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De Novo Generation of Escape Variant-Specific CD8$^+$ T-Cell Responses following Cytotoxic T-Lymphocyte Escape in Chronic Human Immunodeficiency Virus Type 1 Infection

Todd M. Allen,[1]† Xu G. Yu,[1]† Elizabeth T. Kalife,[1] Laura L. Reyor,[1] Mathias Lichterfeld,[1] Mina John,[3] Michael Cheng,[1] Rachel L. Allgaier,[1] Stanley Mui,[1] Nicole Frahm,[1] Galit Alter,[1] Nancy V. Brown,[1] Mary N. Johnston,[1] Eric S. Rosenberg,[1] Simon A. Mallal,[3] Christian Brander,[1] Bruce D. Walker,[1,2] and Marcus Altfeld[1]*

Partners AIDS Research Center and Infectious Diseases Unit, Massachusetts General Hospital, and Division of AIDS, Harvard Medical School, Boston, Massachusetts 02129[1]; Howard Hughes Medical Institute, Chevy Chase, Maryland 20815[2]; and Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital, Perth, Western Australia 6000, Australia[3]

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Human immunodeficiency virus type 1 (HIV-1) evades CD8$^+$ T-cell responses through mutations within targeted epitopes, but little is known regarding its ability to generate de novo CD8$^+$ T-cell responses to such mutants. Here we examined gamma interferon-positive, HIV-1-specific CD8$^+$ T-cell responses and autologous viral sequences in an HIV-1-infected individual for more than 6 years following acute infection. Fourteen optimal HIV-1 T-cell epitopes were targeted by CD8$^+$ T cells, four of which underwent mutation associated with dramatic loss of the original CD8$^+$ response. However, following the $G_{357}S$ escape in the HLA-A11-restricted $Gag_{349–359}$ epitope and the decline of wild-type-specific CD8$^+$ T-cell responses, a novel CD8$^+$ T-cell response equal in magnitude to the original response was generated against the variant epitope. CD8$^+$ T cells targeting the variant epitope did not exhibit cross-reactivity against the wild-type epitope but rather utilized a distinct T-cell receptor Vβ repertoire. Additional studies of chronically HIV-1-infected individuals expressing HLA-A11 demonstrated that the majority of the subjects targeted the $G_{357}S$ escape variant of the $Gag_{349–359}$ epitope, while the wild-type consensus sequence was significantly less frequently recognized. These data demonstrate that de novo responses against escape variants of CD8$^+$ T-cell epitopes can be generated in chronic HIV-1 infection and provide the rationale for developing vaccines to induce CD8$^+$ T-cell responses directed against both the wild-type and variant forms of CD8 epitopes to prevent the emergence of cytotoxic T-lymphocyte escape variants.

Virus-specific CD8$^+$ T-cell responses have been shown to play an important role in the control of many viral infections, including human immunodeficiency virus type 1 (HIV-1) infection. CD8$^+$ T cells recognize viral antigens (epitopes) presented by HLA class I molecules on the surface of infected cells through specific binding of their T-cell receptor (TCR) with the HLA class I-epitope complex. A number of studies have demonstrated that viruses can evade CD8$^+$ T-cell-mediated immune pressure through the selection of sequence variations within targeted regions (5, 23, 24, 29, 32). Studies of HIV-1 and simian immunodeficiency virus (SIV) infections have shown selection of cytotoxic T-lymphocyte (CTL) escape variants during both primary and chronic infections (1, 2, 6, 10–12, 16, 20, 21, 26, 37, 40, 42). A subset of studies has furthermore demonstrated that the selection of viral escape variants during chronic SIV and HIV-1 infections can result in loss of immune control and disease progression (6, 14, 21). Thus, the ability of HIV-1 to escape from virus-specific CD8$^+$ T-cell responses has been proposed as an important obstacle for the maintenance of protective immune responses (5, 24, 32), and likewise the viral diversity resulting from CTL-driven viral evolution represents a major hurdle for HIV-1 vaccine design (47).

