The functional CD8 T cell response to HIV becomes type-specific in progressive disease

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High levels of HIV-specific CD8 T cells are demonstrable throughout HIV disease using laboratory assays that measure responses to consensus epitopes. In acute infection, the dynamics of the antiviral CD8 T cell response correlate well with the decline in viremia. However in chronic infection, although responses are detected against a broader spectrum of epitopes, virus-specific CD8 T cells are apparently unable to control viral replication. To investigate whether CD8 T cells responding to consensus epitopes may have lost their in vivo relevance in the chronic phase because of viral evolution driven by immune pressure, we compared the CD8 T cell response to CD4 T cell targets infected with either lab-adapted HIV_{IIIB} or the patient's own virus. The magnitude of the IFN-γ response declined with disease progression, especially to autologous virus. T cell receptor (TCR) clonotypes of HIV_{IIIB} and autologous virus–responding cells were determined by sequencing TCR β chain variable (TCRBV) genes. In two of three asymptomatic donors, the dominant clonotypes overlapped, whereas in five symptomatic patients, the TCR clonotypes responding to HIV_{IIIB} virus were completely different from those responding to autologous virus. Moreover, in cytolytic assays, T cell lines derived from IFN-γ^+ cells responding to lab-adapted or autologous virus cross-recognized target cells infected with either virus in asymptomatic subjects with shared TCR clonotypes but not in progressors with differing clonotypes. Therefore, in advanced-stage patients, viral-specific CD8 T cells recognizing consensus epitopes persist from an earlier response but no longer effectively recognize autologous virus.

J. Clin. Invest. **110**:1339–1347 (2002). doi:10.1172/JCI200216028.

Introduction

Studies of immune responses generated in HIV-infected individuals suggest that CD8 T cells play an important role in host defense against the virus. This has been very effectively shown in acute HIV infection where the appearance of virus-specific CD8 T cells is temporally associated with a decline in plasma viremia (1–3). At the peak of the acute response, up to 5% of CD8 T cells specific for a single immunodominant epitope have been detected by MHC/peptide tetramer staining (4). Evidence for the vast mobilization of virus-specific CD8 T cells during primary infection is also provided by the substantial expansion of particular CD8 T cell BV subsets containing HIV-specific CTLs (5). Direct evidence for the role of CD8 T cells in viral control comes from the closely related simian immunodeficiency virus model in Rhesus macaques, wherein elimination of CD8 T cells results in higher viral burden and rapid disease progression (6, 7).

Received for publication May 29, 2002, and accepted in revised form September 3, 2002.

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Conflict of interest: No conflict of interest has been declared.

Nonstandard abbreviations used: phytohemagglutinin (PHA); HIV_{IIIB} (IIIB); T cell receptor (TCR); TCR β chain variable (TCRBV); Centers for Disease Control (CDC); long-term nonprogressor (LTNP); complementarity-determining region 3 (CDR3).

Significant expansions of Vβ subsets containing CD8 T cells directed against the virus are also present during chronic infection (8–12). In fact, the CTL response, which is highly focused toward a few epitopes during primary HIV infection, broadens during the chronic phase (13). Paradoxically, despite the mobilization of this broad virus-specific CD8 T cell repertoire, most untreated chronically infected subjects are unable to control viral replication. The reasons for the inability of CD8 T cells to control viral replication are not completely understood. HIV-specific CD8 T cells that are dominant in chronic infection may not be the ones associated with the dramatic clearance of virus in acute infection, because Vβ families that are significantly expanded during primary infection are rapidly downsized and even eliminated (14). This is also borne out by recent studies showing substantial differences in the CTL epitopes targeted in primary versus chronic HIV infection (15). Persistence of virus may be facilitated by sequence mutations in key conserved epitopes targeted by $CD8^+$ CTLs (16–24). The apparent breadth and intensity of $CD8^+$ T cells in chronic infection could in fact reflect attempts of the immune system to contain the virus by targeting new substituted epitopes to control multiple epitope variants. In such a scenario, CD8 T cells generated before sequence mutations may persist as memory cells even when they are no longer relevant. In fact, use of assays that measure frequencies of HIV-specific cells by MHC/peptide tetramer staining or functional responses to relatively conserved consensus epitopes may result in misleading interpretations.

Table 1
Clinical characteristics of study subjects

Subject	CDC disease stage[A]	CD4 count (cells/mm^3)	HIV RNA copies/ml plasma	Rx
CW15	A1 (LTNP)	1,102	3,400	None
CW5	A1 (LTNP)	590	23,565	None
606	A2	615	<50	HAART
343	A2	550	<500	HAART
203	A2	490	<500	AZT, ddI
307	B2	283	8,633	HAART
204	B3	440	8,300	ddC
214	B2	220	14,510	AZT, ddC
216	C3	50	5,975	AZT
BW3	C3	40	<400	HAART
BW7	C3	50	<400	HAART

[A]Stage A, asymptomatic; stage B, minor symptoms present (e.g., thrush); stage C, major opportunistic infections present. Patients were classified by their lowest recorded CD4 counts. LTNPs were defined by infection for more than 5 years with CD4 T cell counts higher than 500 cells/mm^3 in the absence of antiretroviral drugs. Rx, treatment; HAART, highly active antiretroviral therapy. ddI, dideoxyinosine (Bristol-Myers Squibb, New York, New York, USA); ddC, dideoxycystosine (Hoffman La Roche, Basel, Switzerland).

Most studies analyzing the CTL response to HIV-1 have relied on the use of recombinant vaccinia–infected or synthetic peptide–pulsed targets. These conventional targets express antigens in excess, which may not be representative of antigen expression on physiologically relevant CD4 T cell targets in vivo. This may be particularly important in HIV infection because MHC class I downmodulation by Nef may lower epitope densities on infected cells to levels below the threshold of recognition by low-avidity CD8 T cells (25, 26). Moreover, most laboratory assays measure responses to viral gene products of laboratory strains of HIV, which may differ substantially from the patient's viral strain.

