determine a relationship with viral outcome (data not shown). The degree of functionality is more clearly evident when the CD8+ T-cell responses from all functional combinations within a group are summed together (Fig. 4). Despite variations in frequency, the dominant responses in patients 114, 101, and 102 exhibited a higher degree of functionality than the responses observed in patients 119 and 124. Further cells from patient 103 were not available for analysis.

Memory phenotyping of polyfunctional CD8+ T-cell populations according to CD27, CD45RO, and CD57 expression failed to reveal any consistent patterns that could add discriminatory power to our analyses; indeed, substantial phenotypic heterogeneity was apparent within the polyfunctional HIV-specific CD8+ T-cell subset (data not shown).

Despite the limited number of patients in this study, the observation that a higher degree of virus-specific CD8+ T-cell functionality prevails in individuals with reduced levels of plasma viremia in the absence of ART suggests that qualitative aspects of the HIV immune response may help predict virological outcome after SIT. It remains to be determined whether this relationship is causal.

Viral sequence variation and immune recognition within targeted CD8+ T-cell epitopes. The mutability of HIV, especially in the absence of ART, can enable the virus to escape immune surveillance through alterations in antigen presentation and recognition. An effective HIV-specific CD8+ T-cell response, however, may be sufficient to suppress viral replication and limit the emergence of escape mutations. It is possible, therefore, that SIT individuals with poorer virological outcomes in the absence of therapy have developed escape mutations capable of subverting the HIV-specific CD8+ T-cell response. To determine the extent of sequence variation in targeted viral epitopes during the SIT and post-SIT periods, and to measure the ability of HIV-specific CD8+ T cells to recognize these epitopes, longitudinal sequence analysis of plasma virus RNA followed by high-resolution peptide mapping was performed. Changes that emerged within stretches of sequences containing one or more CD8+ T-cell epitopes are shown in Fig. 5. Despite various levels of rebounding plasma virus over the SIT period, most immunodominant epitope regions failed to show evidence of significant sequence variation. In addition, the emergence of new variants was not observed during the post-SIT period when pVL was slowly increasing or plateauing. Sequence analysis of molecular clones derived from HIV proviral DNA from patients 101 and 114 confirmed the presence of viral variants similar to those found in the plasma (data not shown); this suggests that archived virus had