Viral escape from CD8$^+$ T-cell-mediated immune pressure predominantly occurs through the selection of sequence variations in positions within targeted CD8$^+$ T-cell epitopes that are crucial for the binding to HLA class I molecules (anchor positions) (1, 11) or positions that are responsible for the interaction between the TCR and the peptide-HLA class I complex (TCR sites). Both mechanisms result in the loss of recognition of the variant form of the epitope. More recently, the extent to which mutations both within targeted CD8$^+$ T-cell epitopes and within regions flanking these epitopes can impair processing of the respective epitope (1, 11, 48) is becoming better understood. As a consequence, both processing mutations and mutations within HLA-binding anchor residues of CD8$^+$ T-cell epitopes have the capacity to impair the recognition of infected cells by all epitope-specific CD8$^+$ T cells, irrespective of their TCR usage. In contrast, amino acid changes in positions of a CD8$^+$ T-cell epitope that are respon-

* Corresponding author. Mailing address: Partners AIDS Research Center, Massachusetts General Hospital, 149 13th Street, Boston, MA 02129. Phone: (617) 724-2461. Fax: (617) 724-8586. E-mail: maltfeld@partners.org.

† T.M.A. and X.G.Y. contributed equally to this study.

sible for the interaction with the TCR allow for the continuous presentation of the epitope on the surface of infected cells. This persistent presentation of the viral epitope variants on infected cells may therefore enable the recruitment of novel epitope-specific $CD8^+$ T cells utilizing the TCR that are capable of recognizing the variant epitope.

Here, we studied the evolution of HIV-1-specific $CD8^+$ T-cell responses and autologous viral sequences in an infected individual identified during acute HIV-1 infection to determine whether following viral escape $CD8^+$ T-cell responses are capable of recognizing the escaped CTL epitope. We observed viral escape from CTL-mediated immune pressure in multiple targeted epitopes, which was followed by a dramatic decline in the respective epitope-specific $CD8^+$ T-cell responses. However, following the selection of a $G_{357}S$ CTL escape variant in an HLA-A11-restricted $CD8^+$ T-cell epitope within HIV-1 p24 $Gag_{349–359}$ (ACQGVGGPGHK) and the decline of wild-type-specific $CD8^+$ T-cell responses, a new $CD8^+$ T-cell response equal in magnitude to the original T-cell response did develop that was specific for the $G_{357}S$ CTL escape variant and did not cross-react with the wild-type epitope. These data demonstrate that de novo epitope-specific $CD8^+$ T-cell responses directed against CTL escape variants can be generated in chronic adult HIV-1 infection and indicate that vaccine strategies aiming to induce $CD8^+$ T-cell responses directed against both the wild-type epitope and the CTL escape variant of an epitope should be possible in order to help prevent the emergence of CTL escape variants.

MATERIALS AND METHODS

Study subjects. A total of 14 individuals followed at the Partners AIDS Research Center at Massachusetts General Hospital in Boston were enrolled in this study. All individuals expressed the HLA class I allele HLA-A11. Study subjects included subject AC-04, who was identified during acute HIV-1 infection, prior to HIV-1 seroconversion (27, 45), and 13 individuals identified during chronic infection. Chronically infected individuals were infected for more than 2 years. The study was approved by the institutional review board and was conducted in accordance with the human experimentation guidelines of the Massachusetts General Hospital.

IFN-γ ELISPOT assay. HIV-1-specific $CD8^+$ T-cell responses were quantified by gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay (4) using overlapping peptides spanning the HIV-1 clade B consensus sequence (15) and a panel of peptides corresponding to previously described optimal clade B CTL epitopes (9). Peripheral blood mononuclear cells (PBMC) were plated at 100,000 per well with peptides at a final concentration of 14 μg/ml in 96-well plates and processed as previously described (4). PBMC were incubated with medium alone (negative control) and phytohemagglutinin (positive control). Specific IFN-γ-secreting T cells were counted using an automated ELISPOT reader (AID, Strassburg, Germany) and calculated by subtracting the average negative control value and expressed as the number of spot-forming cells (SFC) per 10^6 input cells. Negative controls were always ≤30 SFC per 10^6 input cells. A response was considered positive if ≥50 SFC per 10^6 input cells and at least three times greater than the mean background activity. Comparisons of recognition of epitope variants were performed by ELISPOT assay using serial dilutions of truncated peptides as previously described (3).