To understand how representative the group-specific responses against lab-strain viruses are of the response to the patient's own viruses, we used a novel assay to assess the response to HIV-infected primary CD4 T cell targets (27). We used these targets to quantitate the IFN-γ response of circulating CD8 T cells against lab-strain and autologous HIV virus and to capture IFN-γ–producing cells to determine whether the dominant T cell clonotypes in cells responding to autologous virus overlap with those responding to lab-strain virus.

Methods

Subjects. The work was carried out on a cross section of HIV-infected subjects (Table 1). The study was approved by the Institutional Review Committee of the Center for Blood Research. Blood was drawn after obtaining informed consent, and PBMCs were isolated by Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, New Jersey, USA) density-gradient centrifugation. Samples were either freshly obtained or cryopreserved using a programmed cell freezer (model 9000; Gordinier Electronics Inc., Roseville, Michigan, USA).

Generation of viral stocks. To generate autologous virus, CD4 T cells in PBMCs from each subject were positively selected with immunomagnetic beads (Miltenyi Biotec, Auburn, California, USA) and stimulated with phytohemagglutinin (PHA) (4 μg/ml) in RPMI 1640 supplemented with 15% heat-inactivated FCS and 60 IU/ml recombinant IL2 (Chiron Corp., Emeryville, California, USA) to promote virus production. Supernatants were tested for p24 production using an HIV-1 p24 ELISA kit (NEN Life Science Products, Boston, Massachusetts, USA), and samples were used for infection when the p24 level was over 100 ng/ml. In some cases, virus production was amplified by coculture with PHA blasts from HIV-seronegative subjects. HIV_{IIIB} (IIIB) stock was generated in H9 cells from virus originally obtained from Robert Gallo's laboratory (University of Maryland, Institute of Human Virology, Baltimore, Maryland, USA). HIV_{NL4-3} viral stock was generated from 293T cells transfected with the plasmid construct pNL4-3, obtained from the Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, NIH (Bethesda, Maryland, USA).

Generation of uniformly infected primary CD4 T cell targets for cell stimulation and cytotoxicity assays. CD4 T cells isolated from PBMCs by selection with immunomagnetic beads were stimulated with PHA (4 μg/ml). The CD4 PHA blasts were cultured in the presence of antiretroviral drugs (500 nM Azido deoxythymidine [AZT] [Sigma Chemical Co., St. Louis, Missouri, USA], 30 nM Saquinavir [Hoffman La Roche, Basel, Switzerland], and 15 nM Ritonavir [Abbott Laboratory, Abbott Park, Illinois, USA]) for 1–2 weeks to guarantee that there was no autologous viral contamination. The cells were then infected with HIV strain IIIB, NL4-3, or autologous virus at an moi of 0.01–0.1 or were used as uninfected controls. After 3–5 days of culture, the infection status and MHC class I expression were verified by surface staining with HLA-ABC PE (DAKO Corp., Foster City, California, USA) and intracellular staining with p24 FITC-conjugated p24 antibody (Immunotech, Westbrook, Maine, USA). The infected cells were enriched by CD4 depletion as described (28).

Detection and capture of IFN-γ–producing HIV-specific CD8 T cells. CD4-depleted PBMCs (2×10^6 cells) were stimulated overnight (16–18 hours) with IIIB or autologous virus–infected CD4 T cells at an effector/target ratio ranging from 5:1 to 10:1. Control cultures were stimulated with uninfected CD4 blasts. The cultures were then treated with an IFN-γ catch reagent (Miltenyi Biotec) to generate an affinity matrix on the cell surface. After incubation at 37°C for 1 hour to allow capture of secreted IFN-γ onto the affinity matrix, the cells were labeled with IFN-γ detection antibody conjugated to PE. An aliquot of the cells was costained with anti-CD8 mAb and analyzed by flow cytometry. Finally, IFN-γ^+ cells were isolated using anti-PE microbeads (Miltenyi Biotec) per the manufacturer's instructions.

Identification of T cell receptor β chain variable and complementarity-determining region 3 sequences. RNA was purified from IFN-γ–producing cells using a commercial RNA extraction kit (QIAGEN Inc., Valencia, California,

USA), and cDNA was synthesized using the SMART RACE cDNA amplification kit (CLONTECH Laboratories Inc., Palo Alto, California, USA), which provides an RT enzyme that adds a 5′ oligo(dC) tail. An anchor oligonucleotide in the mix, which has a dG3′ tail, allows incorporation of the anchor sequence into the 5′ end of the cDNA. The first-strand cDNA was used for anchored PCR with the upstream anchor primers provided by the manufacturer and a T cell receptor–specific (TCR-specific) Cβ outer Kd25 (5′-GGCCAGGCACACCAGTGTGGCCTTTTGGGT-3′) and a Cβ inner primer, Kd24 (5′-TTCTGATGGCTCAAACACAGGAC-3′). Second strand cDNA was synthesized using a touchdown PCR for five cycles at 94°C for 5 seconds, 72°C for 2 minutes, followed by 5 cycles of PCR at 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 2 minutes. Finally, specific amplification of TCR β chain variable (TCRBV) genes was done with 25 cycles at 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 2 minutes. Subsequently, amplification reactions were diluted 50-fold with reaction buffer and a second round of PCR was performed with inner primers for another 20 cycles at 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 2 minutes. Amplified PCR products were analyzed on 1.5% agarose gels and purified with the QIAquick PCR purification kit (QIAGEN Inc.). The purified PCR products were cloned into TA cloning vector pCR2.1 (Invitrogen Corp., Carlsbad, California, USA). For each sample, plasmid DNA was purified from multiple clones using a plasmid purification kit (QIAGEN Inc.). TCRBV genes were sequenced from the cloned DNA using an automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

Amplification of individual BV genes by RT-PCR. Total RNA from IFN-γ^+ cells was also used for amplification of individual Vβ families. cDNA was synthesized from total RNA using the TaqMan reverse transcription kit (Applied Biosystems). Aliquots of cDNA were amplified with 26 different 5′ sense Vβ-specific primers and a common 3′ antisense Cβ primer using sequences described by Choi et al. (29). A sample with no cDNA served as negative control, and a sample with 5′ and 3′ Cβ oligonucleotides for amplification of the Cβ region served as positive control.

