Antigen Load and Viral Sequence Diversification Determine the Functional Profile of HIV-1–Specific CD8+ T Cells

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Antigen Load and Viral Sequence Diversification Determine the Functional Profile of HIV-1–Specific CD8+ T Cells

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Abbreviations: CI, confidence interval; CTL, cytotoxic T lymphocyte; HAART, highly active antiretroviral therapy; IC50, 50% stimulatory concentration; IFN, interferon; IL, interleukin; MFI, median fluorescence intensity; MIP, macrophage inflammatory protein; nt, nucleotide(s); PBMC, peripheral blood mononuclear cell; SD, standard deviation; SFC, spot-forming cell; TNF, tumor necrosis factor; WT, wild type

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ABSTRACT

Background

Virus-specific CD8+ T lymphocytes play a key role in the initial reduction of peak viremia during acute viral infections, but display signs of increasing dysfunction and exhaustion under conditions of chronic antigen persistence. It has been suggested that virus-specific CD8+ T cells with a "polyfunctional" profile, defined by the capacity to secrete multiple cytokines or chemokines, are most competent in controlling viral replication in chronic HIV-1 infection. We used HIV-1 infection as a model of chronic persistent viral infection to investigate the process of exhaustion and dysfunction of virus-specific CD8+ T cell responses on the single-epitope level over time, starting in primary HIV-1 infection.

Methods and Findings

We longitudinally analyzed the polyfunctional epitope-specific CD8+ T cell responses of 18 patients during primary HIV-1 infection before and after therapy initiation or sequence variation in the targeted epitope. Epitope-specific CD8+ T cells responded with multiple effector functions to antigenic stimulation during primary HIV-1 infection, but lost their polyfunctional capacity in response to antigen and up-regulated programmed death 1 (PD-1) expression with persistent viremic infection. This exhausted phenotype significantly decreased upon removal of stimulation by antigen, either in response to antiretroviral therapy or by reduction of epitope-specific antigen load in the presence of ongoing viral replication, as a consequence of in vivo selection of cytotoxic T lymphocyte escape mutations in the respective epitopes. Monofunctionality increased in CD8+ T cell responses directed against conserved epitopes from 49% (95% confidence interval 27%–72%) to 76% (56%–95%) (standard deviation [SD] of the effect size 0.71), while monofunctionality remained stable or slightly decreased for responses directed against escaped epitopes from 61% (47%–75%) to 56% (42%–70%) (SD of the effect size 0.18) ($p < 0.05$).

Conclusion

These data suggest that persistence of antigen can be the cause, rather than the consequence, of the functional impairment of virus-specific T cell responses observed during chronic HIV-1 infection, and underscore the importance of evaluating autologous viral sequences in studies aimed at investigating the relationship between virus-specific immunity and associated pathogenesis.

The Editors' Summary of this article follows the references.

Introduction

During acute viral infections, antigen-specific CD8$^+$ T cells control viral replication, leading to a clearance or significant reduction of viremia with subsequent development of robust and fully differentiated memory CD8$^+$ T cells. However, in some cases, the immune response is unable to completely clear a viral infection during acute infection. This inability leads to viral persistence, which is often associated with the subsequent development of chronic viral disease. Under conditions of chronic antigen persistence, virus-specific CD8$^+$ T cells gradually lose their ability to proliferate, to secrete a diverse profile of cytokines, and they display progressively dysfunctional cytotoxic activity [1–4], ultimately leading to exhaustion or even anergy of these cells [5]. The functional exhaustion of virus-specific T cells has been best characterized in lymphocytic choriomeningitis virus (LCMV) infection of mice [5–7], and has also been described in several chronic human infections, such as HIV, hepatitis B virus (HBV), or hepatitis C virus (HCV) infection [8–10]. Therefore, CD8$^+$ T cell responses in chronic viral infections are characterized by an increasing degree of functional impairment, and this defect has been proposed as one reason for the incapability of the host to clear the persisting infection.

In HIV-1 infection, numerous studies have described the accumulation of functional impairments of virus-specific CD8$^+$ T cells from early to chronic stages of infection. Besides a skewed maturation process from effector memory to effector cells [8,11], significant differences in the ability of HIV-1–specific CD8$^+$ T cells to proliferate and to secrete cytokines have been reported between patients with progressive versus nonprogressive HIV-1 disease [12–15]. Comparison of virus-specific CD8$^+$ T cell responses between HIV-1–infected individuals with persistently low viral loads (nonprogressors) and HIV-1–infected individuals with ongoing viremia (progressors) have furthermore suggested that "polyfunctional" CD8$^+$ T cells with the ability to mediate up to five different effector functions (CD107a, interleukin [IL]-2, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and macrophage inflammatory protein [MIP]-1β) in response to stimulation by antigen may form the basis of a more effective CD8$^+$ T cell response [16,17]. Similarly, differences between antigen-specific CD8$^+$ T cells from HIV-1 progressors and nonprogressors with respect to up-regulation of programmed death-1 (PD-1, also called CD279) [18–21] and down-modulation of the IL-7 receptor in chronic viral infection (CD127) [22,23] have been demonstrated. While PD-1 has been described as a marker for activation and susceptibility to apoptosis [24] and CD127 down-modulation as a marker for a lack of transition into memory T cells [23], both markers have been linked to the functional exhaustion of CD8$^+$ T cells [18,20,21,24,25].

Based on these recent studies suggesting that loss of "polyfunctional" virus-specific CD8$^+$ T cells and up-regulation of PD-1 on those cells are correlates of immune failure in chronic viral infection, we hypothesized that antigen persistence directly affects the functionality of antigen-specific CD8$^+$ T cells during the transition from acute to chronic infection. We therefore used HIV-1 infection as a model of chronic persistent viral infection to investigate the process of exhaustion and dysfunction of virus-specific CD8$^+$ T cell responses on the single-epitope level over time, starting in primary infection.

Material and Methods

Participants

Longitudinal peripheral blood mononuclear cell (PBMC) samples from 18 HIV-1–infected individuals with either acute or early HIV-1 infection were investigated. Eleven individuals with acute HIV-1 infection, as defined by either a negative HIV-1 p24 ELISA or an evolving HIV-1 Western blot (less than three bands), and seven individuals with early HIV-1 infection, defined as HIV-1 seroconversion within 6 mo prior to study enrollment, were recruited from Massachusetts General Hospital and the Fenway Community Health Center in Boston, Massachusetts. Table 1 shows the clinical characteristics of study participants. The respective institutional review boards approved the present study, and each participant provided written, informed consent for participation in the study.

Peptides

Peptides corresponding to previously described HLA-matched optimal CD8$^+$ T cell epitopes were used in this study [26] and synthesized at the Massachusetts General Hospital Peptide Synthesis Core Facility.

HLA Tissue Typing

High- and intermediate-resolution HLA class I typing was performed by sequence-specific PCRs according to standard procedures. DNA was extracted from PBMCs using Purgene DNA Isolation Kit for blood (Gentra Systems).

Determination of HIV Gene Sequences and Viral Subtypes

HIV RNA was extracted from 500 µl of frozen plasma using a guanidinium-based lysis buffer followed by isopropanol and ethanol washes. A negative (PBS buffer) control was included in each extraction run. HIV *gag* (codons 1–501, HXB2 nucleotides (nt) 789-2293), *protease* (codons 1–99, nt 2253–2549), *reverse transcriptase* (codons 1–400, nt 2550–3749), *env gp160* (nt 6225–8795), and *nef* (codons 1–206; nt 8797–9414) were amplified by nested RT-PCR using sequence-specific primers. Amplified products were "bulk" sequenced in both 5′ and 3′ directions on an automated DNA sequencer. HIV sequence data were analyzed using Sequencher (Genecodes). Nucleotide mixtures were called if the height of the secondary peak exceeded 30% of dominant peak height. Sequence data were aligned to HIV-1 subtype B reference strain HXB2 (GenBank [http://www.ncbi.nlm.nih.gov/sites/entrez] accession no. K03455) using a modified NAP algorithm [26,27], insertions stripped out, and alignments visually inspected. Neighbor-joining phylogenetic trees were constructed to guard against potential sample mixups or contamination events, and to ensure that longitudinal samples from the same participant clustered together, as described [28].