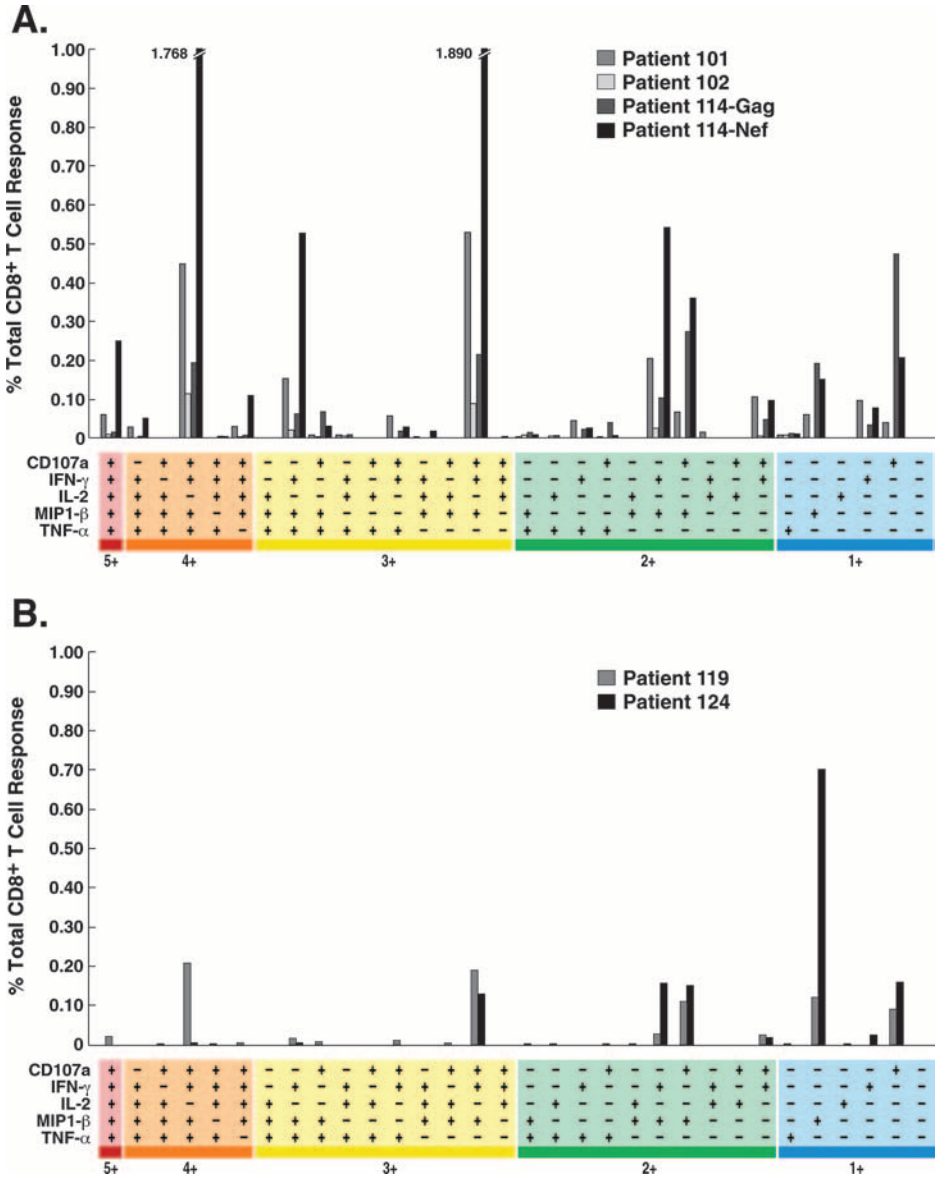

FIG. 3. Functional characterization of HIV-specific $CD8^+$ T-cell populations during the cessation of ART. PBMCs from patients 101 (week 124), 102 (week 112), and 114 (week 124) (A) and from patients 119 (week 100) and 124 (week 100) (B) were stimulated for 6 h with the relevant peptides and stained with a panel of monoclonal antibodies to examine degranulation (CD107a), cytokine production (IFN-γ, TNF-α, and IL-2), and chemokine production (MIP1β). The 31 possible positive responses that can be discerned from the simultaneous examination of these five functional parameters are shown on the *x* axis and are color-coded according to the number of functions expressed per group. The total frequency of $CD8^+$ T cells displaying each particular functional profile is shown on the *y* axis.

not changed significantly during SIT with attendant periods of substantial viral replication.

High-resolution epitope-mapping experiments were performed with saturating concentrations of synthetic peptides consisting of individual 11-mer peptides overlapping by 10 amino acids. Peptides were derived from autologous and consensus HxB2 virus sequences (http://hiv-web.lanl.gov) for each HIV epitope region included in the sequence analysis (Fig. 5). The pattern of targeted $CD8^+$ T-cell epitopes observed in individuals who responded well during the post-SIT period consisted of high-frequency responses to multiple HLA class I-restricted epitopes within and between immunodominant regions. A list of optimal $CD8^+$ T-cell epitopes and their HLA class I restrictions is shown in Table 4.