Analysis of HIV-1-specific $CD8^+$ T-cell function by multiparameter flow cytometry. HLA-A11 peptide tetramers refolded with epitopic peptides corresponding to the wild-type sequence of the HIV-1 p24 $Gag_{349–359}$ $CD8^+$ T-cell epitope ACQGVGGPGHK and the $G_{357}S$ variant of this epitope ACQGVGG PSHK (the difference is underlined) were purchased from a commercial supplier (Beckman Coulter) with phycoerythrin (PE) or allophycocyanin (APC) fluorescence conjugation, respectively. Five hundred thousand to 1 million cells were initially incubated with A11-tetramer complexes (Beckman Coulter, San Diego, CA) refolded with the wild-type peptide or the mutant $AK11_{G357S}$ peptide for 30 min at room temperature as described previously (22). After two washes with phosphate-buffered saline, the wild-type or mutant A11-AK11 peptide (2 μg/ml) was added together with anti-CD28 antibodies, anti-CD49d antibodies (1 μg/ml each; BD Biosciences, San Jose, CA), and PE-Cy5-labeled CD107a antibodies (10 μl/ml; BD Biosciences). Brefeldin A (10 μg/ml; Sigma) and monensin (6 μg/ml; BD Biosciences) were added after the initial hour of incubation. After an additional 5 h of incubation, cells were washed with phosphate-buffered saline–1% bovine serum albumin and stained with surface antibodies (CD8 APC-Cy7, CD3 APC-Cy5.5, CD45RA Alexa Fluor 405, and CCR7 PE-Cy7). Cells were then washed again, fixed, and permeabilized using the Caltag Fixation-Permeabilization kit (Caltag, Burlingame, CA) and stained for intracellular expression of cytokines (interleukin-2–PE and IFN-γ–fluorescein isothiocyanate). In a subset of experiments, PBMC were only incubated with both the A11 tetramers refolded with the wild-type epitope peptide and the escape variant peptide and anti-CD8 and anti-CD4. Cells were acquired on an LSRII flow cytometer (BD Biosciences) using the FACSDiVa software. Control conditions were established by the use of autologous PBMC which had not been stimulated with peptide but otherwise had been treated identically. Flow data were analyzed with the FlowJo software package (Treestar, Ashland, OR).

Characterization of TCR Vβ chain usage of epitope-specific $CD8^+$ T cells. Epitope-specific $CD8^+$ T cells were identified by intracellular cytokine staining as described above, using anti-CD8 and anti-IFN-γ monoclonal antibodies (Becton Dickinson), and also stained with a panel of 24 fluorescein isothiocyanate- or PE-conjugated monoclonal antibodies against TCR Vβ chains (Beckman Coulter) most frequently used by $CD8^+$ T cells and processed as described above. A specific TCR Vβ chain usage was defined when more than 2% of the epitope-specific $CD8^+$ T cells were stained positive with the respective TCR Vβ chain antibody.

Sequencing of autologous virus. Autologous virus was sequenced from plasma RNA using population and clonal sequencing as described previously (3). Viral RNA was isolated from plasma, and a nested PCR was conducted using a set of described primers specific for HIV-1 (3). First-round PCR cycling conditions were 94°C for 2 min; 35 to 50 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C; and a final extension of 68°C for 20 min, and nested PCRs were shortened to a 1-min extension time. PCR fragments were then gel purified and sequenced directly or cloned (TOPO TA Cloning kit; Invitrogen, Carlsbad, CA). Plasmid DNA was isolated by miniprep (QiaPrep Turbo Miniprep) and sequenced bidirectionally on an ABI 3100 PRISM automated sequencer. Sequencher (Gene Codes Corp., Ann Arbor, MI) and MacVector 4.1 (Oxford Molecular) were used to edit and align sequences.

Assessment of statistically significant associations between viral sequences and HLA class I alleles. The autologous (pretreatment) HIV-1 sequences from 230 individuals in the Western Australian HIV Cohort Study (35) were examined in the segments within specified $CD8^+$ T-cell epitopes targeted by study subject AC-04. Bulk sequencing of plasma RNA was performed using previously described methods (38). HLA-A, -B, and -C genotypes were also determined for all individuals by standard sequence-based genotyping. Each individual in the population therefore contributed a single HIV-1 sequence and an HLA class I genotype to the analysis. Statistical associations between viral sequences and HLA class I genotypes were examined using the Epipop program as previously described (35). Multivariate logistic regression models were carried out for each viral position of interest with the outcome set as a specified amino acid and the HLA-A, -B, and -C genotypes as covariates, generating an odds ratio and a *P* value for each HLA allele-viral sequence association.