Assessing Early and Late T Cell Responses by IFN-γ ELISPOT Assay
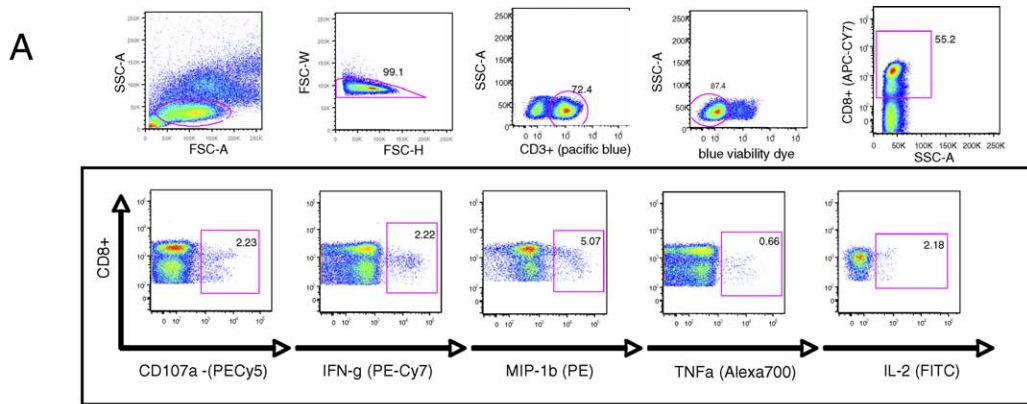
CD8$^+$ T cell responses were assessed by IFN-γ ELISPOT at the 2 mo time point after diagnosis with primary HIV-1 infection (± 10 d). PBMC were plated in 96-well polyvinylidene difluoride–backed plates (MAIP S45; Millipore) that had
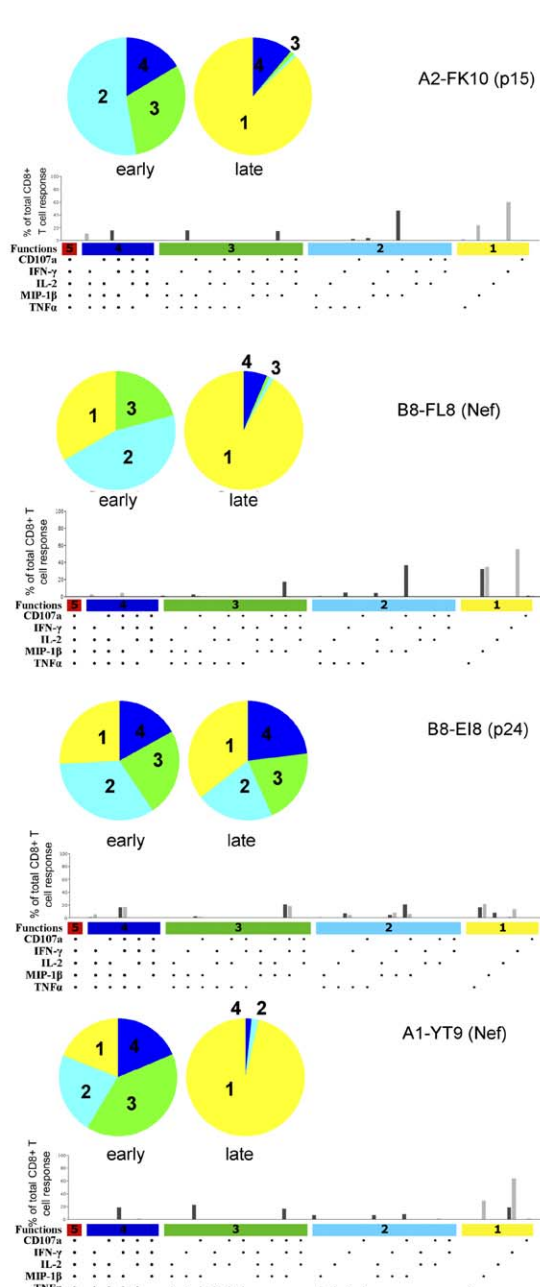
Table 1. Characteristics of Study Participants

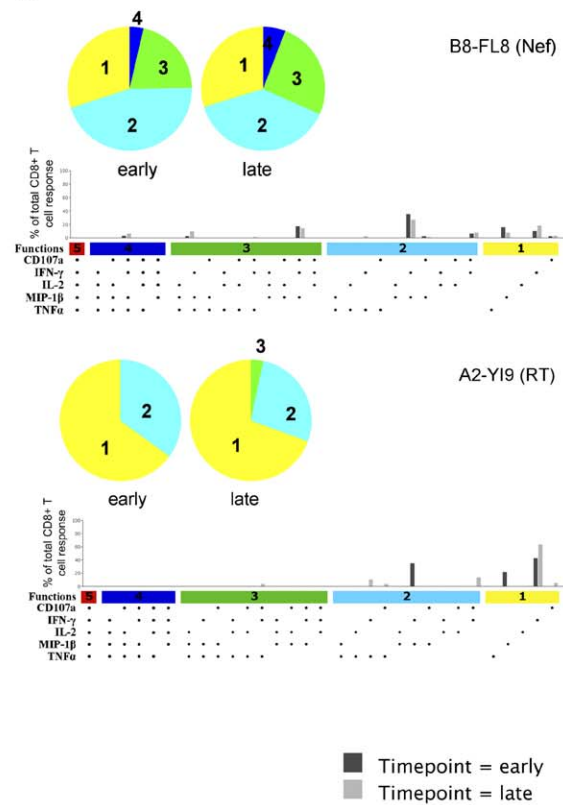| Patient ID	Sex	Race	Clinical Stage of HIV Infection	HIV Anti-p24 ELISA	Western Blot[a]	Baseline Presentation			First Time Point		Second Time Point	
						Viral Load[b] (Copies/ml)	CD4+ Count[c] (cells/µl)	CD8+ Count[c] (cells/µl)	Viral Load (Copies/ml)	CD4+ Count (cells/µl)	Viral Load (Copies/ml)	CD4+ Count (cells/µl)
Ac-211	Male	White	Acute	Negative	Negative	29,700,000	357	447	25,200	765	35,400	439
Ac-160	Male	White	Acute	Positive	Indeterminate	468,000	856	529	14,300	974	6,800	870
Ac-153	Male	White	Acute	Negative	Negative	>500,000	929	1,070	12,400	929	<50	1,371
Ac-38	Male	White	Acute	Negative	Negative	1,500	697	664	1,760	438	1,112	588
Ac-76	Male	Hispanic	Acute	Positive	Negative	>750,000	384	1,261	396,000	302	<400	172
Ac-78	Male	White	Acute	Negative	Negative	573,000	353	428	227,000	223	<400	368
Ac-83	Male	White	Acute	Negative	Negative	>500,000	411	ND	60,500	423	83,700	374
Ac-84	Male	White	Acute	Negative	Negative	624,000	434	576	20,300	445	<50	494
Ac-85	Male	White	Acute	Indeterminate	Indeterminate	318,000	519	749	198,000	559	<50	989
Ac-96	Male	White	Acute	Negative	Negative	>750,000	581	ND	32,100	412	28,700	354
Ac-98	Male	White	Acute	Negative	Negative	>750,000	390	629	5,733	390	18,300	332
Ac-158	Male	White	Early	Positive	Positive	160,000	452	815	160,000	452	<50	702
Ac-166	Male	White	Early	Positive	Positive	243,000	405	977	188,000	405	128,000	281
Ac-177	Male	White	Early	Positive	Positive	26,800	363	971	26,800	363	427,000	217
Ac-183	Male	White	Early	Positive	Positive	35,400	598	1,088	6,140	545	21,800	500
Ac-193	Male	White	Early	Positive	Positive	4,920	434	769	4,920	434	30,300	273
Ac-199	Male	Hispanic	Early	Positive	Positive	486,867	1,204	1,807	147,000	841	176,000	455
Ac-55	Male	Asian	Early	Negative	Positive	161,000	204	ND	12,900	244	<50	329

Figure 1. Multiparameter Flow Cytometric Analysis of the Impact of Antiretroviral Therapy Initiation on the Functional Response Pattern of Antigen-Specific CD8+ T Cells

(A) Gating scheme for identification of multifunctional CD8+ T cell responses. Single function gates were set based on negative control (unstimulated) samples and were placed consistently across samples. Initial gating was on the lymphocyte population. Doublets were removed in a forward scatter width (FSC-W) versus height (FSC-H) plot and live/dead discrimination was done subsequently with exclusion of high blue viability dye expression (UV). Sequentially CD3+ and CD8+ events were defined and gates for each respective function using combinations that provided optimal separation were made. After the gates for each function were created, we used the Boolean gate platform to create the full array of possible combinations, equating to 32 (2^5) response patterns when testing five functions. Shown here are the responses of patient Ac-160 to the HLA-B27-restricted epitope KK10 in p24 Gag. Data are reported after background correction.

(B) Representative multifunctional composition of epitope-specific CD8+ T cell responses in an untreated patient identified during primary HIV-1 infection (Ac-177). Epitope-specific CD8+ T cell responses against four HIV-1 epitopes were studied during primary (early) and chronic (late) HIV-1 infection in this patient. All 32 possible combinations of the five antigen-specific functions studied for each epitope are shown on the x-axis and the contribution of each epitope-specific CD8+ T cell population exhibiting the respective combination of functions to the total epitope-specific response are indicated as bars. Responses are grouped and color-coded according to the number of functions (1 = yellow, 2 = cyan, 3 = green, 4 = blue, 5 = red). Dark grey bars show the results from the early sample; light grey bars show the results from the later samples. The data are summarized by pie charts in which each slice of the pie represents the fraction of the total epitope-specific response that consists of CD8+ T cells with the respective number of functions. Pie charts on the left represent the fraction of the respective epitope-specific CD8+ T cell responses during primary HIV-1 infection (early), while pie charts on the right represent the later time point (late).