Generation of HIV-specific CTL lines. Bulk cell lines were generated by culturing the immunomagnetically selected IFN-γ^+ cells in the presence of irradiated allogeneic PBMC feeder cells and recombinant IL15 (25 ng/ml; R&D Systems Inc., Minneapolis, Minnesota, USA) for 10–14 days for use as effectors in cytotoxicity assays. Culture was kept to the minimum to avoid altering the original clonal repertoire.

Chromium release assays. IIIB and autologous virus–infected CD4 T cell targets, generated as described above, were labeled with ^{51}Cr, washed, and distributed in round-bottom 96-well plates (5×10^3 cells in 100 μl). The bulk cultures from IIIB– and autologous virus–responding cells were used as effectors in triplicate wells at effector/target ratios ranging from 1:1 to 5:1. The effector and target cells were incubated for 4 hours at 37°C. Supernatants (40 μl) from the cultures were harvested and g counts measured on a Packard Microplate reader (Microplate scintillation counter; Packard Instrument Company, Downers Grove, Illinois, USA). Percent specific cytotoxicity was calculated from the average cpm as (average cpm minus spontaneous release/total release minus spontaneous release) × 100.

Results

Frequencies of IFN-γ–producing cells responding to primary CD4 T cell targets infected with lab-strain and autologous HIV decline with disease progression. HIV-infected CD4 T cells rapidly downmodulate CD4 expression, so homogeneously infected CD4 T cells can be selected for use as targets by immunomagnetic depletion of CD4-expressing uninfected cells from infected cultures (28, 30). Because infected cells are significantly enriched, the method eliminates variability in infectivity, making it possible to compare results from different samples and viral strains. We used this method to generate primary CD4 T cell targets infected with autologous or IIIB virus for use as stimulators to detect and capture functional IFN-γ–secreting, HIV-specific CD8 T cells. CD4 T cell blasts from each patient were treated with antiretroviral drugs for 7 days to suppress endogenous virus. The cells were then infected with either IIIB or autologous virus, and after 3–5 days, were negatively enriched for infected cells by CD4 depletion. The level of enrichment of infected cells and the mean fluorescence intensity of HIV p24 staining were comparable between CD4 stimulator cells infected with autologous virus and CD4 stimulator cells infected with IIIB virus. We also compared MHC class I expression between autologous and HIV_{IIIB} virus–infected targets, because the viral Nef protein is known to induce downmodulation of class I expression. Figure 1a depicts representative data showing comparable p24 and MHC class I expression in CD4 T cells infected and enriched for autologous virus, IIIB, or another lab-adapted virus, NL4-3. As HIV_{IIIB} has been reported to contain multiple viral clones, some of which have dysfunctional *nef* genes (26, 31), we also PCR-amplified and cloned the *nef* region of the IIIB strain used in the study and compared it with a IIIB strain recently obtained from the NIH AIDS repository. Although 50% of the clones derived from NIH IIIB viral isolate contained truncated *nef* sequences, fortuitously, all of the sequences from the clones derived from the viral stock that we used for the study contained intact *nef* sequences (not shown).

To measure IFN-γ production by CD8 T cells, PBMCs were stimulated with uninfected CD4 T cells or CD4 T cells infected with either IIIB or autologous virus. In all cases, negligible numbers of IFN-γ–producing cells were observed when uninfected CD4 blasts were used as stimulators (<0.1% IFN-γ^+ CD8 T cells) (Figure 2a). Thus, after retroviral treatment, endogenous virus is effectively suppressed and does not interfere with

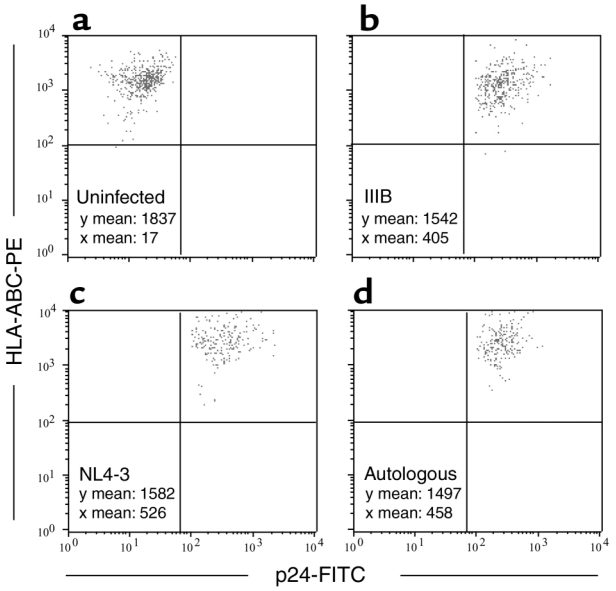

Figure 1
Flow cytometric analysis of MHC class I and p24 expression on HIV-infected primary CD4 T cells used as stimulator cells. CD4 PHA blasts from representative subject 307 were used as uninfected control (**a**) or were infected with HIV_{IIIB} (**b**), NL4-3 (**c**), or autologous virus (**d**) for 3 days and then enriched for infected cells by immunomagnetic depletion of cells that had not downmodulated CD4 expression. Cells were stained externally with PE-conjugated anti–HLA-ABC antibody (MHC class I framework) and internally with FITC-conjugated anti-p24 antibody.