Statistical analysis. Statistical analysis and graphical presentation were done using SigmaPlot 5.0 (SPSS Inc., Chicago, IL). Results are given as a mean ± a standard deviation or a median with a range. Statistical analysis of significance (*P* values) was based on two-tailed *t* tests and Fischer's exact test.

Nucleotide sequence accession numbers. The sequence data determined in this study were submitted to GenBank and assigned accession no. DQ127534 to DQ127536 and DQ127172 to DQ127224.

RESULTS

Evolution of viral sequence variations within targeted $CD8^+$ T-cell epitopes. Viral escape from CTL responses has been shown to be a hallmark of SIV and HIV-1 infections (1, 2, 6, 10–12, 16, 20, 21, 26, 37, 40, 42). In order to better understand the impact of viral sequence variations within targeted $CD8^+$ T-cell epitopes on the evolution of epitope-specific $CD8^+$ T-cell responses, we performed detailed longitudinal studies of HIV-1-specific T-cell responses and autologous viral se-

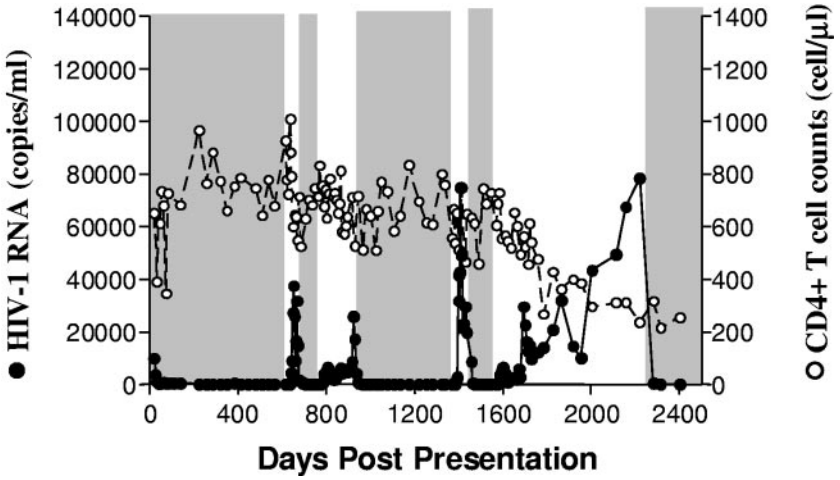

FIG. 1. Clinical course of HIV-1 infection in study subject AC-04. Viral loads and $CD4^+$ T-cell counts in AC-04 are shown, starting at the time of acute HIV-1 infection. Shaded regions denote time under treatment with HAART.

quences in an infected individual identified during acute HIV-1 infection. This study subject (AC-04) presented during symptomatic acute infection with a negative HIV-1 p24 Gag enzyme-linked immunosorbent assay, a viral load of 10×10^6 copies of HIV-1 RNA per ml of plasma, and a $CD4^+$ T-cell count of 652 cells per μl (27, 45). The study subject was treated with highly active antiretroviral therapy (HAART) within 7 days of presentation, and the viral load was suppressed below the limit of detection within 286 days. After 617 days of successful treatment (day 624 postpresentation [pp]), AC-04 underwent four cycles of structured treatment interruptions (Fig. 1). The viral load rebounded during each treatment interruption, resulting in a continuous decline in $CD4^+$ T cells during the periods of viral replication, reaching 237 $CD4^+$ T cells per μl at day 2,240 pp, when HAART was reinitiated.