In patient 101, the highest-frequency $CD8^+$ T-cell responses were directed against two separate immunodominant regions in Gag containing the known epitopes HLA A3 [KIRLRPGG $K_{(18-26)}$] (KK9), HLA A3/B7 [RLRPGGKKK$_{(20-28)}$] (RK9), and HLA B7 [RPGGKKKYK$_{(22-30)}$] in p17. The KK9 and RK9 epitopes were previously reported for individuals with nonprogressive disease (31) and individuals treated early during acute infection (2). An escape mutation identified in individuals during acute infection was not found within the autologous virus pool from patient 101 (2, 56). In addition, the KK9 and RK9

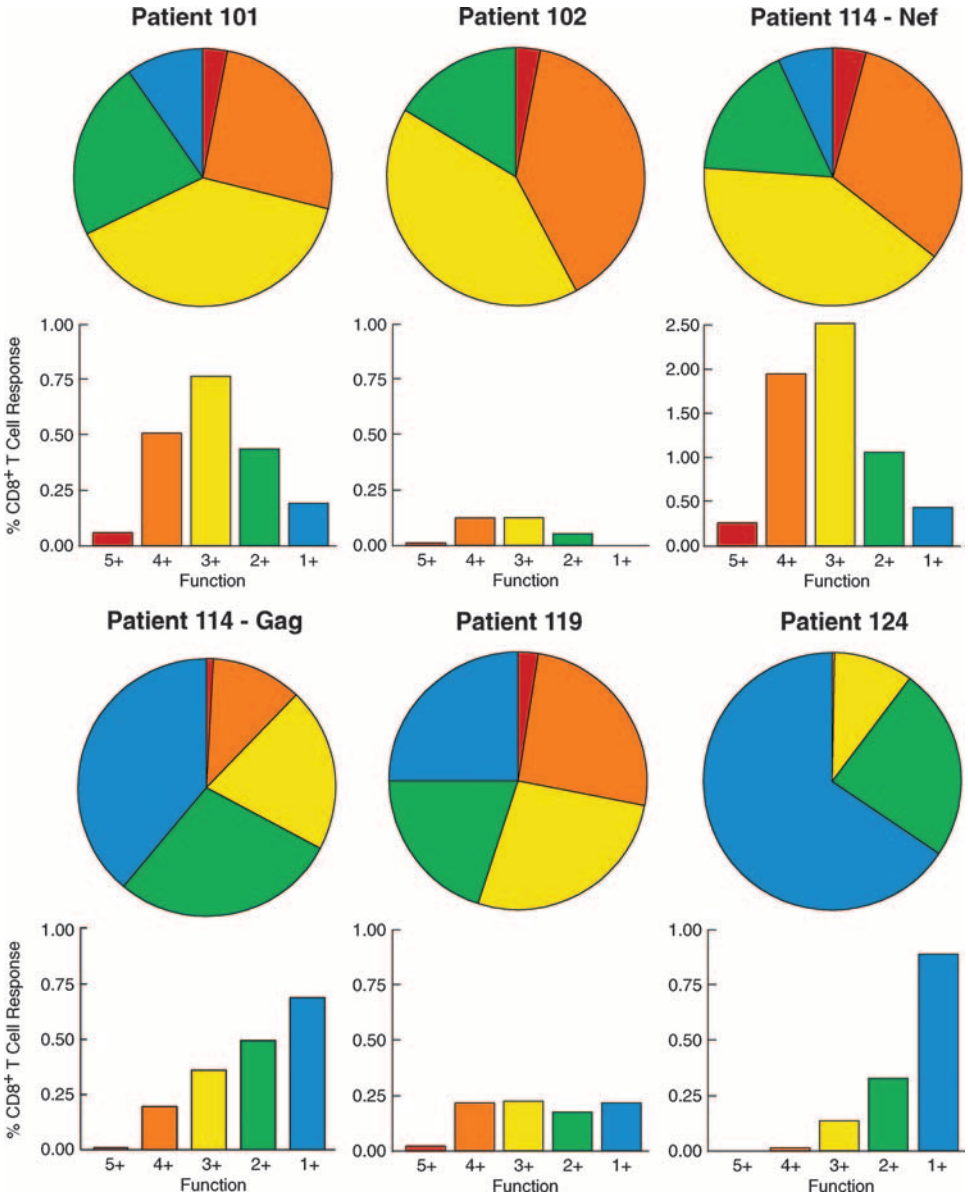

FIG. 4. Functional profiles of HIV-specific CD8+ T-cell populations. Individual combinations specific for each functional category (5+, 4+, and 3+, etc.) are expressed as a percentage of the total response and ordered from highest functionality to lowest. The majority of Gag- and Nef-specific CD8+ T cells from the immunodominant populations in patients 101 and 114, respectively, expressed predominantly 4+ and 3+ functions, as did patient 102. In contrast, Gag-specific CD8+ T cells from patients 119 and 124 had more limited functionality; the subdominant Gag-specific CD8+ T-cell population from patient 114 exhibited similarly restricted functionality.

epitopes appeared to be recognized by virus-specific CD8+ T cells from patient 102 and patient 119, even though these individuals had different functional profiles and virological outcomes off ART.

In patient 114, virus-specific CD8+ T cells targeted the highly conserved p24 Gag epitope ETINEEAAEW$_{(203-212)}$ (EW10) that is restricted by the HLA-A25 allele (35) and conserved epitopes in Nef with overlapping HLA-restriction to A, B, and C alleles (Fig. 5 and Table 4). Interestingly, while responses to consensus and autologous viral variants were similar in magnitude for the EW10 Gag epitope, the autologous variants targeted in Nef elicited higher-frequency responses than consensus peptides. For example, the autologous HLA A24-restricted Nef epitope IYSQKRQDI$_{(101-109)}$ (II9A) was exclusively targeted over the consensus IHSQKKQDI$_{(101-109)}$ (II9) epitope. Previous studies demonstrated that the optimal anchor residues for binding to HLA A24 molecules are tyrosine (Y), phenylalanine (F), tryptophan (W), and methionine (M) at position 2 and F, leucine (L), isoleucine (I), and W at the carboxy terminus of the 9-mer peptide (36, 38). Therefore, it is possible that an amino acid change from histidine (H) to Y within the autologous II9 epitope alters the affinity of the HLA class I-peptide interaction. A similar pattern of immunodominant CD8+ T-cell recognition was observed for the