(C) Representative multifunctional composition of epitope-specific CD8+ T cell responses of a patient, who received HAART during primary HIV-1 infection (AC-85). Strategy of analysis and figure layout is same as described for (B). The functionality of epitope-specific CD8+ T cell responses against the HLA-B8-restricted epitope FL8 in Nef and HLA-A2-restricted epitope YI9 in RT remained stable or even slightly increased from a time point before (early) to a time point after (late) antiretroviral treatment initiation.

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been coated previously with 100 µl of an anti–IFN-γ mAb 1-D1k (2 µg/ml; Mabtech) overnight at 4 °C. Peptides were added directly to the wells at a final concentration of 14 µg/ml. Cells were added to the wells at 50,000–100,000 cells/well. The plates were incubated at 37 °C, 5% CO_2 overnight (14–16 h) and then processed as described [29]. IFN-γ–producing cells were counted using the AID ELISPOT reader and are expressed as spot-forming cells (SFCs) per 10^6 cells. The number of specific IFN-γ–secreting T cells was calculated by subtracting the negative control value from the established SFC count. The negative controls were always < 30 SFCs/10^6 input cells. A response was considered positive if > 55 SFC per 10^6 and at least three times greater than mean background activity. The positive control consisted of incubation of 100,000 PBMCs with phytohemagglutinin.

Assessing Functional Avidity of Epitope-Specific T Cells Using Serial Peptide Dilutions

Functional avidity of epitope-specific CD8+ T cells was investigated by using serial 1:10 peptide titrations in an ELISPOT assay and was defined as peptide concentration that resulted in 50% maximum (IC50) IFN-γ production, as described previously [30].

Assessing Antigen-Specific T Cell Function by Multiparameter Flow Cytometry

Cryopreserved PBMCs of the 2 mo and following time point used for ELISPOT assays (as described above) were thawed, resuspended to 1–2 × 10^6 cells/ml in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 5.5 ml of HEPES buffer; pH 7.2 ± 0.2), and rested for 1–2 h at 37 °C; 5% CO_2. PBMCs were then examined for viability by Trypan blue exclusion (typically 80%–90% viable) and adjusted to 1 × 10^6 cells/ml. Co-stimulatory antibodies (CD28 and 49d, 1 µg/ml; BD Biosciences) and CD107a-PE-Cy5 (BD Biosciences) were added and the cells aliquoted at 1 ml to each tube containing 2 µg/ml of each peptide. An unstimulated (R10 only) and a positive control (2 µl of phorbol-12-myristate-13-acetate 1mg/ml and 1 µl of ionomycin 1 mg/ml [AG Scientific]) were included in each assay. Cells were incubated for 30 min

at 37 °C, 5% CO_2, and monensin (Golgistop, 0.7 µl/ml; BD Biosciences) and brefeldin A (10 µg/ml; Sigma-Aldrich) were then added. Following a total incubation of 6 h, the cells were washed with PBS and stained for intracellular amine groups in order to discriminate between live and dead cells (blue viability dye, Invitrogen). Cells were washed again and then stained with anti-CD3-Pacific blue (BD Biosciences), anti-CD8-APC-Cy7 antibodies (BD Biosciences). Cells were then fixed in 1% paraformaldehyde (Fix Perm A), washed with PBS, and then permeabilized (Fix Perm B solution) (Caltag Laboratories). Cells were intracellularly stained using a panel of Il-2-FITC (BD Biosciences), IFN-γ-PE-Cy7 (BD Biosciences), TNF-α-Alexa700 (BD Biosciences), and MIP-1β-PE (BD Biosciences). Between 150,000 and 500,000 events were collected per sample. All data were collected on a BD LSRII (BD Biosciences) flow cytometer and analyzed using FlowJo 8.3.3 software (TreeStar). Initial gating was on the lymphocyte population and then used a forward scatter width (FSC-W) versus height (FSC-H) plot to remove doublets (see Figure 1A). Subsequently, the events were gated through a side scatter (SSC) versus blue viability dye (UV) and sequentially gated on CD3+ and CD8+ events. Following identification of CD8+ T cells, a gate was made for each respective function using combinations that provided optimal separation. After the gates for each function were created, we used the Boolean gate platform to create the full array of possible combinations, equating to 32 response patterns when testing five functions. Data are reported after background correction and the percent of epitope-specific CD8+ T cell responses had to be at least >2-fold higher than background for individual cytokines or CD107a before using the Boolean gate platform to be considered as a positive response.

Assessing CD127 Frequency and PD-1 Expression on Epitope-Specific (Tetramer-Positive) CD8+ T Cells

Cryopreserved PBMCs were processed as described above. Cells were then washed with PBS and stained for intracellular amine groups in order to discriminate between live and dead cells (blue viability dye, Invitrogen). After an additional wash with PBS, PE-labeled HLA class I tetramers or pentamers (Beckman-Coulter, NIH, Proimmune) refolded with the

respective CD8$^+$ T cell epitopes were added. Following 20 min incubation at room temperature, cells were stained with the surface antibodies anti-CD3-Pacific blue (BD Biosciences), anti-CD8-APC-Cy7 (BD Biosciences), anti-CD127-biotinylated cascade yellow (BD Biosciences/Caltag, Invitrogen), and anti-PD-1 Alexa 647 (Gordon Freeman) for 20 min and after additional washing steps fixed with 1% paraformaldehyde. Data collection and analysis was performed as described above.

Statistical Analysis

Unless otherwise noted, all data were background-subtracted and a lower threshold corresponding to at least twice above background was built for individual cytokine, chemokine, and CD107a expression in the flow cytometry-based experiments. Values below this threshold were set to 0. PD-1 expression was assessed by median fluorescence intensity (MFI). We used the general linear model to test for differences between observations at the respective time points, and two-way interaction effects were modeled to assess differences between observation periods for different groups. The standard errors of the estimate were adjusted for clustering by participant. Statistical analyses were performed using Graph Pad Prism 4.0, Excel (Microsoft Office), and STATA 9. A p-value below 0.05 ($p < 0.05$) was considered statistically significant.

Results

Influence of Antigen Persistence and Elimination on Virus-Specific CD8$^+$ T Cell Function

Viral antigen has been associated with both the development and expansion of virus-specific CD8$^+$ T cells during acute infection and their functional impairment in the setting of persistent antigenemia during chronic viral infection. To determine the impact of antigen on the functionality of virus-specific CD8$^+$ T cell responses, we compared CD8$^+$ T cell responses directed against eight optimal HIV-1 epitopes in seven patients identified during primary HIV-1 infection before and after initiation of treatment with highly active antiretroviral therapy (HAART), as well as responses against 26 optimal HIV-1 epitopes in 11 patients with primary HIV-1 infection who remained untreated (Table 2). The median viral load before initiation of HAART among untreated patients was lower (25,200 copies/ml [range 1,760–188,000 copies/ml]) when compared to treated patients (160,000 copies/ml [range 12,400–396,000]), although this difference did not achieve statistical significance ($p = 0.12$). Among untreated patients, viral loads increased slightly over study follow-up (median of 30,300 copies/ml [1,112 – 427,000 copies/ml]), while viral load dropped below the level of detection in all treated patients at the follow-up time point (Table 1). Mean follow-up times for untreated and treated patients were 282 and 141 d, respectively.

A comprehensive functional profile of the studied epitope-specific CD8$^+$ T cells was assessed in all study participants at the 2 mo time point ($\pm$ 10 d) and follow-up time points by multiparameter flow cytometry using a panel quantifying five different antigen-specific functions, including CD107a, a marker for T cell degranulation, IFN-γ, IL-2, TNF-α, and MIP-1β. The bulk response of HIV-1–specific CD8$^+$ T cells directed against the eight studied epitopes in the treatment group decreased (average $\pm$ SD: 2.3% $\pm$ 3.2% to 0.89% $\pm$ 1.0%, $p = 0.21$) following initiation of HAART, as described previously [31–34], but this decrease in HIV-1-specific CD8$^+$ T cell frequencies was not statistically significant. In contrast, the bulk antigen-specific CD8$^+$ T cell responses did not change in the untreated group during the study period (average $\pm$ SD: 0.82% $\pm$ 1.34% to 0.72% $\pm$ 0.73%).

We next investigated the changes in the functional profile of HIV-1–specific CD8$^+$ T cells in the untreated and treated groups, using the five functional readouts described above. Gates for all five different functions were created (Figure 1A) and the Boolean gate platform was used, as described [16], to generate the full array of possible combinations, equating to 32 response patterns when testing five functions. Subsequently, the fraction of the total epitope-specific CD8$^+$ T cells able to respond to antigenic stimulation with one, two, three, four, or five function(s), respectively, was calculated, as described previously [17]. Initially, we longitudinally assessed the functional qualities of virus-specific CD8$^+$ T cell responses in patients who did not receive any antiretroviral treatment (Figure 1B). Even though all patients were studied within 2 mo of presentation with primary HIV-1 infection, the functionality of epitope-specific CD8$^+$ T cell responses differed substantially among the patients. Interestingly, the functionality of epitope-specific CD8$^+$ T cell responses differed not only between patients but also substantially within the same patient and ranged from monofunctional to four-functional responses for different epitopes studied in the same patient (Figure 1B).