HIV-1-specific $CD8^+$ T-cell responses in subject AC-04 were assessed using an IFN-γ ELISPOT assay and peptides corresponding to optimal CTL epitopes described for the individual's HLA class I type (A2, A11, B18, B44, Cw5, and Cw12). Over the 2,400-day study period, including 1,410 days on HAART and 990 days off HAART, AC-04 developed T-cell responses to 14 described optimal $CD8^+$ T-cell epitopes (Table 1). In addition, comprehensive assessment of $CD8^+$ T-cell responses using overlapping peptides spanning the entire HIV-1 clade B consensus sequence identified eight additional $CD8^+$ T-cell responses against epitopes not yet optimally defined (data not shown). Sequencing of the autologous virus during primary infection (55 days pp), as well as 2,227 days pp, demonstrated sequence evolution within 4 of the 14 optimal $CD8^+$ T-cell epitopes targeted (Table 1). In each case, viral sequence evolution within these four targeted epitopes resulted in an in vivo decline of $CD8^+$ T-cell responses directed against peptides corresponding to the respective wild-type sequence (Fig. 2). In order to examine in vitro the impact of these variant epitopes on $CD8^+$ T-cell recognition, the peptides corresponding to the escape variants of the respective epitopes were tested for recognition using PBMC. In three out of the four cases, the variant epitopes were not recognized by early wild-type-specific $CD8^+$ T-cell responses (Fig. 2A to C), while significant cross-recognition existed between the wild-type and escape variant peptides of the HLA-Cw12-restricted Tat epitope when maximal peptide concentrations were used in the ELISPOT assay (Fig. 2D). In this case, however, the use of serial peptide dilutions of the wild-type and escape variant

TABLE 1. HIV-1-specific $CD8^+$ T-cell epitopes targeted in subject AC-04

Epitope	Peptide sequence	No. of SFC/10^6 PBMC[a]	Sequence variation[b]
A11-AK11 $Gag_{349–359}$	ACQGVGGPGHK	1,280	ACQGVGGPGHK --------S--
A2-AL9 $Vpr_{59–67}$	ALIRILQQL	950	ALIRILQQL --t-S----[c]
Cw12-CC8 $Tat_{30–37}$	CCFHCQVC	750	CCFHCQVC --M-----
Cw5-SL9 $Rev_{67–75}$	SAEPVPLQL	1,000	SAEPVPLQL ----G----
B44-AW11 $Gag_{306–316}$	AEQASQDVKNW	2,310	None
A11-AK9 $Pol_{314–322}$	AIFQSSMTK	640	None
A11-QK9 $Pol_{425–433}$	QIYAGIKVK	530	None
A11-IK10 $Pol_{497–506}$	IYQEPFKNLK	730	None
A11-QK9 $Pol_{676–684}$	QIIEQLIKK	320	None
B18-LY10 $Vif_{102–110}$	LADQLIHLHY	1,930	None
A11-QK10 $Nef_{73–82}$	QVPLRPMTYK	820	None
A11-AK9 $Nef_{84–92}$	AVDLSHFLK	2,129	None
B18-YY9 $Nef_{135–143}$	YPLTFGWCY	240	None
A2-LV10 $Nef_{137–146}$	LTFGWCFKLV	250	None

[a] Peak epitope-specific IFN-γ $CD8^+$ T-cell responses (SFC/10^6 PBMC).
[b] Autologous viral sequences during primary infection (day 55 pp) and day 2,227 pp. Dashes indicate parts of sequences that match those of the lines above.
[c] A lowercase letter indicates a mixed-based population at that residue.