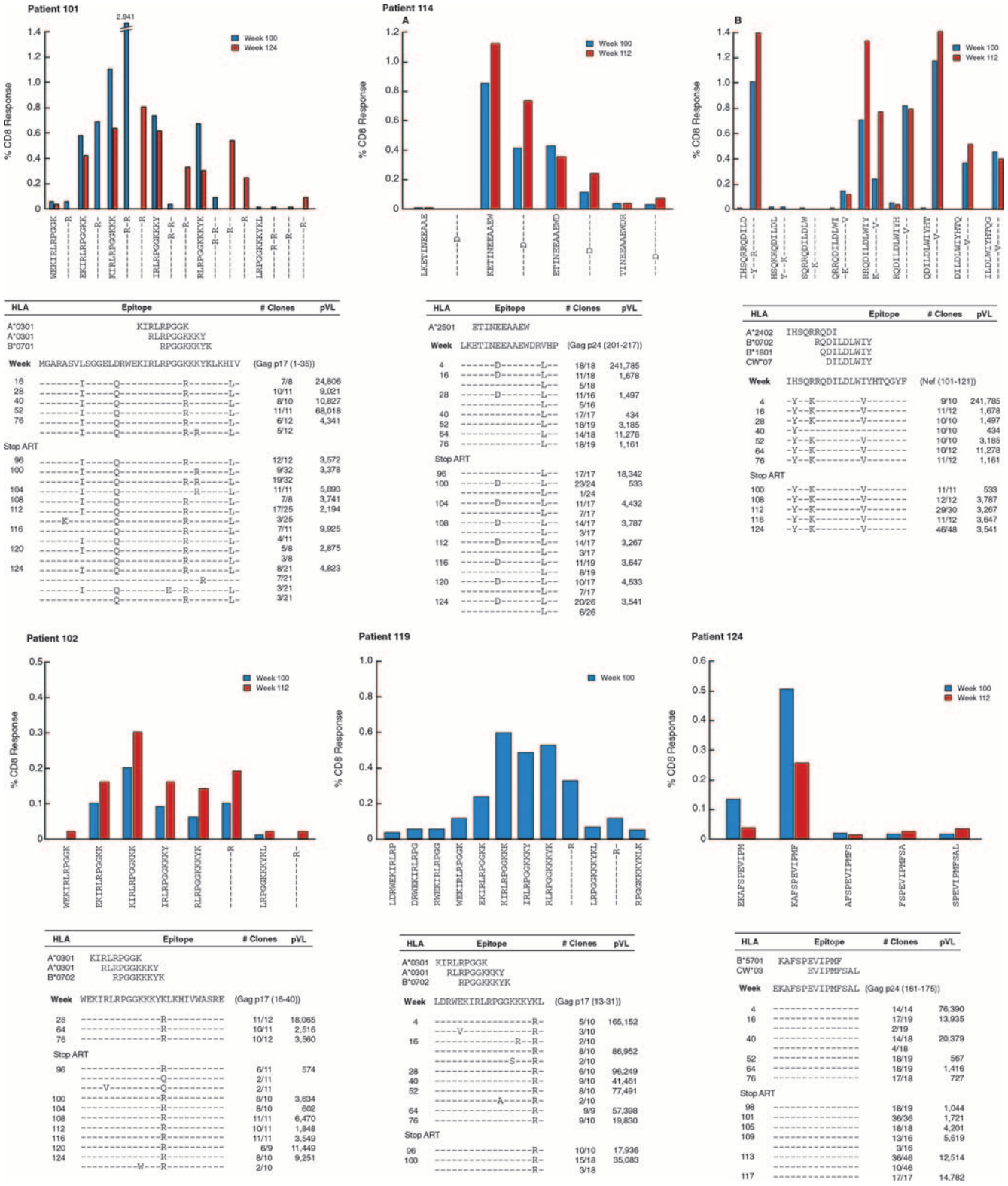

FIG. 5. Sequence evolution and recognition of targeted epitopes by HIV-specific CD8$^+$ T cells. Longitudinal sequence analyses of immunogenic regions showed limited evolution within viral populations during SIT and the post-SIT period off ART. HIV-specific CD8$^+$ T-cell recognition of viral epitopes was determined using overlapping 11-mer peptides offset by 1 amino acid from consensus and autologous sequences for each targeted region. Only the dominant viral epitopes are shown for clarity. CD8$^+$ T cells from patients 101 and 114 recognized multiple HIV epitopes restricted by different HLA class I alleles. Of note, the highest-frequency responses consistently targeted autologous over consensus peptides.

TABLE 4. CD8+ T-cell recognition of optimal epitopes within immunodominant regions of Gag and Nef[a]

Patient	Immunodominant region	Optimal epitope	HLA	Location (positions)
101	WEKIRLRPGGKKKYKL	KIRLRPGGK	A3	p17 (18–26)
	----------R-R---	**--------R**		