Despite this variability, there was a similar pattern of changes in virus-specific CD8$^+$ T cell functionality from early to chronic HIV-1 infection in the patients who did not receive antiretroviral treatment, as exemplified for patient Ac-177 (Figure 1B). The functional profile of CD8$^+$ T cells directed against the four HIV-1 epitopes studied in this patient substantially changed over a study period of 163 d. While the CD8$^+$ T cells against the HIV-1 epitopes FLKEKGGL (B8-FL8) in Nef, YFPDWQNYT (A1-YT9) in Nef, and FLGKIWPSHK (A2-FK10) in p15 Gag showed a three- and four-functional response to antigenic stimulation at the first time point studied during primary infection, these epitope-specific CD8$^+$ T cell responses became predominantly monofunctional at the time point studied 166 d later. The functionality of the CD8$^+$ T cell response against the HLA-B8-restricted epitope EIYKRWII (B8-EI8) in p24 Gag, which consisted of up to four functions during primary infection, also decreased, but less prominently compared to the other three epitope-specific responses. There was no clear pattern in the order by which individual CD8$^+$ T cell functions disappeared. However, TNF-α was never present as a monofunctional response, in line with previous studies describing the early loss of this effector function [1,35]. These data indicate that the ability of CD8$^+$ T cells to respond to antigenic stimulation with cytokine secretion and degranulation differs between epitopes in the same patient, but decreases from acute to chronic HIV-1 infection, resulting in a predominantly monofunctional response toward the chronic stage of infection in patients with persistent viral replication.

In contrast to this overall reduction in the functional profile of epitope-specific CD8$^+$ T cells during untreated

Table 2. Characteristics of Studied Epitopes

Group	Patient	Epitopes	Sequence, First Time Point	Sequence, Second Time Point	Days Between First and Second Time Point	Days between Last Detected WT Sequence and Assessment of Second Time Point	Antiretroviral Therapy
Patients on HAART	Ac-55	Cw8-AL9 (Nef)	AVDLSHFLK	—	100	n/a	yes
	Ac-76	Cw5-SL9 (Rev)	SAEPVPLQL	—	203	n/a	yes
	Ac-84	B18-FK10 (p24)	FRDYVDRFYK	—	95	n/a	yes
	Ac-85	A2-YI9 (RT)	YTAFTIPSI	—	141	n/a	yes
		B8-FL8 (Nef)	FLKEKGGL	—	—	n/a	—
	Ac-78	B8-WM8 (Nef)	WPTVRERM	—	126	n/a	yes
	Ac-153	B57-TW10 (p24)	TSTLQEQIGW	—	61	n/a	yes
	Ac-158	B7-GL9 (p24)	GPKVKQWPL	—	261	n/a	yes
Average					*141*		
Untreated Patients with no Epitope Variant at Second Time Point	Ac-166	A11-ALK9 (Nef)	AVDLSHFLK	AVDLSHFLK	409	n/a	no
	Ac-211	B8-EI8 (p24)	EIYKRWII	EIYKRWII	350	n/a	no
		Cw7-KY11 (Nef)	KRQDILDLWVY	KRQDILDLWVY	—	n/a	—
	Ac-177	B8-FL8 (Nef)	FLKEKGGL	FLKEKGGL	163	n/a	no
		B8-EI8 (p24)	EIYKRWII	EIYKRWII	—	n/a	—
		A1-YT9 (Nef)	FFPDWQNYT	FFPDWQNYT	—	n/a	—
		A2-FK10 (p15)	FLGKIWPSHK	FLGKIWPSHK	—	n/a	—
	Ac-96	B8-FL8 (Nef)	FLKEKGGL	FLKEKGGL	364	n/a	no
	Ac-183	A1-YT9 (Nef)	YFPDWQNYT	YFPDWQNYT	215	n/a	no
		B44-AW11 (p24)	AEQASQDVKNW	AEQASQDVKNW	—	n/a	—
	Ac-98	A3-RK9 (p17)	RLRQGGRKK	RLRQGGRKK	511	n/a	no
Average					*335*		
Untreated Patients with Epitope Variants at Second Time Point	Ac-166	B8-FL8 (Nef)	FLKEKGGL	FLRKEGGL	409	90	no
		A1-QY9 (gp41)	QRGWEVLKY	LRGWEILKY	—	90	—
	Ac-211	A3-RK9 (p17)	RLRPGGKKK	RLRPGGKKR	350	206	no
		B8-FL8 (Nef)	FLKEKGGL	FLKEQGGL	—	83	—
	Ac-183	A1-WH10 (Nef)	WRFDSRLAHH	WKFDSLLAHH	215	173	no
		B27-KK10 (p24)	KRWIIIGLNK	KRWII[I/M]GLNK	—	173	—
	Ac-38	A3-RK9 (p17)	RLRPGGKKK	RLRPGGKKQ	68	68	no
	Ac-98	Cw8-AL9 (Nef)	AAVDLSHFL	AA[F/L]DLSYFL	511	367	no
		B35DL9 (gp120)	DPNPQEVVL	DP[N/S]PQEI[A/V]L	—	186	—
	Ac-83	A2-YI9 (RT)	YTAFTIPSI	YTAFTIPS[I/A/T/V]	182	182	no
		B40-KL9 (Nef)	KEKGGLEGL	[K/R]EKGGLEGL	—	182	—
		B51-TI8 (RT)	TAFTIPSI	TAFTIPS[I/A/T/V]	—	182	—
	Ac-160	B27-KK10 (p24)	KRWIIIGLNK	KRWIIMGLNK	61	40	no
	Ac-193	A3-RK9 (p17)	RLRPGGKKK	RLRPGGKKT	405	405	no
	Ac-199	A2-SL9 (p17)	SLYNTVATL	SLYNTVAVL	330	330	no
Average					*281*	*184*	

Variant amino acids within the epitope are indicated by underline. Mixed bases are indicated as [X/Y].
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viremic infection, the functional profile of HIV-1–specific CD8$^+$ T cells in individuals who were treated with HAART was largely maintained or even expanded, as shown in Figure 1C for patient Ac-85. CD8$^+$ T cell responses against the HLA-A2–restricted epitope YTAFTIPSI (A2-YI9) in RT showed a mainly mono- and dual-functional cytokine secreting profile at baseline, while responses against the HLA-B8-restricted epitope in Nef FLKEKGGL (B8-FL8) were dominated by a dual- to tri-functional responses (Figure 1C). In this patient, viral load was suppressed below the level of detection by HAART, and HIV-1–specific CD8$^+$ T cell responses were again characterized after 141 days of antiretroviral treatment. While the overall magnitude of the specific responses declined, HIV-1–specific CD8$^+$ T cell responses maintained or even slightly improved in their ability to secrete multiple

cytokines and chemokines for both epitopes, in contrast to the substantial decrease in the functional repertoire observed in untreated patient Ac-177.