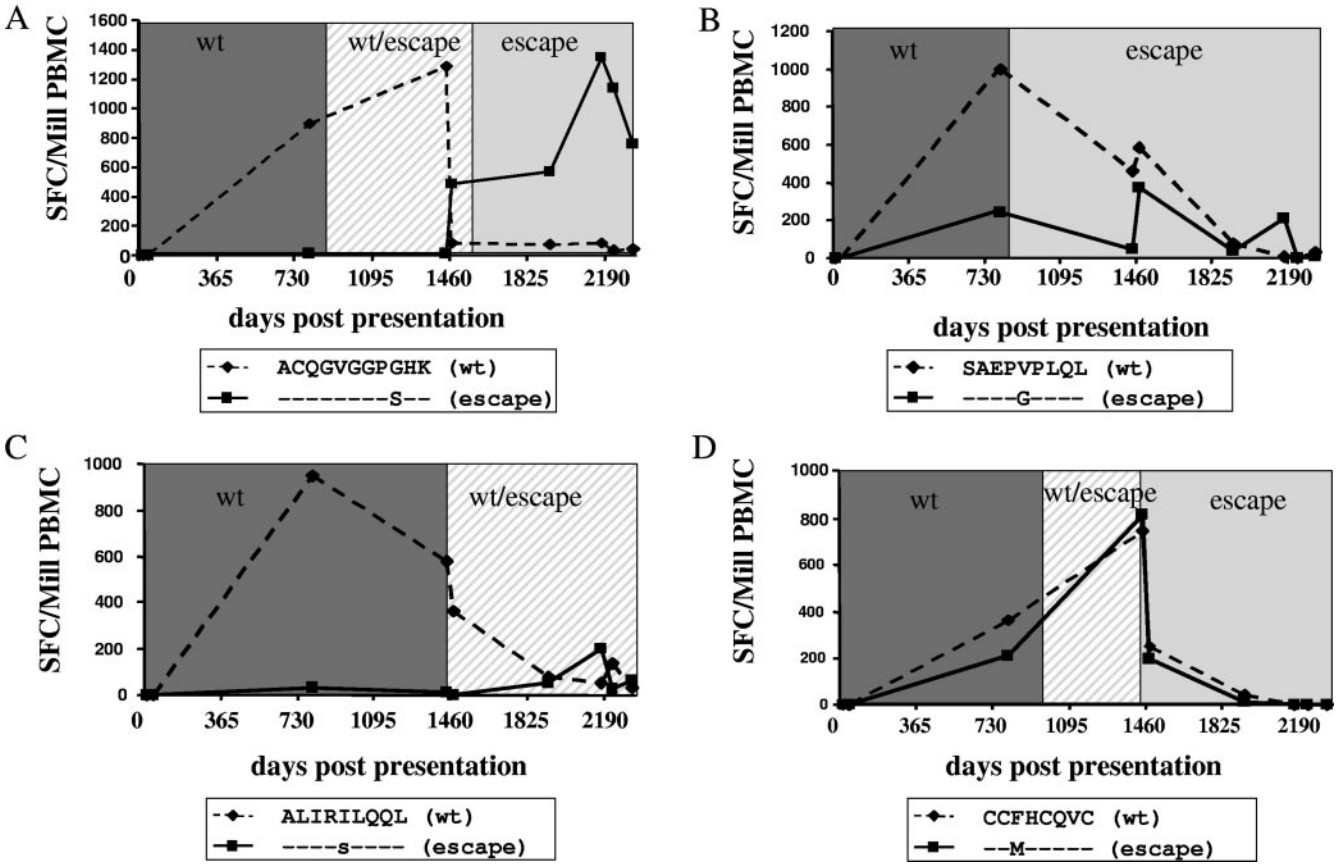

FIG. 2. Changes in epitope-specific $CD8^+$ T-cell responses in relationship to the emergence of viral sequence variations in AC-04. Changes in the magnitude of epitope-specific $CD8^+$ T-cell responses measured by IFN-γ ELISPOT assay are shown for the four epitopes targeted by study subject AC-04 that exhibited viral sequence variations over time. Panels: A, HIV-1 $Gag_{349–359}$; B, HIV-1 $Rev_{67–75}$; C, HIV-1 $Vpr_{57–63}$; D, HIV-1 $Tat_{30–37}$. Shaded regions denote periods during which the autologous viral sequence reflected wild-type (wt) sequences, escape variant sequences, or a mixture of both, as determined by the sequencing of individual viral clones. Mill, million.

peptides demonstrated that the wild-type peptide was recognized at 100-fold lower concentrations (data not shown), suggesting that the sequence variations also resulted in reduced recognition of this epitope. Taken together, these data demonstrate the development of robust, HIV-1-specific $CD8^+$ T-cell responses in subject AC-04 that resulted in viral escape from CTL-meditated immune pressure in 4 out of the 14 optimal $CD8^+$ T-cell epitopes targeted and a decline in the frequency of each wild-type-specific $CD8^+$ T-cell response.