analysis. The frequency of cells producing an IFN-γ response to virus-infected targets was assessed in 11 seropositive subjects: five Centers for Disease Control (CDC) stage A subjects, including two long-term nonprogressors (LTNPs); three CDC stage B subjects; and three CDC stage C subjects. The frequency of IFN-γ–producing CD8 T cells ranged from 0% to 10.6% (Figure 2b). The frequency of IFN-γ^+ CD8 T cells responding to autologous and IIIB virus–infected targets in subjects in whom response against both viruses could be evaluated suggests a decline in functional HIV-specific IFN-γ–producing cells in more advanced donors (mean 4.4% ± 3.6% against autologous vs. 4.1% ± 2.8% against IIIB in 3 CDC stage A subjects, 1.3% ± 1.9% against autologous vs. 2.5% ± 1.9% against IIIB in three CDC stage B subjects, and 0.37% ± 0.3% against autologous vs. 0.8% ± 0.75% in three CDC stage C subjects). In two additional CDC stage A subjects, the percentages of CD8 T cells producing an IFN-γ response to HIV_{IIIB} were 10.6% and 4.3% of CD8 T cells, but no autologous virus could be generated in these patients despite several attempts. The decline in frequencies of IFN-γ–secreting cells was statistically significant when the magnitude in the CDC stage A subjects was compared with that in the more advanced CDC stage B and C subject group ($P < 0.02$ for IIIB-specific response and $P < 0.04$ for autologous virus–specific response). Surprisingly, twice as many IFN-γ^+ cells responded to lab-adapted IIIB virus than responded to autologous virus in CDC stage B and stage C subjects (median 1.15 vs. 0.55, respectively; $P < 0.03$).

Oligoclonal and nonoverlapping TCRBV usage in IFN-γ^+ CD8 T cells responding to targets infected with lab-strain and autologous virus. We also characterized the clonotypic composition of the responding cells by using anchored PCR to determine the TCRBV region and complementarity-determining region 3 (CDR3) gene sequences in immunomagnetically selected IFN-γ^+ populations. IFN-γ–secreting cells were captured by immunomagnetic selection with αPE beads after external staining with PE-conjugated anti–IFN-γ (Figure 3). In most cases, more than 90% of the captured cells stained for IFN-γ. As the isolated cells were not fixed, they could be used for RNA isolation as well as for further culture. Anchored PCR was used for BV gene analysis because

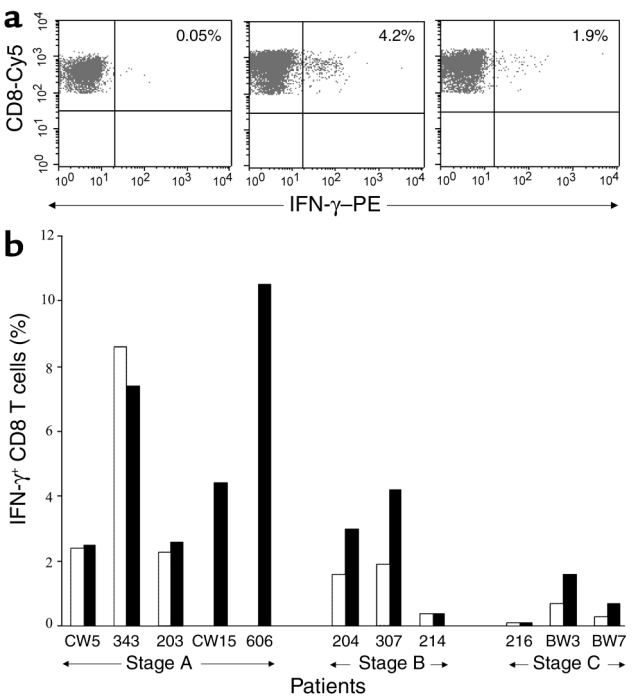

Figure 2
Frequency of IFN-γ–producing cells responding to HIV-infected targets declines in more advanced disease stages. (**a**) Representative flow cytometric analysis of surface IFN-γ and CD8 staining on gated CD8 T cells in PBMCs from subject 307 after overnight stimulation with uninfected CD4 T cells (left) or HIV_{IIIB} (middle) and autologous (right) virus–infected CD4 T cell targets and capture of secreted IFN-γ on the cell surface. The numbers shown are the percentages of CD8 T cells producing IFN-γ. (**b**) The percentage of CD8 T cells producing IFN-γ in response to IIIB (black bars) and autologous (white bars) virus–infected targets are shown for subjects at various disease stages after subtracting background values for uninfected targets. In subjects 606 and CW15, the response was measured only against IIIB virus because no autologous virus could be generated.

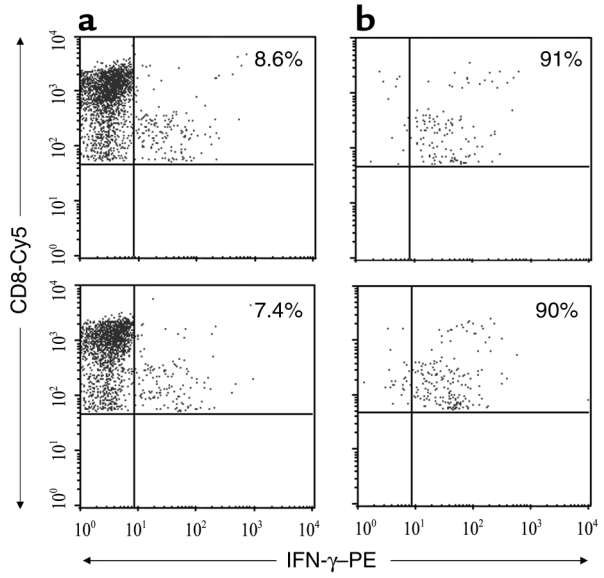

Figure 3
Immunomagnetic selection of IFN-γ–secreting HIV-specific CD8 T cells. PBMCs from donor 343 were stimulated with autologous (upper panels) or IIIB (lower panels) virus–infected primary CD4 T cells, stained for IFN-γ, and immunomagnetically isolated. Cells before (**a**) and after (**b**) selection were stained with anti–CD8-Cy5 antibody and analyzed for IFN-γ$^+$ cells within a CD8-gated population.