We next compared for the entire group of untreated and treated study patients the functional profile of all epitope-specific CD8$^+$ T cell responses detected at an early time point before treatment initiation and a late time point after treatment initiation or a respective time point in the untreated group (Table 1). Each fraction of function was calculated as a percentage of the total CD8$^+$ T cell response as described above. In the untreated patients, the functional profile of epitope-specific CD8$^+$ T cell responses decreased over time, while the fraction of virus-specific CD8$^+$ T cells with a single cytokine/chemokine function increased (Figure 2A and Table S1). In contrast, the functional profile of
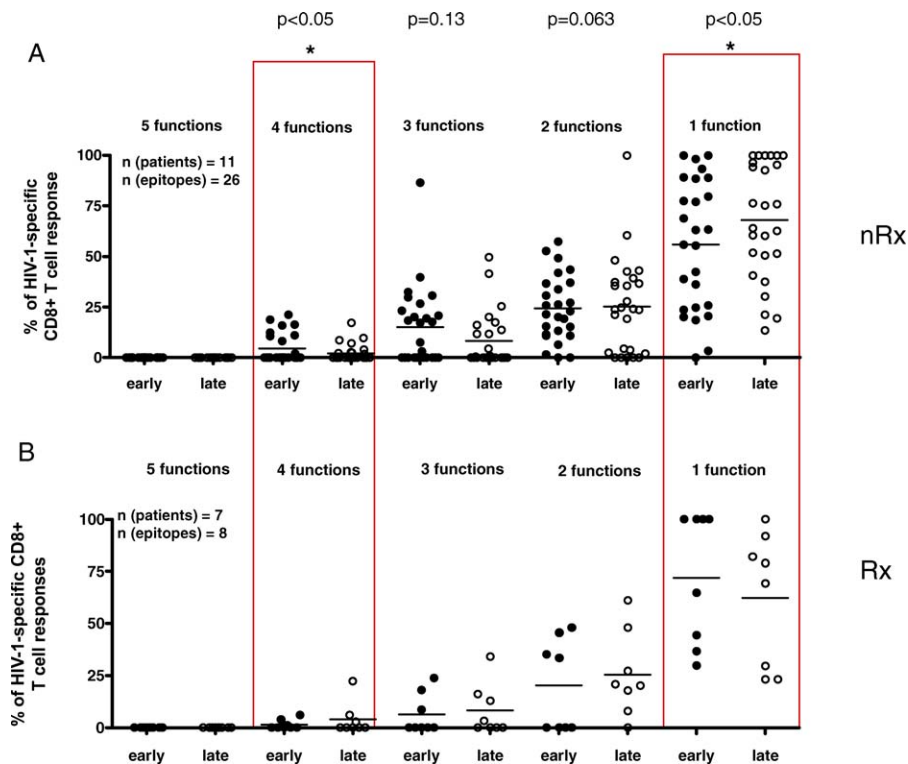
Figure 2. Loss of Epitope-Specific CD8+ T Cell Functionality during Antigen Persistence
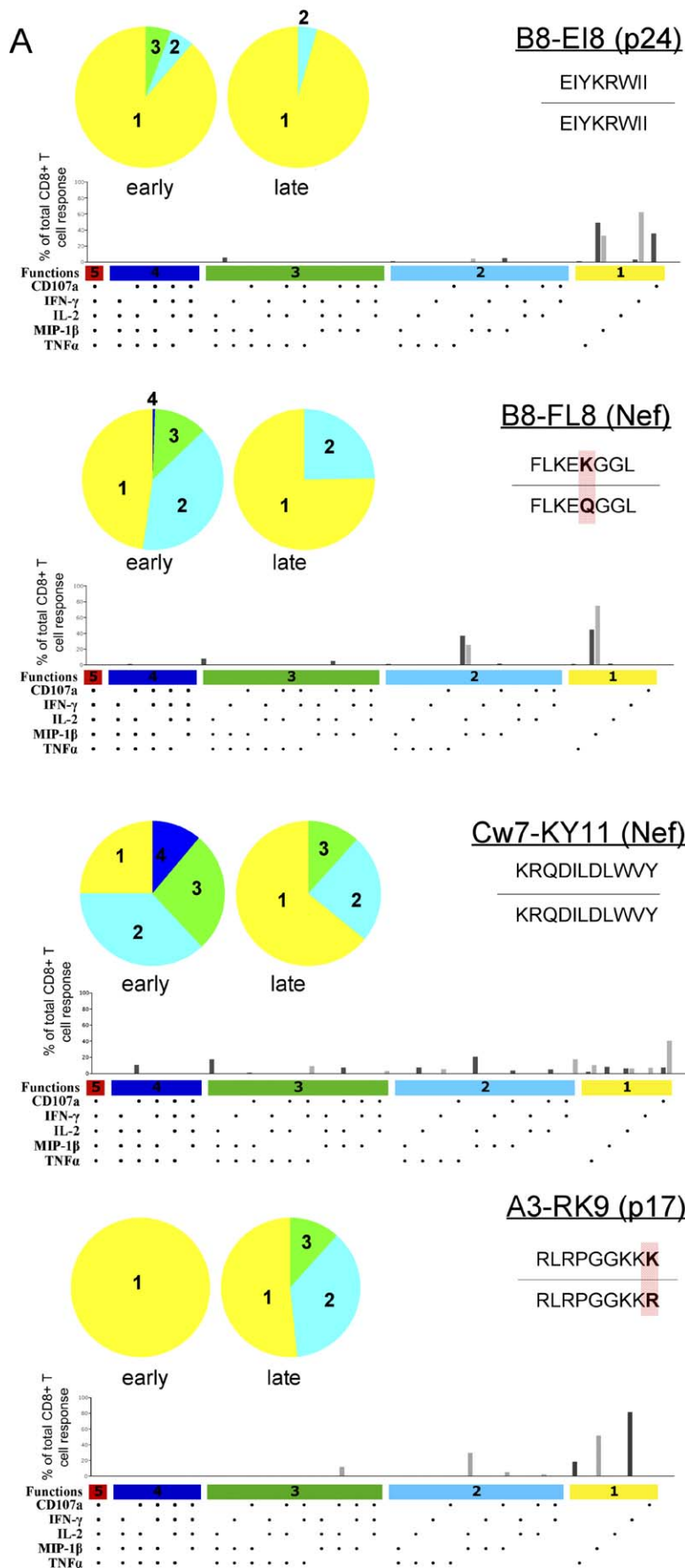
(A) Summary of fractions of epitope-specific CD8+ T cell responses with one, two, three, four, and five antigen-specific functions in 11 untreated patients (nRx); (B) summary of fractions of eight epitope-specific CD8+ T cell responses for seven patients before and after receiving antiretroviral therapy (Rx). Frequencies of different combination of epitope-specific CD8+ T cell functions were quantified with Boolean gates using FlowJo software, and fractions were calculated from total epitope-specific CD8+ T cell response. Each of the combination of epitope-specific functions (1–5) is shown separately before ("early") and after ("late") antiretroviral treatment initiation for the treated study group (Rx) or for a corresponding early and late time point in the untreated group (nRx), respectively. The monofunctional fraction of the HIV-1–specific CD8+ T cell response increased significantly ($p < 0.05$) in comparison to a decrease of the monofunctional fraction of the HIV-1–specific CD8+ T cell response in the treated group (*). In addition, the increase of the four-functional fraction of the HIV-1–specific CD8+ T cell responses in the treated group was significant ($p < 0.05$) in comparison to the decrease of the four-functional fraction in the untreated group (*). We used the general linear model to test two-way interaction effects between observation period and group (nRx versus Rx) to calculate significance, and standard errors of the estimate were adjusted for clustering by patient.
doi:10.1371/journal.pmed.0050100.g002

epitope-specific CD8+ T cells in treated study patients remained stable or broadened (Figure 2B and Table S1). A significant ($p < 0.05$) two-way interaction effect was detected between treatment and observation period, where the effect of observation period was expected to produce opposite effects by group. In this case, an appreciable increase of the single cytokine/chemokine function was observed in untreated patients (Figure 2A), contrasting the decrease of the monofunctional responses in the treated patients (Figure 2B, $p < 0.05$). Similarly, the contribution of four-functional CD8+ T cell responses decreased in the untreated group, while it slightly increased in the treated group ($p < 0.05$). In contrast to the significant changes in the functionality of HIV-1–specific CD8+ T cells in the presence or absence of HIV-1 antigen, CMV-, Flu-, or EBV-specific CD8+ T cell functions did not change significantly over time (see Figure S1), indicating that HIV-1 antigenemia itself was the driving force for the qualitative changes in the HIV-1–specific CD8+ T cell functionality. The more restricted functional profile of CEF-specific CD8+ T cell responses in our study compared to a previous publication [16] might be due to the more restrictive definition of a positive individual cytokine response (twice above background) before Boolean gating

used here, and substantial difference in the clinical characteristics of the study cohorts (primary versus chronic HIV-1 infection). Taken together, these longitudinal studies of HIV-1–specific CD8+ T cell functionality in individuals with treated and untreated primary HIV-1 infection demonstrate that the quality of an epitope-specific CD8+ T cell response remains stable or improves in the absence of antigen following initiation of antiretroviral therapy but decreases in the presence of persistent antigen.

Amino Acid Substitutions within the Targeted Epitope Influence the Functional Ability of the Respective Epitope-Specific CD8+ T-Cell Response
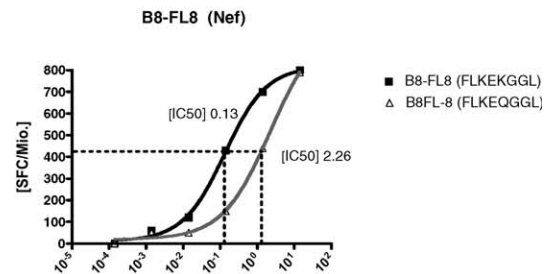
The above data indicate that changes in the functional profile of HIV-1–specific CD8+ T cells largely depend on the presence or absence of antigen. Previous studies have demonstrated the ability of HIV-1 to evade CD8+ T cell–mediated immune pressure by selection of escape variants, where sequence mutations within targeted T cell epitopes can have a significant impact on the avidity of the interactions between the TCR and the HLA class I–presented epitope [36–38]. To investigate the impact of HIV-1 sequence variations over time on the functional profile of epitope-specific CD8+ T cells, we longitudinally sequenced plasma virus RNA of all

A

Ac 211

■ Timepoint = early
□ Timepoint = late

B8-EI8 (p24)

EIYKRWII
—————
EIYKRWII

B8-FL8 (Nef)

FLKE**K**GGL
—————
FLKE**Q**GGL

Cw7-KY11 (Nef)

KRQDILDLWVY
—————
KRQDILDLWVY

A3-RK9 (p17)

RLRPGGKK**K**
—————
RLRPGGKK**R**

B

B8-FL8 (Nef)

[IC50] 0.13
[IC50] 2.26

■ B8-FL8 (FLKEKGGL)
△ B8FL-8 (FLKEQGGL)

B

A3-RK9 (p17)

[IC50] 0.012
[IC50] 29.95

■ A3 -RK9 (RLRPGGKKK)
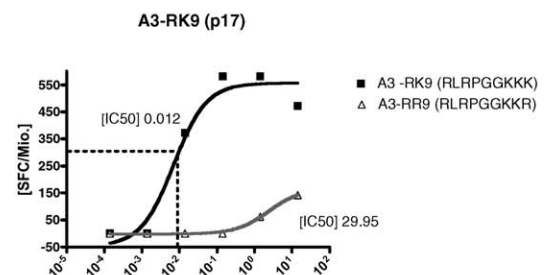△ A3-RR9 (RLRPGGKKR)