	------------R---	RLRPGGKKK	A3, B7	p17 (20–28)
		------R-R		
		RPGGKKKYK	B7	p17 (22–30)
		------R--		
	WIILGLNKIVRMYS	IILGLNKI	A2, A3	p24 (266–273)
	--M----------	**-M------**		
		GLNKIVRMY	B15	p24 (269–277)
	DCKTILKALGPAATLE	KALGPAATL	B15,Cw3	p24 (335–343)
102	WEKIRLRPGGKKKYK	KIRLRPGGK	A3	p17 (16–30)
	--------------R	RLRPGGKKK	A3, B7	p17 (20–28)
		RPGGKKKYK	B7	p17 (22–30)
		--------R		
114	LKETINEEAAEWDRVHP	ETINEEAAEW	A25	p24 (203–212)
	------D----------	**----D-----**		
	IHSQRRQDILDLWIYHTQG	IHSQRRQDI	A24	Nef (101–109)
	-Y--K--------V-----	**-Y--K----**		
		RQDILDLWI	B7	Nef (106–114)
		--------V		
		QDILDLWIY	B18	Nef (107–115)
		-------V-		
		DILDLWIY	Cw7	Nef (108–115)
		------V-		
119	LDRWEKIRLRPGGKKKYKL	KIRLRPGGK	A3	p17 (13–31)
	-----------------R-	RLRPGGKKK	A3, B7	p17 (20–28)
		RPGGKKKYK	B7	p17 (22–30)
		--------R		
	LVQNANPDCKTILKALG	VQNANPDCK	A3	p24 (323–331)
		NPDCKTIL	B8	p24 (327–334)
124	EKAFSPEVIPMFSAL	KAFSPEVIPMF	B57	p24 (162–172)
		EVIPMFSAL	Cw3	p24 (167–175)