Development of de novo $CD8^+$ T-cell responses directed against a CD8 T-cell viral escape variant. We observed that none of the sequence variations in the four escaping $CD8^+$ T-cell epitopes in subject AC-04 occurred within HLA binding anchor positions that would likely prevent the epitope from reaching the cell surface, but rather within amino acid positions potentially responsible for the interaction with the TCR of the respective $CD8^+$ T cells (9, 13, 34, 44). This prompted us to follow the evolution of $CD8^+$ T-cell responses to these escape variants longitudinally in order to determine whether new $CD8^+$ T-cell responses developed that were capable of recognizing the variant epitopes. While $CD8^+$ T-cell responses to the Rev, Tat, and Vpr epitopes continuously declined following the development of the escape mutations, a new $CD8^+$ T-cell response specific to the $G_{357}S$ escape variant of the HLA-A11-restricted p24 $Gag_{349–359}$ epitope (ACQGVGG PSHK [the variation is underlined]; $A11\text{-}AK11_{G357S}$) emerged at day 1,471 (Fig. 2A), approximately 674 days following the first detection of autologous virus harboring the p24 Gag $G_{357}S$ mutation (Table 2). This $A11\text{-}AK11_{G357S}$-specific $CD8^+$ T-cell response subsequently increased in frequency, reaching 1,400 SFC/10^6 PBMC at day 2,164 pp (Fig. 2A), at a time when 100% of the cloned sequence harbored the p24 Gag $G_{357}S$ mutation (Table 2). Interestingly, this novel $CD8^+$ T-cell response was specific for the $A11\text{-}AK11_{G357S}$ escape variant but did not demonstrate significant cross-reactivity with the A11-AK11 wild-type sequence (Fig. 2A). This lack of detectable cross-reactivity of the novel variant-specific $CD8^+$ T-cell response with the wild-type sequence was reconfirmed using HLA-A11 tetramers refolded with either the wild-type A11-AK11 peptide or the $A11\text{-}AK11_{G357S}$ escape variant peptide. While $CD8^+$ T cells isolated at day 1,427, at the peak response against the wild-type epitope, only bound to HLA-A11 tetramers refolded with the wild-type epitope, $CD8^+$ T cells derived from day 2,323 only bound the HLA-A11 tetramer refolded with the $A11\text{-}AK11_{G357S}$ escape variant peptide (Fig. 3). Taken together, these data demonstrate the development of

TABLE 2. Population and clonal p24 $Gag_{349–359}$ sequences in subject AC-04

Days pp	Population sequence	Clonal sequence	Clonal frequency	% $G_{357}S$ variant
	ACQGVGGPGHK	ACQGVGGPGHK		
55	----------	ND[a]	ND	ND
660	----------	ND	ND	ND
797	--------S--	----------	4/18	78
		--------S--	14/18	
930	--------S--	----------	7/12	42
		--------S--	5/12	
1,670	--------S--	----------	0/12	100
		--------S--	12/12	
2,227	--------S--	----------	0/11	100
		--------S--	10/11	
		-----W--S--	1/11	

[a] ND, not done.

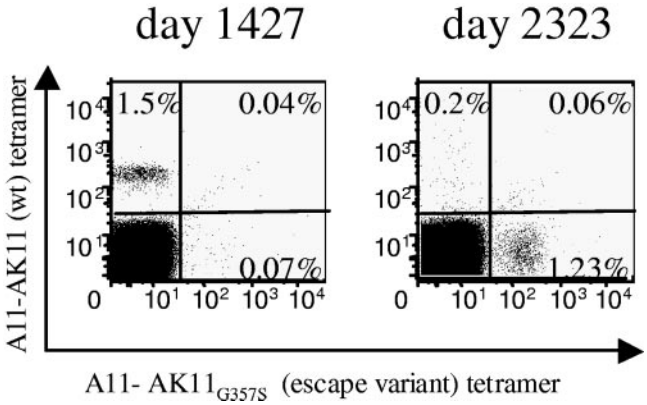

FIG. 3. Lack of cross-reactivity between A11-AK11-specific and A11-$AK11_{G357S}$-specific $CD8^+$ T cells. Dot plots showing the flow cytometric analysis of HIV-1-specific $CD8^+$ T-cell responses in AC-04 using MHC class I-peptide tetramer complexes specific for the A11-AK11 peptide (PE labeled) or for the A11-$AK11_{G357S}$ escape variant (APC labeled). Tetramer-positive $CD8^+$ T cells were quantified at day 1,427 pp and day 2,323 pp. wt, wild type.

novel $CD8^+$ T-cell responses specific for the CTL escape variant of the A11-AK11 epitope that do not cross-react with the original wild-type sequence.