Figure 3. Representative Multifunctional Composition of Epitope-Specific CD8+ T Cell Responses against Conserved Epitopes or Epitopes with Sequence Variations in an Untreated Patient (Ac-211)

(A) Epitope-specific CD8+ T cell responses against four HIV-1 epitopes were studied during primary and chronic HIV-1 infection in this patient (Ac-211). All 32 possible combinations of the five antigen-specific functions studied for each epitope are shown on the x-axis, and the contribution of each epitope-specific CD8+ T cell population exhibiting the respective combination of functions to the total epitope-specific response are indicated as bars. Responses are grouped and color-coded according to the number of functions (1 = yellow, 2 = cyan, 3 = green, 4 = blue, 5 = red). Dark grey bars show the results from the early sample; light grey bars show the results from the later samples. The data are summarized by pie charts in which each slice of the pie represents the fraction of the total epitope-specific response that consists of CD8+ T cells with the respective number of functions. Pie charts on the left represent the fraction of the respective epitope-specific CD8+ T cell responses during primary HIV-1 infection (early), while pie charts on the right represent the later time point (late). Amino acid sequences of each studied epitope during the early and late time point are indicated next to each graph. Amino acid substitutions within the epitope are highlighted in red shading. The functionality of the CD8+ T cell responses against the epitopes B8-EI9 in p24, B8-FL8 in Nef and Cw7-KY11 in Nef decreased to a primarily monofunctional composition, while the fraction of monofunctional epitope-specific CD8+ T cells decreased, and two- and three functional responses increased, for the epitope A3-RK9 in p17. The amino acid sequence of the A3-RK9 epitope displayed a substitution at the anchor residue position 9 from lysine (K) → arginine (R).

(B) Comparison of the functional avidity of CD8+ T cells specific for two epitopes and their respective variants within the same individual (Ac-211). Upper graph: Intraindividual comparison of the recognition of the B8-FL8 (Nef) wild type (WT) and the B8-FL8 K5Q variant measured by serial log dilution of both peptides. IC50 was calculated and is indicated in the figure. The variant peptide was recognized with similar functional avidity at all concentrations to the B8-FL8 WT peptide. Lower graph: Intraindividual comparison of the recognition of the A3-RK9 WT versus the A3-RK9 K9R variant was measured by serial log dilutions of both peptides. IC50 was calculated and is indicated in the figure. The variant peptide was recognized with much lower functional avidity at all concentrations than was the A3-RK9 WT peptide.

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study participants who did not receive antiretroviral therapy. Among the 11 untreated patients, we identified 15 epitopes that developed amino acid substitutions at the later time point and, for comparison, 11 epitopes within the same patients that remained conserved (Table 2).

As exemplified for one untreated study patient in Figure 3A (Ac-211), the CD8+ T cell response against the HLA-Cw7–restricted epitope KRQDILDLWVY (Cw7-KY11) in Nef lost the capability for a "polyfunctional" response and became predominantly (64%) monofunctional (Figure 3A). Similarly, the T cell responses against the HLA-B8-restricted epitopes FLKEKGGL (B8-FL8) in Nef and EIYKRWII (B8-EI8) in p24 Gag included at the first time point a mixture of CD8+ T cells able to exhibit up to three different functions following antigenic stimulation, but became predominantly monofunctional (75% and 95% respectively) at the later time point. In contrast, the response against the HLA-A3–restricted epitope RLRPGGKKK (A3-RK9) in p17 Gag increased from a 100% monofunctional response to a dual- and triple-functional response in the same patient. Sequence analysis within these epitopes revealed a conservation of the sequences within the Cw7-KY11 and B8-EI8 epitopes during the study period, while the B8-FL8 and the A3-RK9 epitopes revealed amino acid substitutions (A3-RK9 at position 9 from K to R, and B8-FL8 at position 5 from K to Q; see Figure 3A and Table 2). While the change from lysine to glutamine in B8-FL8 had no significant impact on the recognition by CD8+ T cells and was recognized with comparable functional avidity to the wild-type sequence, the K-to-R mutation in the A3-RK9 epitope resulted in a much lower functional avidity, in line with previous studies (see Figure 3B) [27]. These data in a single individual suggest that sequence variations within a targeted CD8+ T cell epitope that lead to lost or reduced recognition of the epitope can result in an increase in the functional profile of the respective epitope-specific CD8+ T cell population.

We next extended this analysis to all the 15 epitopes that developed amino acid substitutions and 11 epitopes that remained conserved and compared changes in the functional profile of epitope-specific CD8+ T cells between these two groups (Figure 4; Table S1). Interestingly, the above described decrease in the functional profile of HIV-1–specific CD8+ T cells during untreated infection (Figure 2A), resulting in an

increase of monofunctional epitope-specific CD8+ T cells, was much more pronounced and increased from 49% (95% confidence interval [CI] 27%–72%) to 76% (56%–95%) (standard deviation [SD] of the effect size 0.71) when only the 11 conserved epitopes were included and epitopes that developed amino acid substitutions were excluded (Figure 4A). In striking contrast, CD8+ T cells directed against the 15 HIV-1 epitopes that showed amino acid substitutions from the first to the second time point maintained a more diverse functional profile over time and the monofunctionality remained stable or slightly decreased for responses directed against escaped epitopes from 61% (95% CI 47%–75%) to 56% (42%–70%) (SD of the effect size 0.18). This change in epitope-specific CD8+ T cell functionality over time between the two groups was significant for changes in three-functional responses ($p < 0.05$) and monofunctional responses ($p < 0.05$). Functional avidity studies for several of these epitope-specific CD8+ T cell responses using serial dilutions of peptides corresponding to either the wild-type or the variant sequence of the respective epitope showed that the poly-functionality of epitope-specific CD8+ T cells increased specifically for those epitopes that exhibited sequence variations resulting in a substantial decrease in the functional avidity (see Figure S2). The increase in epitope-specific CD8+ T cell functionality (defined as the percent increase in epitope-specific CD8+ T cells with more than one function) was furthermore significantly correlated ($R = 0.5$, $p = 0.025$) to changes in functional avidity (defined as the change in the IC50 for epitopes that developed sequence variations, see Figure S2, and set to 0 for those epitopes that remained conserved during the study period). These data demonstrate that the functional quality of an epitope-specific CD8+ T cell response can be specifically modulated by sequence variation within the targeted epitope, and is not a simple consequence of overall viral load.

Decrease in PD-1 Expression on Epitope-Specific CD8+ T Cells Exhibiting a Change in the Viral Sequence of the Targeted Epitope

Recently it has been shown that PD-1 expression on CD8+ T cells is closely correlated with the functional exhaustion of CD8+ T cells in HIV-1–infected patients, and might serve as a correlate of protective immunity [19,20,39]. Therefore, we
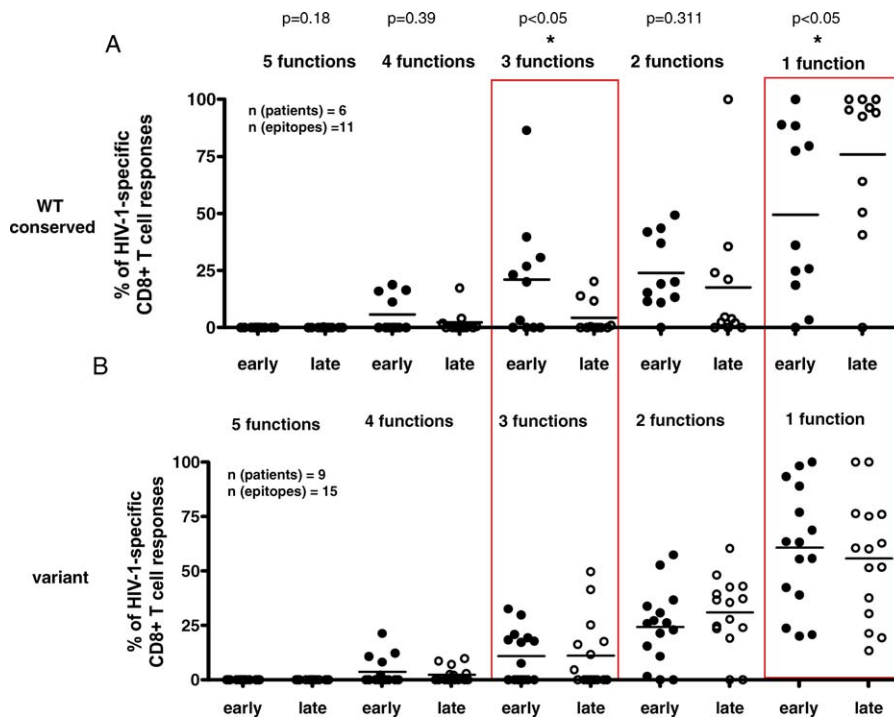
Figure 4. Expansion of the Functional Profile of Epitope-Specific CD8$^+$ T Cells after the Emergence of Sequence Variations within the Respective Targeted Epitopes

Fractions of epitope-specific CD8$^+$ T cell responses with one, two, three, four, and five antigen-specific functions for conserved epitopes (6 patients; $n = 11$ epitopes) (A) are summarized in comparison to epitopes, which exhibited amino acid substitutions (9 patients; $n = 15$ epitopes). Frequencies of different combination of cytokine production were assessed similarly to that in Figure 2A and 2B. Each of the functional profiles (1–5) are shown separately before ("early") or after ("late") the development of an escape mutation in the targeted epitope or for CD8$^+$ T cell responses targeting a conserved epitope at the same time points ("early" or "late" respectively). While the monofunctional fraction increased in the targeted conserved epitopes, it remained stable for the targeted epitopes developing a sequence variation. The interaction effect between group and period for conserved and evolving epitopes was significant for three-functional responses ($p < 0.05$) (*) and for the monofunctional responses ($p < 0.05$) (*). We used the general linear model to test two-way interaction effects between observation period and group (WT versus variant) to calculate significance, and standard errors of the estimate were adjusted for clustering by patient.