[a] Autologous epitopes are in bold type.

HLA B18 [QDILDLWIY$_{(107-115)}$] and HLA Cw7 [DILDL WIY$_{(108-115)}$] epitopes. The autologous variant (I changed to V immediately prior to the C terminus) (Table 4) for each HLA allele was again recognized exclusively over the consensus sequence. These data confirm previous studies suggesting that autologous epitopes are recognized at higher frequencies than consensus peptides (3, 20). More importantly, it appears that immunodominant CD8+ T-cell recognition in two individuals with favorable virological outcomes occurs within multiple conserved regions of Gag (patient 101) or Nef (patient 114), restricted by distinct HLA alleles. In individuals with poorer virological outcomes (patients 102, 124, and 119), CD8+ T-cell recognition of optimal epitopes occurred at much lower frequencies overall and perhaps with a lesser degree of "epitope overlap" (Table 4 and Fig. 5). However, in the absence of any significant evidence for mutational immune escape in any of the individuals studied here, it is difficult to invoke a role for such targeting patterns in relation to virological outcome.

DISCUSSION

In this study, we examined quantitative and qualitative aspects of the HIV-specific CD8+ T-cell response during and after SIT to define the immunologic associates of virological outcome. The principal findings were (i) that the magnitude and breadth of the HIV-specific CD8+ T-cell response was unable to account for differences in viremic control, (ii) that significant sequence evolution within immunodominant targeted CD8+ T-cell epitopes over time did not occur regardless of virological outcome, and (iii) that the range of effector functions elicited by CD8+ T cells in response to antigen encounter was associated with the control of viral replication after cessation of ART.

Recent advances in immunotechnology have allowed a more comprehensive quantitative assessment of the magnitude and breadth of the HIV-specific immune response (1, 8, 9, 14). In addition, the use of matrices composed of overlapping peptides derived from HIV consensus or autologous viral sequences has enabled detailed analyses of CD8+ T-cell specificity in conjunction with sensitive quantitative techniques (1, 3, 14, 55). However, despite this enhanced ability to characterize HIV-specific T-cell responses directly ex vivo, neither pangenomic analyses nor more specific epitope-centered studies have been able to identify reliable correlates of protection in chronic HIV infection. Similar data have been reported for individuals undergoing various SIT regimens (6, 24, 47). In the