this technique amplifies all TCRBV genes without qualitative or quantitative biases, unlike PCR amplification of individual TCRBV families. After PCR amplification, the product was analyzed by gel electrophoresis (Figure 4a). In each case, TCRBV genes were sequenced from 30–50 clones derived from the amplified PCR products (Table 2). To ensure the validity of the TCRBV gene sequences identified, in two subjects the analysis was performed in two independent experiments and yielded similar results (data not shown). For five samples, the authenticity of the analysis was also verified by direct PCR amplification of the reverse-transcribed RNA using a panel of upstream Vβ primers and a common downstream Cβ primer. Although the specific BV$^+$ usage of the IFN-γ–producing cells could not be verified by antibody staining immediately after stimulation because of TCR downmodulation that accompanies activation (data not shown), we could demonstrate the presence of the specific BV$^+$ cells in the IFN-γ–selected population after a brief period of culture. Figure 4, b and c, shows the confirmatory studies for one subject for which all 50 clones sequenced from the captured cells responding to autologous virus had an identical TCRBV sequence belonging to the 7S1 family. In that case, the PCR analysis amplified only a single 7S1 band, and the antibody staining after culture showed that 90% of the cells stained for 7S1 antibody.

The TCR usage of CD8 T cells responding to lab-strain HIV was analyzed in ten subjects (Table 2). The response ranged from monoclonal to polyclonal, although in most subjects, one or two clonotypes dominated the response, with other minor clonotypes contributing less than 10% to the response. Because the specificity of the capture method is about 90% (see Figure 1), it is difficult to tell whether clonotypes present at frequencies of below 10% are contaminating cells or are participating in the specific response. A polyclonal response with no clear dominant clonotype was seen in only one subject. This CDC stage A subject (no. 606), with 13 TCR clonotypes participating in the response, had the highest frequency of IFN-γ–producing cells (10.6%). All seven of the CD8 T cell clones generated from the IFN-γ$^+$ cells from this subject were HIV-specific, suggesting that the TCR clonotypes identified were not because of contamination with nonspecific cells (data not shown). In the two LTNPs analyzed, the TCRBV gene usage was monoclonal.

A similar clonotypic analysis was performed for CD8 T cells responding to autologous virus–infected targets

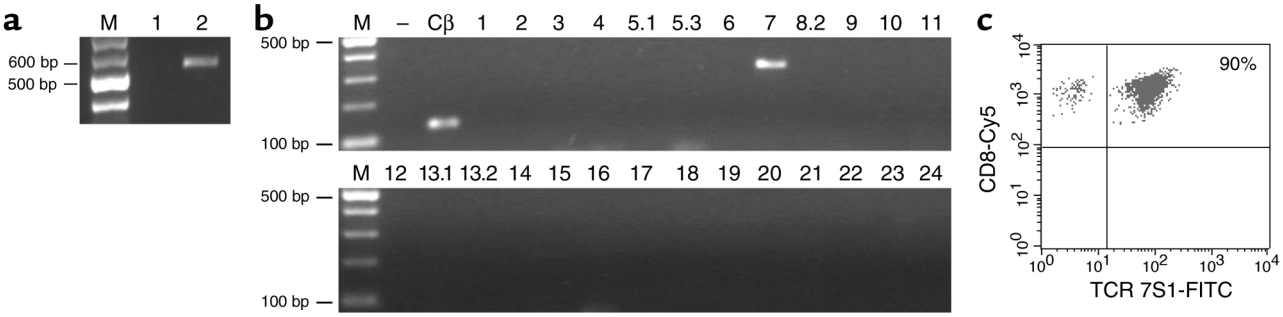

Figure 4
Representative analysis of TCRBV usage in CD8 T cells responding to HIV-infected targets. (**a**) Agarose gel analysis of anchored PCR amplification of TCRBV genes from immunomagnetically captured IFN-γ$^+$ cells stimulated with autologous virus from representative subject 307. TCRBV and CD3 region sequencing of 50 cloned PCR products revealed a single 7S1 clonotype (see Table 2). Lanes depict molecular weight markers (M), negative control containing no cDNA template (1), and TCRBV gene amplification product from autologous virus–responding CD8 T cells (2). (**b**) Independent evaluation of TCRBV gene usage by PCR amplification of the same sample with a panel of 5′ Vβ primers and a common 3′ Cβ primer (lane numbers indicate individual Vβ families and decimals indicate subfamilies). (**c**) Flow cytometric analysis of cultured IFN-γ$^+$ CD8 T cells from the same donor stained with TCRBV antibody corresponding to TCRBV gene 7S1.

Table 2
TCRBV and CDR3 amino acid sequences of CD8 T cells producing IFN-γ in response to HIV-infected primary T cell targets