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hypothesized that reduction in the functional avidity of the interaction between CD8$^+$ T cells with their respective epitope resulting from amino acid substitutions will also affect the expression of PD-1 on those cells, indicating weaker activation of these epitope-specific CD8$^+$ T cells. Sufficient specimens were available to longitudinally investigate CD8$^+$ T cell responses directed against five epitopes by tetramer staining, which contained at the first time point the wild-type viral sequence but showed variation in the viral sequence at the second time point that resulted in reduced recognition of the epitope. CD8+ T cell responses directed against seven epitopes in the same patients that remained conserved were used as controls. While the MFI ($\pm$ standard deviation [SD]) of PD-1 on epitope-specific tetramer-positive CD8$^+$ T cells increased for the seven conserved epitopes over time (210 $\pm$ 156 to 309 $\pm$ 334; see Figure S3), PD-1 expression significantly decreased on the five epitope-specific CD8$^+$ T cells that developed a sequence variation in the targeted epitope (399 $\pm$ 123 to 248 $\pm$ 103, $p = 0.006$ paired t-test) (Figure 5), but was still higher than the PD-1 negative control. In contrast, the expression of CD127 on epitope-specific cells was very low, as described previously [22,40] and not significantly impacted by viral escape (unpublished data). The reduction of PD-1 expression levels following escape further support our previous results, which indicate a general

improvement of epitope-specific CD8$^+$ T cell functionality and quality upon reduction of the specific antigen (epitope) level that is independent of the overall level of viral replication.

Discussion

The identification of correlates of protective immunity in HIV-1 infection has largely remained elusive [9]. Recent studies have reported that effective virus-specific CD8$^+$ T cells display a "polyfunctional" capacity, including the ability to secrete multiple cytokines and chemokines in response to antigenic stimulation in HIV-1–infected individuals with chronic long-term nonprogressive infection [16]. These data suggest an important role for the ability of antigen-specific CD8$^+$ T cells to secrete multiple cytokines/chemokines in the control over viral replication. However, the preserved functionality of HIV-1–specific CD8$^+$ T cells that is observed in chronically infected individuals who control HIV-1 viremia could represent either the cause of control over viral replication or the consequence of low viremia. The longitudinal studies in individuals identified during primary HIV-1 infection presented here suggest that the functional profile of an epitope-specific CD8$^+$ T cell response is largely
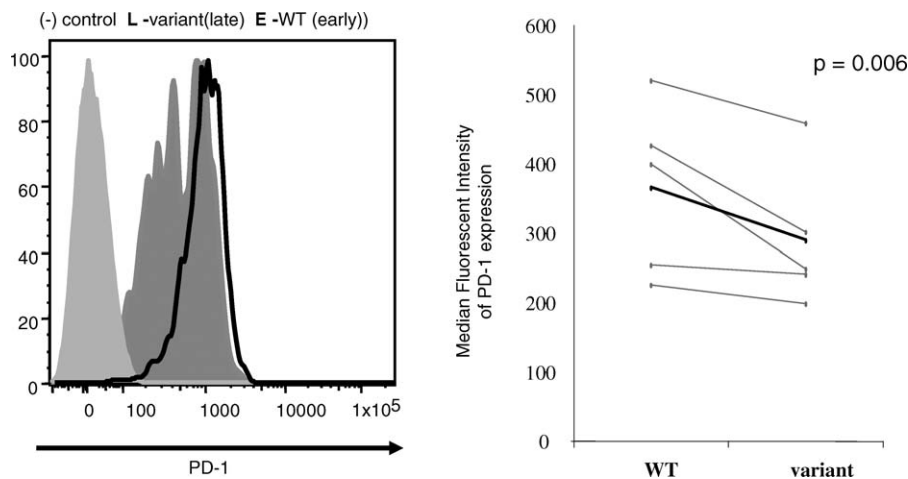
Figure 5. Reduction in PD-1 Expression on Epitope-Specific CD8+ T Cells after Sequence Evolution in the Respective Epitopes

Primary flow data (for the HLA-A3-restricted epitope A3-RK9 in p17 Gag [left]) and combined data for five individuals (right) for PD-1 expression on epitope-specific CD8+ T cells before ("WT–early") and after ("variant–late") the selection of amino acid substitutions within the targeted epitopes are shown. PD-1 is expressed as MFI on tetramer-positive CD8+ T cells. In the graph on the left, data from the early time point are shown by the black tracing, while data for the late time point are shown by grey shading. A fluorescent minus one control is shown in comparison for both stainings. In the graph on the right, grey lines represent changes within patients between time periods ("WT," early; "variant," late); the black line represents the median PD-1 expression change on all five studied tetramer-positive CD8+ T cell populations between time periods. The decrease of the PD-1 expression on tetramer-positive CD8+ T cells before and after the development of sequence variations within epitopes was statistically significant ($p = 0.006$; paired t-test).

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determined by the duration and intensity of antigenic exposure, and is therefore mainly a consequence of viremia.

In this study we investigated whether the presence or absence of antigen has an impact on the quality and functionality of epitope-specific CD8+ T cells during the transition from acute to chronic HIV-1 infection. Our longitudinal data demonstrate that HIV-1–specific CD8+ T cell responses directed against the individual epitopes studied showed a very heterogeneous functional profile, but more "polyfunctional" effector CD8+ T cells were present in the early phase than in later stages of HIV-1 infection. In the presence of continued antigen stimulation during untreated HIV-1 infection, the ability of HIV-1–specific CD8+ T cells to respond to antigenic stimulation with a combination of degranulation and secretion of multiple cytokines and chemokines was reduced, such that the later stages of untreated HIV-1 infection were characterized by a predominantly monofunctional cytokine or chemokine response toward the same epitope. This result is in line with previous studies indicating a sequential loss of effector functions of virus-specific CD8+ T cells, as was first shown in the LCMV model [5,6] but also reported in progressive chronic HIV-1 infection [12,35,41–43]. Further support for this selective impairment of antigen-specific T cell function in chronic infection is provided by the observation here that the loss of functionality was restricted to HIV-1–specific CD8+ T cells, whereas no significant changes occurred in the functionality of CD8+ T cell responses toward EBV, CMV, and influenza antigens in the same participants. This observation suggests that the impairment of a functional HIV-1–specific CD8+ T cell response during the transition from acute to chronic infection likely represents the consequence of ongoing specific antigenic stimulation. In line with this model, the loss of functionality from acute to chronic HIV-1 infection was arrested or even reversed in patients receiving antiretroviral treatment, resulting in a general reduction of antigen

as shown previously [44,45]. Although the total number of HIV-1–specific CD8+ T cells decreased with antiretroviral treatment, the ability of the remaining individual epitope-specific CD8+ T cells to secrete multiple cytokines/chemokines in response to stimulation was maintained or increased for HIV-1–specific responses, indicating that the functional impairment of antigen-specific CD8+ T cells is dynamic and can be partially restored.

To more precisely investigate the impact of antigen on the functionality of CD8+ T cells, we longitudinally studied epitope-specific CD8+ T cell responses directed against conserved and escaping CTL epitopes in the same study participant. This approach allowed us to directly compare, within the same study participant, epitope-specific CD8+ T cell populations that were continuously driven by antigen (conserved CTL epitopes) to those, where the specific antigen/epitope evaded by sequence variation (escaped CTL epitopes). In line with the above data, studies of the epitope-specific CD8+ T cells directed against conserved epitopes revealed a reduction in the functional profile of these continuously activated CD8+ T cells. In contrast, epitope-specific CD8+ T cells regained functional capacities toward the original "wild-type" epitope following the development of sequence variations, and also significantly down-regulated the expression of PD-1, a marker of activation and exhaustion of T cell responses. The biphasic pattern of the decrease of PD-1 expression (Figure 5) suggests that PD-1-positive epitope-specific CD8+ T cell responses that are highly exhausted either become apoptotic and die, while PD-1low epitope-specific CD8+ T cell subsets expand following CTL escape, or that PD-1high cells are partially down-regulating PD-1 expression on the cell surface. Similar findings were recently reported in SIV-infected macaques, in which PD-1 expression gradually declined on CD8+ T cells specific for SIV-derived epitopes that had undergone mutational escape,

while PD-1^{high} expressing CD8+ T cell were highly susceptible to cell death [24].