setting of SIT, while the overall HIV-specific T-cell response has been observed to increase with repeated exposure to bursts of autologous viral replication, neither the magnitude nor the breadth of the HIV-specific $CD8^+$ T-cell response could be correlated with levels of plateau pVL after SIT. Our data suggest a similar pattern. In two individuals who maintained consistently lower levels of pVL in the absence of ART, one (patient 101) exhibited a high-frequency HIV-specific $CD8^+$ T-cell response directed against a broad array of Gag epitopes, while the other (patient 114) developed a narrowly focused response, predominantly to Nef. A third individual (patient 124) with a more modest reduction in viremia off ART exhibited a low-frequency HIV-specific $CD8^+$ T-cell response that was more narrowly targeted to Gag. Conversely, the individual with the worst virological response in the absence of ART (patient 103) exhibited the broadest antiviral $CD8^+$ T-cell response, which targeted predominantly epitopes derived from the Gag protein. Thus, in agreement with numerous SIT studies that concluded that there is rarely an immunologic or virological benefit to this type of therapy (5, 21, 23, 24), our data suggest that the frequency and breadth of the HIV-specific $CD8^+$ T-cell response observed in the absence of ART in individuals with chronic infection are not necessarily beneficially altered by prolonged periods of SIT with respect to virological outcome.

Detailed sequence analysis of targeted HIV epitopes in our cohort revealed little variation during the course of SIT and the post-ART period. A higher level of variation suggests possible selection pressure exerted by $CD8^+$ T cells and attempts by the virus to escape recognition. However, this was not observed regardless of virological outcome off ART. Indeed, when we examined $CD8^+$ T-cell recognition of autologous HIV epitopes present during and after SIT in two virological responders, we found that a high percentage of $CD8^+$ T cells recognized multiple conserved epitopes in Gag (patient 101) or Nef (patient 114) using distinct HLA-restricted class I alleles. These observations suggest that separate HLA-restricted populations of HIV-specific $CD8^+$ T cells may prevent viral escape through the focused recognition of immunodominant epitopes (39, 54). Additionally, the lack of viral evolution in the majority of targeted immunodominant epitopes over time despite the presence of strong $CD8^+$ T-cell responses may be due to a high degree of sequence conservation necessitated by biological constraints. The HLA A3-KK9 and -RK9 epitopes in Gag p17, the HLA B15-GY9 epitope in Gag p24, and the RQDILDLW $IY_{(106\text{-}115)}$ region in Nef have all been identified as being conserved regions in HIV that elicit strong $CD8^+$ T-cell responses (2, 4, 16, 19, 25, 27, 28, 55). Many of these epitopes are more than 90% conserved among clade B sequences in the Los Alamos Sequence Database, and some are highly conserved across other clades, suggesting constraints on sequence evolution within these regions.

Due to the limited predictive value provided by simple quantitative measures of the HIV-specific $CD8^+$ T-cell immune reactivity, we sought to investigate whether the quality of the response was a better indicator of viral replication kinetics in the absence of ART. It is well established that $CD8^+$ T cells primed to eliminate virus-infected targets are capable of a multitude of functions that include various combinations of cytolytic activity together with cytokine and chemokine expression. Previous reports suggested that individuals with progressive HIV disease produce less TNF-α and IL-2, while long-term nonprogressors retain the ability to make both of these antiviral cytokines within their HIV-specific $CD8^+$ T-cell populations. In addition, the expression of MIP1β, but not IFN-γ, has been found to dominate the HIV-specific $CD8^+$ T-cell response regardless of the targeted HIV antigen and the total frequency of the response (13). When we tested the functionality of virus-specific $CD8^+$ T-cell populations in individuals with good virological outcomes, we found that despite differences in the magnitudes and breadths of the total HIV-specific response, these individuals maintained a higher level of function overall than did individuals with poorer outcomes. It remains to be determined, however, if HIV-specific $CD8^+$ T-cell functionality is a consequence of low levels of plasma viremia or if these cells actively restrict viral replication. A detailed analysis of the contribution of each individual functional response within polyfunctional HIV-specific $CD8^+$ T-cell populations may elaborate the causal nature of host-virus interactions. However, it is likely that $CD8^+$ T-cell functionality alone cannot account for changes in the replication kinetics of virus in the absence of therapy. Despite the small number of patients and the inherent issues associated with the interpretation of observational studies of humans, these data suggest that functional attributes of the HIV-specific $CD8^+$ T-cell response might be important correlates of virological outcome after exposure to SIT regimens and could represent useful biological parameters to measure in the clinical context.

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