	IFN-γ⁺ CD8 T cells responding to HIV_{IIIB}						IFN-γ⁺ CD8 T cells responding to autologous virus					
Subject	Frequency (%)	TCRBV		CDR3		TCRBJ	Frequency (%)	TCRBV		CDR3		TCRBJ
CDC stage A												
343	**40**	**5S6**	**CAS**	**SLDFEGRVF**	**FG**	**1S4**	**45**	**6S7**	**CAS**	**SSTMGQGRSPLH**	**FG**	**1S6**
	35	**6S7**	**CAS**	**SSTMGQGRSPLH**	**FG**	**1S6**	**35**	**13S1**	**CAS**	**SYSMATGGNIQY**	**FG**	**2S4**
	10	17S1	CAS	SWGTAGTDTQY	FG	2S3	**10**	**5S6**	**CAS**	**SLDFEGRVF**	**FG**	**1S4**
	5	**13S1**	**CAS**	**SYSMATGGNIQY**	**FG**	**2S4**	5	5S3	CAS	SSIAGGLAGELF	FG	2S2
	5	5S1	CAS	SVQGAEAF	FG	1S1	5	13S3	CAS	RAGGGSGANVLT	FG	2S6
	5	14S1	CAS	SLSGGYSNQPQH	FG	1S5						
203	**58**	**6S3**	**CAS**	**SLLDGGRDTQY**	**FG**	**2S3**	**48**	**6S3**	**CAS**	**SSAQGAPLNWLF**	**FG**	**1S4**
	32	**6S3**	**CAS**	**SSAQGAPLNWLF**	**FG**	**1S4**	**44**	**6S3**	**CAS**	**SLLDGGRDTQY**	**FG**	**2S3**
	6	2S1	CSA	GSGTSGSNEQ	FG	2S1	4	14S1	CAS	SLTGSTPLKWF	FG	2S3
	4	13S1	CAS	GQLGDYT	FG	1S2	4	22S1	CAS	SPFVLDRGGERLF	FG	2S4
CW5	100	6S4	CAS	SLLLSGRSDTQY	FG	2S3	96	18S1	CAS	SPPRTGAPYGYT	FG	1S2
							4	2S1	CSA	PNAVGELF	FG	2S2
CW15	100	3S1	CAS	SSVYAVNSPLH	FG	1S6	ND					
606	10	6S7	CAS	SPDSQGIYEQY	FG	2S7	ND					
	10	4S1	CSV	GQGYGYT	FG	1S2						
	10	2S1	CSA	NRGVSGELF	FG	2S2						
	10	17S1	CAS	SGASYGYT	FG	1S2						
	10	18S1	CAS	SPRTGNYGYT	FG	1S2						
	10	6S7	CAS	SSRTSGGFTQY	FG	2S3						
	8	5S5	CAS	SSIAGGLAGELF	FG	2S2						
	8	5S1	CAS	SLEAGLTEAF	FG	1S1						
	8	14S1	CAS	SLTGSLGNQPQH	FG	1S5						
	4	9S3	CAS	SQGRWGGTDTQY	FG	2S3						
	4	12S2	CAI	SDQTGTSAEAF	FG	1S1						
	4	9S1	CAS	SQDSAQPQY	FG	2S3						
	4	5S1	CAS	SLGGTEAF	FG	1S1						
CDC stage B												
307	60	3S1	CAG	GSGTNYGYT	FG	1S2	100	7S1	CAS	SQRGGSYNEQF	FG	2S1
	40	6S4	CAS	SVTAPPDTQY	FG	2S3						
214	100	3S1	CAS	SLEVGYYEQY	FG	2S7	82	15S1	CAS	SKTSGKPNEQF	FG	2S1
							18	3S1	CAS	SSIAGMGETQY	FG	2S5
204	100	7S3	CAS	SQGTGVSTEAF	FG	1S1	62	6S4	CAS	SGEYSNGPQH	FG	1S5
							34	1S1	CAS	SAPTRLYEQY	FG	2S7
							4	6S2	CAS	SHLEGYEQY	FG	2S7
CDC stage C												
BW3	50	21S3	CAS	SPINTPNTGELF	FG	2S2	64	9S1	CAS	GDGTGDEQY	FG	2S7
	22	6S3	CAS	GTTGEAYGYT	FG	1S2	20	7S6	CAS	SLRLASGNTIY	FG	1S3
	18	15S7	CAT	SAGGRTGELF	FG	2S2	16	18S1	CAS	SPSGLDTEAF	FG	1S1
	10	6S4	CAS	SPTDGLNTEAF	FG	1S1						
BW7	100	5S2	CAS	SLAGQYTGELF	FG	2S2	90	15S1	CAS	SAGTGGNTIY	FG	1S1
							10	7S3	CAS	SQGRTASGPPGYT	FG	1S2

Bold text shows shared clonotypes in response to IIIB and autologous HIV virus–infected cells. ND, not done because autologous virus could not be generated. TCRBJ, TCR β chain joining.

in the eight of the subjects for whom autologous virus could be generated (Table 2). The response to autologous virus was also oligoclonal, with one or two clonotypes dominating the response in most subjects. We compared the TCR clonotypes responding to lab-strain virus with those recognizing the patient's own virus to determine whether group-specific epitopes in HIV_{IIIB} contribute to the dominant response in vivo. In two of three CDC stage A subjects, the immunodominant TCR clonotypes against lab-adapted HIV_{IIIB} virus and autologous virus overlapped to a large extent, suggesting a focusing of the response toward such conserved epitopes (Table 2). In both patients, clonotypes exclusive to autologous virus or lab-adapted virus constituted less than 10% of the response. However, in all other subjects analyzed (CDC stage A, $n = 1$; stage B, $n = 3$; and stage C, $n = 2$), the dominant TCR clonotypes used in response to autologous virus and lab-adapted IIIB virus were completely different.