Additional analysis of the functional avidity of CD8+ T cells in response to peptides corresponding to the wild-type epitope and the escape variant furthermore showed that the functional profile of epitope-specific CD8+ T cells was restored only under conditions in which the selected epitope variant resulted in poor recognition. Thus, changes in the functional profile of CD8+ T cells and the expression levels of PD-1 can serve as strong indicators of whether a sequence variation detected in a targeted viral epitope truly resulted in reduced recognition by CD8+ T cells. These studies comparing the functionality of continuously activated and "escaped" CD8+ T cell responses directed against the same pathogen clearly highlights the dominant role of antigen persistence on T cell functionality. This observation demonstrating a direct link between the sequence evolution of a targeted epitope and the "functionality" of the ex vivo response furthermore emphasizes the necessity for the concomitant evaluation of autologous viral sequences in studies aimed at correlating CD8+ T cell function, including PD-1 expression, with markers of HIV-1 disease progression.

Overall, these data support a model in which antigen-specific CD8+ T cell function in chronic HIV-1 infection, as defined by the ability of epitope-specific CD8+ T cells to activate multiple functional pathways ex vivo following stimulation, is largely defined by the previous history of in vivo antigenic stimulation. Similarly, these data support a model in which continued recognition of specific antigen is one of the major forces driving the functional impairment of virus-specific CD8+ T cells during chronic persistent infections. Importantly, this study underscores the relevance of evaluating autologous viral sequences when interpreting data on the functionality of virus-specific immune responses.

Supporting Information

Figure S1. Preservation of the Functional Profile of CD8+ T Cell Response towards CMV, EBV, or Influenza Indicate Important Role of Antigen Load in Determining the Quality of CD8+ Responses

Summary of fractions of CMV-, EBV-, or influenza-specific CD8+ T cell responses with one, two, three, four, and five antigen-specific functions in eight untreated patients (three did not have a detectable response) (A) and seven patients starting antiretroviral therapy (B). Data are shown before ("early") and after ("late") receiving antiretroviral therapy or a corresponding early ("early) or late ("late") time point for untreated patients. Frequencies of different combinations of epitope-specific CD8+ T cell functions were quantified with Boolean gates using FlowJo software and fractions were calculated from total epitope-specific CD8+ T cell responses. Each of the functional profiles (1–5) are shown separately before and after antiretroviral treatment initiation for the treated study group or for a corresponding early and late time point in the untreated group, respectively. The functional profile of the EBV-, CMV-, and influenza-specific CD8+ T cell responses at the first time point showed a large percentage of dual-, triple-, and quadruple-functional responses, did not differ between the treated and the untreated groups, and did not change over time in the treated and the untreated groups. None of the interaction effects between period and treatment group reached significance for any of the outcomes (1–5 functions).

Found at doi:10.1371/journal.pmed.0050100.sg001 (102 KB PPT).

Figure S2. Serial Peptide Titration Curves Comparing Recognition of Peptides Corresponding to Wild-Type Sequences and to Evolving Variants Identified by Sequencing of the Autologous Virus by Epitope-Specific CD8+ T Cells over Time in the Indicated Individuals

Peptide concentrations are shown in micrograms per milliliter. The monofunctional fraction of epitope-specific CD8+ T cells decreased and the multifunctional fraction increased in cases in which the IC50 of the variant epitope showed a substantial decrease. The corresponding monofunctional fraction for each epitope-specific CD8+ T cell response changed as follows: B51-TI8 (RT) 23.8% to 37.8%; Cw8 AL9 (Nef) 68.8% to 76%; A3-RK9 (p17) 68.2% to 21.2%; B27-KK10 (p24) 98.3% to 62.7%; A2-SL9 (p17) 55% to 76%; B27-KK10 (p24) 42.5% to 19.5%.

Found at doi:10.1371/journal.pmed.0050100.sg002 (100 KB PPT).

Figure S3. Changes of PD-1 Expression on Epitope-Specific CD8+ T Cells Directed against Conserved Epitopes at an Early and Late Time Point

Displayed is the MFI of different epitope-specific (tetramer+) CD8+ T cells. The graphs illustrate changes of the PD-1 MFI of all investigated tetramer-positive CD8+ T cells directed against conserved epitopes at an early and late time point. The median of PD-1 expression on all seven tetramer-positive CD8+ T cells is shown in bold. The increase of the PD-1 expression on tetramer-positive CD8+ T cells at an early and late time point did not reach statistical significant ($p = 0.07$ paired t-test).

Found at doi:10.1371/journal.pmed.0050100.sg003 (61 KB PPT).

Table S1. Polyfunctional Profile of Each Individual Epitope Tested

Found at doi:10.1371/journal.pmed.0050100.st001 (29 KB XLS).

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Editors' Summary

Background. Viruses are small infectious agents responsible for many human diseases, including acquired immunodeficiency syndrome (AIDS). Like other viruses, the human immunodeficiency virus 1 (HIV-1; the cause of AIDS) enters human cells and uses the cellular machinery to replicate before bursting out of its temporary home. During the initial stage of HIV infection, a particular group of cells in the human immune system, CD8+ T cells, are thought to be important in controlling the level of the virus. These immune system cells recognize pieces of viral protein called antigens displayed on the surface of infected cells; different subsets of CD8+ T cells recognize different antigens. When a CD8+ T cell recognizes its specific antigen (or more accurately, a small part of the antigen called an "epitope"), it releases cytotoxins (which kill the infected cells) and cytokines, proteins that stimulate CD8+ T cell proliferation and activate other parts of the immune system. With many viruses, when a person first becomes infected (an acute viral infection), antigen-specific CD8+ T cells completely clear the infection. But with HIV-1 and some other viruses, these cells do not manage to remove all the viruses from the body and a chronic (long-term) infection develops, during which the immune system is constantly exposed to viral antigen.

Why Was This Study Done? In HIV-1 infections (and other chronic viral infections), virus-specific CD8+ T cells lose their ability to proliferate, to make cytokines, and to kill infected cells as patients progress to the long-term stages of infection. That is, the virus-specific CD8+ T cells gradually lose their "effector" functions and become functionally impaired or "exhausted." "Polyfunctional" CD8+ T cells (those that release multiple cytokines in response to antigen) are believed to be essential for an effective CD8+ T cell response, so scientists trying to develop HIV-1 vaccines would like to stimulate the production of this type of cell. To do this they need to understand why these polyfunctional cells are lost during chronic infections. Is their loss the cause or the result of viral persistence? In other words, does the constant presence of viral antigen lead to the exhaustion of CD8+ T cells during chronic HIV infection? In this study, the researchers investigate this question by looking at the polyfunctionality of CD8+ cells responding to several different viral epitopes at various times during HIV-1 infection, starting very early after infection with HIV-1 had occurred.

What Did the Researchers Do and Find? The researchers enrolled 18 patients recently infected with HIV-1 and analyzed their CD8+ T cell responses to specific epitopes at various times after enrollment using a technique called flow cytometry. They found that the epitope-specific CD8+ cells produced several effector proteins after antigen stimulation during the initial stage of HIV-1 infection, but lost their polyfunctionality in the face of persistent viral infection. The CD8+ T cells also increased their production of programmed death 1 (PD-1), a protein that has been

shown to be associated with the functional impairment of CD8+ T cells. Some of the patients began antiretroviral therapy during the study, and the researchers found that this treatment, which reduced the viral load, reversed CD8+ T cell exhaustion. Finally, the appearance in the patients' blood of viruses that had made changes in the specific epitopes recognized by the CD8+ T cells to avoid being killed by these cells, also reversed the exhaustion of the T cells recognizing these particular epitopes.

What Do These Findings Mean? These findings suggest that the constant presence of HIV-1 antigen causes the functional impairment of virus-specific CD8+ T cell responses during chronic HIV-1 infections. Treatment with antiretroviral drugs reversed this functional impairment by reducing the amount of antigen in the patients. Similarly, the appearance of viruses with altered epitopes, which effectively reduced the amount of antigen recognized by those epitope-specific CD8+ T cells without reducing the viral load, also reversed T cell exhaustion. These results would not have been seen if the functional impairment of CD8+ cells were the cause rather than the result of antigen persistence. By providing new insights into how the T cell response to viruses evolves during persistent viral infections, these findings should help in the design of vaccines against HIV and other viruses that cause chronic viral infections.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050100.

- Read a related *PLoS Medicine* Research in Translation article
- Learn more from the researchers' Web site, the Partners AIDS Research Center
- Wikipedia has a page on cytotoxic T cells (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)
- Information is available from the US National Institute of Allergy and Infectious Diseases on HIV infection and AIDS
- HIV InSite has comprehensive information on all aspects of HIV/AIDS, including a detailed article on the immunopathogenesis of HIV infection
- NAM, a UK registered charity, provides information about all aspects of HIV and AIDS, including a fact sheet on the stages of HIV infection and on the immune response to HIV
- Information is available from Avert, an international AIDS charity, on all aspects of HIV/AIDS, including information on the stages of HIV infection