Bulk cell lines generated from lab-strain HIV–specific IFN-γ⁺ cells recognize autologous virus–infected targets and vice versa only in subjects with shared TCRBV usage. To test the in vivo relevance of the CD8 T cells responding to lab-strain virus, we evaluated the ability of cell lines derived from immunomagnetically selected HIV_{IIIB}-specific IFN-γ⁺ cells to lyse autologous virus–infected targets (Figure 5). Tissue culture was kept to a minimum to avoid in vitro alterations of the native clonal composition. In CTL assays performed after 10–12 days of culture, HIV_{IIIB}-specific T cell lines cross-recognized autologous virus–infected targets in CDC stage A subjects who shared TCRBV genes. However, bulk cell lines from

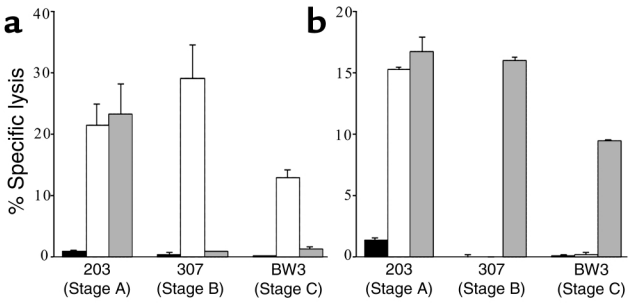

Figure 5
Autologous virus–infected targets are not lysed by IFN-γ+ CD8 T cell lines responding to HIV_{IIIB} virus and vice versa in samples from more advanced donors. Short-term cultures from IFN-γ+ cells responding to IIIB (**a**) or autologous virus (**b**) were tested against uninfected (black bars), IIIB (white bars), or autologous (gray bars) virus–infected primary CD4 T cell targets. Representative data for subjects from each CDC stage are depicted at effector/target ratios ranging from 1:1 to 5:1. Data are presented as mean of experiments performed in triplicate. The cytotoxicity assays confirm the results of TCRBV sequencing. There was cross-recognition of IIIB and autologous virus by two of the stage A donor cell lines, but not by the cell lines from more advanced donors.

CDC stage B and C subjects in whom the TCR clonotypes responding to IIIB and autologous virus were not shared did not cross-recognize autologous virus-infected targets. When bulk lines generated from IFN-γ+ cells responding to autologous virus were tested against HIV_{IIIB}-infected CD4 T cell targets, a similar pattern of cross-recognition was observed. Data from representative subjects are shown in Figure 5.

To confirm that the results obtained with HIV_{IIIB} do not represent an aberrant response to an *nef*-deficient lab-strain virus, we also examined the lytic capability of the bulk cell line generated from IIIB-responsive IFN-γ+ cells against target cells infected with another lab-adapted viral isolate, NL4-3. This viral strain is known to have an intact *nef* gene (32). As shown in Figure 6, the bulk cell line from a CDC stage B subject (no. 307) exhibited comparable lysis of NL4-3 and IIIB virus–infected targets but failed to lyse autologous virus–infected targets. Similar results were obtained with a CTL clone generated from the IIIB-responsive cells from the same subject. These data indicate that the lack of recognition of autologous virus–infected targets was not because of low epitope densities induced by nef-mediated class I downmodulation.

The epitope specificity of this bulk line as well as the clone were mapped to a 10 amino acid region in Nef (LWIYHTQGYF, aa 112–121) using recombinant vaccinia and overlapping 20mer peptide–pulsed B-lymphoblastoid cell lines targets in cytotoxicity assays as described by Lieberman et al. (33) (data not shown). When the *nef* region of the autologous virus was amplified by PCR and sequenced, two amino acid changes were found within the 10mer epitope–containing region (LW**VY**N**T**QGYF) compared with the consensus sequence in the IIIB and NL4-3 strains. This suggests that sequence mutation may be the underlying cause for nonrecognition of autologous virus–infected targets by IIIB-responsive CTLs.

Discussion

This study examines the functional virus-specific CD8 T cell repertoire in the setting of a protracted infection with a virus with a propensity for mutation. We used primary CD4 T cell targets to compare the magnitude and clonotypic composition of CD8 T cells responding to lab-strain HIV_{IIIB} with those responding to autologous viral isolates in HIV-infected individuals at different stages of disease. Our principal findings are that the magnitude of the functional response declines with disease progression and that the TCR clonotypes of CD8 T cells responding to the two viruses overlap in the early stage of infection but completely diverge in later stages of the disease. Consequently, HIV_{IIIB}-responding cells do not recognize autologous virus and vice versa in symptomatic patients. Moreover, the functional CD8 T cell response is oligoclonal in most patients. Taken together, these results suggest that conventional assays that focus on responses to consensus epitopes may not accurately reflect, but rather tend to overestimate, the response to autologous virus. Our results therefore underscore the need to develop approaches that more closely test the relevant in vivo responses.

The stimulator cells we used do not exactly mimic in vivo targets, because CD4 cells are cultured for a brief

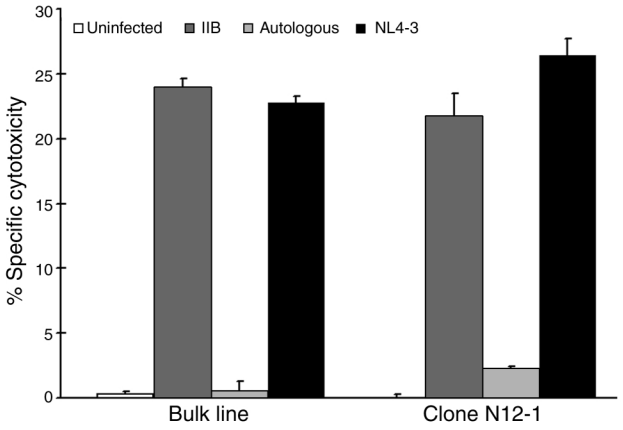

Figure 6
Bulk lines and clones derived from HIV_{IIIB} virus–responsive CD8 T cells recognize IIIB and NL4-3 virus, but not autologous virus–infected CD4 T cell targets. A bulk cell line and clones were generated from CD8 T cells that produced IFN-γ after stimulation with HIV_{IIIB}-infected CD4 T cell targets in a CDC stage B subject (no. 307). The cell line and one clone (N12-1) were then tested for lysis of control uninfected CD4 blasts (white bars) or enriched populations of IIIB (dark gray bars), autologous (light gray bars), or NL4-3 (black bars) virus–infected CD4 blasts at an effector/target ratio of 5:1. Data are presented as mean of experiments performed in triplicate. The epitope recognized by the cell line and clone was mapped to a 10mer region in nef, LWIYHTQGYF (amino acids 112–121). The autologous virus differed from the sequence by two amino acids (LW**VY**N**T**QGYF).

period before use. Nevertheless, they more closely resemble the physiologically relevant targets than do the stimulator cells used in conventional methods. Unlike recombinant vaccinia–infected or synthetic peptide–pulsed targets, infected CD4 T cells, which are the natural targets of the virus, more closely reflect epitope densities achieved on infected targets in vivo (34). This is particularly important in light of reports that the nef protein downmodulates cell-surface MHC class I expression, which may further reduce epitope densities, making virus-infected cells poor targets for CTL lysis and possibly for stimulation of IFN-γ secretion (25, 26).

The decline in frequency of IFN-γ–producing HIV-specific cells in stage B and C disease is unexpected, considering studies that show a high frequency of HIV-specific CD8 T cells until late in the course of HIV disease (35–37). These studies have generally used MHC/peptide tetramer staining or IFN-γ responses after peptide stimulation to identify virus-specific CD8 T cells. One explanation may be that our use of virus-infected targets filters out all of the responses that are not meaningful in the context of infected cells, including low-avidity or subdominant CD8 T cells that may produce IFN-γ only upon stimulation with an excess of peptides. This is also suggested by the predominantly oligoclonal and monoclonal functional response that we observed against both lab-adapted and autologous virus in most of the subjects. Moreover, studies that use MHC/peptide tetramers identify HIV-specific CD8 T cells without regard to their functionality. Many investigators, ourselves included, have reported that a significant fraction of tetramer-positive cells are unable to produce IFN-γ when freshly tested ex vivo (27, 38–40). Functional deficiencies of HIV-specific cells in chronic infection can occur because of their generation or maintenance in a progressively CD4-deficient milieu (41–45). In fact, when we analyzed HIV-specific CD8 T cell frequencies with four different HLA-A2 and B8/peptide tetramers from five subjects in this study who expressed these HLA alleles (subjects CW5, 203, 204, 216, and 307), we found no correlation with the disease stage (ref. 27 and data not shown). Comparable frequencies of gag SLYNVATL/A2.1 tetramer-staining CD8 T cells were seen in samples from an AIDS patient (no. 216) with no detectable IFN-γ^+ CD8 T cells and an LTNP (no. CW5) with over 2% IFN-γ^+ CD8 T cells (1.4% and 1.6%, respectively).

We were surprised to find that the overall frequency of IFN-γ–producing cells was lower in response to autologous virus than to HIV_{IIIB} virus stimulation. This finding suggests that CD8 T cells generated in response to consensus epitopes early in infection persist even after viral mutations make them irrelevant. The responses against any newly presented or substituted epitopes that come up because of viral mutations during chronic infection have to be generated in the setting of HIV-specific CD4 T helper cell deficiency and cytokine imbalance and may be functionally impaired. This may be particularly relevant because the HIV virus selectively targets HIV-specific CD4 T cells (46). Although this needs to be investigated in a larger group of patients, these results suggest that in patients with more advanced disease, measurement of the IFN-γ response to lab-strain virus significantly overstates the functional response to autologous virus.

Although we have used only one lab-strain virus as representative of the response to relatively conserved epitopes, the most striking fact to emerge from the study is the discordance between the CD8 T cell clonotypes responding to autologous virus and lab-strain virus, particularly in more advanced patients. The inability of the bulk cell lines from IIIB-responsive CD8 T cells to cross-recognize autologous virus–infected targets in CTL assays corroborates the TCR sequencing data. Although HIV_{IIIB} virus has been reported to contain multiple viral clones, some of which have dysfunctional *nef* genes (26, 31), our results cannot be explained on the basis of differences in class I expression. Sequence analysis revealed that the HIV_{IIIB} isolate we used for the study did not contain viral clones with a truncated *nef* gene, which was in keeping with our data showing comparable levels of class I expression on autologous and IIIB virus–infected targets (Figure 1). Moreover, in one representative subject, the dichotomy in recognition of autologous and lab-adapted virus could also be confirmed with NL4-3 virus, which has an intact *nef* gene (Figure 6).

One possible explanation for the persistence of virus-specific CD8 T cells that do not recognize the currently circulating autologous virus is that these are residual cells from a previously relevant response that are left behind as the CD8 T cell response diversifies in an effort to cope with viral mutations. In fact, our data showing lower frequencies of IFN-γ^+ CD8 T cells responding to autologous virus compared with IIIB virus-infected targets in CDC stage B and C subjects ($P < 0.03$) also hints at accumulation of cells that may have become irrelevant in the context of the evolving virus. It is well documented that memory T cells can persist indefinitely in mouse models in the absence of antigen (47). In fact, many HIV tetramer–positive cells exhibit memory-like phenotype, suggesting that they have not been recently activated by antigen (27, 48, 49). However, as different viral quasispecies exist in infected individuals, it is also possible that CD8 T cells reacting to group-specific epitopes may be keeping viral quasispecies that have not mutated these epitopes in control. This type of plasticity in the immune response may be a host strategy to abrogate the deleterious consequences of CTL escape (50, 51).

We have provided both functional and molecular evidence suggesting that the dominant CTL response to autologous HIV virus may shift away from group-specific epitopes, particularly in more advanced subjects. Our data suggest that conventional assays that measure responses to consensus sequences from lab-strain viruses may overestimate the breadth and diversity of functioning virus-specific CD8 T cells in vivo. These results also provide insights into how the virus is ultimately able to prevail in the face of an apparently broad

and intense host immune response, as the functional CD8 T cell response to the patient's own viral isolate declines around the time disease symptoms develop.

Acknowledgments

We thank N. Manjunath, D. Zhang, and E. Song for discussions and comments. We also thank M. Lederman, C. Lange, and J. Daily for patient samples. This work was supported by NIH grants AI-49792 and AI-45306 (to P. Shankar).

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