De novo $CD8^+$ T-cell responses directed against the A11-$AK11_{G357S}$ escape variant use a distinct TCR Vβ repertoire but exhibit similar functional properties. To further investigate the differences between the original wild-type (A11-AK11)-specific $CD8^+$ T-cell responses and the de novo responses directed against the escape variant (A11-$AK11_{G357S}$), we used TCR Vβ-specific antibodies to characterize the TCR usage of the A11-AK11- and A11-$AK11_{G357S}$-specific $CD8^+$ T cells. While 82% of the A11-AK11-specific $CD8^+$ T cells identified by IFN-γ production following stimulation with the A11-AK11 wild-type peptide used TCR Vβ1, A11-$AK11_{G357S}$-specific $CD8^+$ T cells did not stain with the TCR Vβ1 antibody (1.7%) but rather with a TCR Vβ9-specific antibody (31%) (Fig. 4). These data demonstrate that study subject AC-04 recruited a new subset of $CD8^+$ T cells specific for the CTL escape variant that did not cross-react with the original wild-type sequence and utilized TCRs expressing a different Vβ chain.

We subsequently used multiparameter flow analysis to characterize the functional properties of the A11-AK11-specific $CD8^+$ T-cell responses primed during primary HIV-1 infection and the A11-$AK11_{G357S}$-specific responses generated during chronic infection. As described previously (19, 22), only a subset of the tetramer-positive $CD8^+$ T cells produced IFN-γ following stimulation with the respective epitopic peptide (10.8% of the A11-AK11-specific $CD8^+$ T cells on day 839 pp and 20.9% of the A11-$AK11_{G357S}$-specific $CD8^+$ T cells at day 2,010). Similarly, only 10.9% and 33.5% of the A11-AK11-specific and A11-$AK11_{G357S}$-specific $CD8^+$ T cells, respectively, expressed the degranulation maker CD107a following stimulation. Overall, tetramer-specific $CD8^+$ T cells were predominantly of a $CCR7^{neg}$ $CCD45RA^{neg}$ effector memory phenotype (88.2% for the A11-AK11-specific and 87.1% for the A11-$AK11_{G357S}$-specific $CD8^+$ T cells), while 6.6% and 7.8%, respectively, expressed markers characteristic for terminally differentiated effector cells ($CCR7^{neg}$ $CD45RA^{pos}$). Taken together, these data suggest that the de novo $CD8^+$ T-cell responses specific for the escape variant (A11-$AK11_{G357S}$), and arising during chronic infection, did not differ significantly in their function from wild-type-specific $CD8^+$ T cells with the assays described.

The A11-$AK11_{G357S}$ variant epitope is frequently recognized during chronic infection. The above data demonstrate that de novo responses to the $G_{357}S$ escape variant of the HLA-A11-restricted p24 $Gag_{349–359}$ $CD8^+$ T-cell epitope can be generated in natural HIV-1 infection. An analysis of 101 HIV-1 clade B sequences published in the Los Alamos HIV-1 database demonstrated that while positions P1 to P8 and P10 to P11 of the A11-AK11 epitope were highly conserved in these sequences (99%), suggesting functional constraints in this region of p24, at position 9, where the mutation arose in AC-04, serine (S) represented a common polymorphism (25%) (Table 3). An analysis of statistical associations between viral sequences and HLA class I genotypes in the Western Australian HIV Cohort Study using the Epipop program (35) demonstrated that this $G_{357}S$ mutation was significantly associated with the expression of HLA-A11 in HIV-1-infected individuals (55% of sequences; odds ratio = 3.7; $P = 0.004$).

Because these data suggested that the $G_{357}S$ mutation evolves frequently in chronically HIV-1-infected individuals expressing HLA-A11, we next examined the frequency by which the A11-$AK11_{G357S}$ escape variant peptide was recognized by $CD8^+$ T cells from chronically HIV-1-infected individuals expressing HLA-A11. Of 13 chronically HIV-1-infected individuals tested expressing HLA-A11, 5 had no detectable responses against either the A11-AK11 wild-type peptide or the A11-$AK11_{G357S}$ escape variant peptide. Surprisingly, the remaining eight subjects all had detectable $CD8^+$ T-cell responses directed against the nonconsensus A11-$AK11_{G357S}$ variant form of the epitope, ranging from 100 to 900 SFC/10^6 PBMC (median, 170 SFC/10^6 PBMC), with two of these subjects exhibiting at the same time responses directed against the A11-AK11 wild-type peptide of 50 and 640 SFC/10^6 PBMC, respectively ($P = 0.03$). Sequence data for the autologous virus were available for 8 of the 13 subjects (data not shown). In only one case was the wild-type sequence of the A11-AK11 epitope detected, while the A11-$AK11_{G357S}$ escape variant sequence

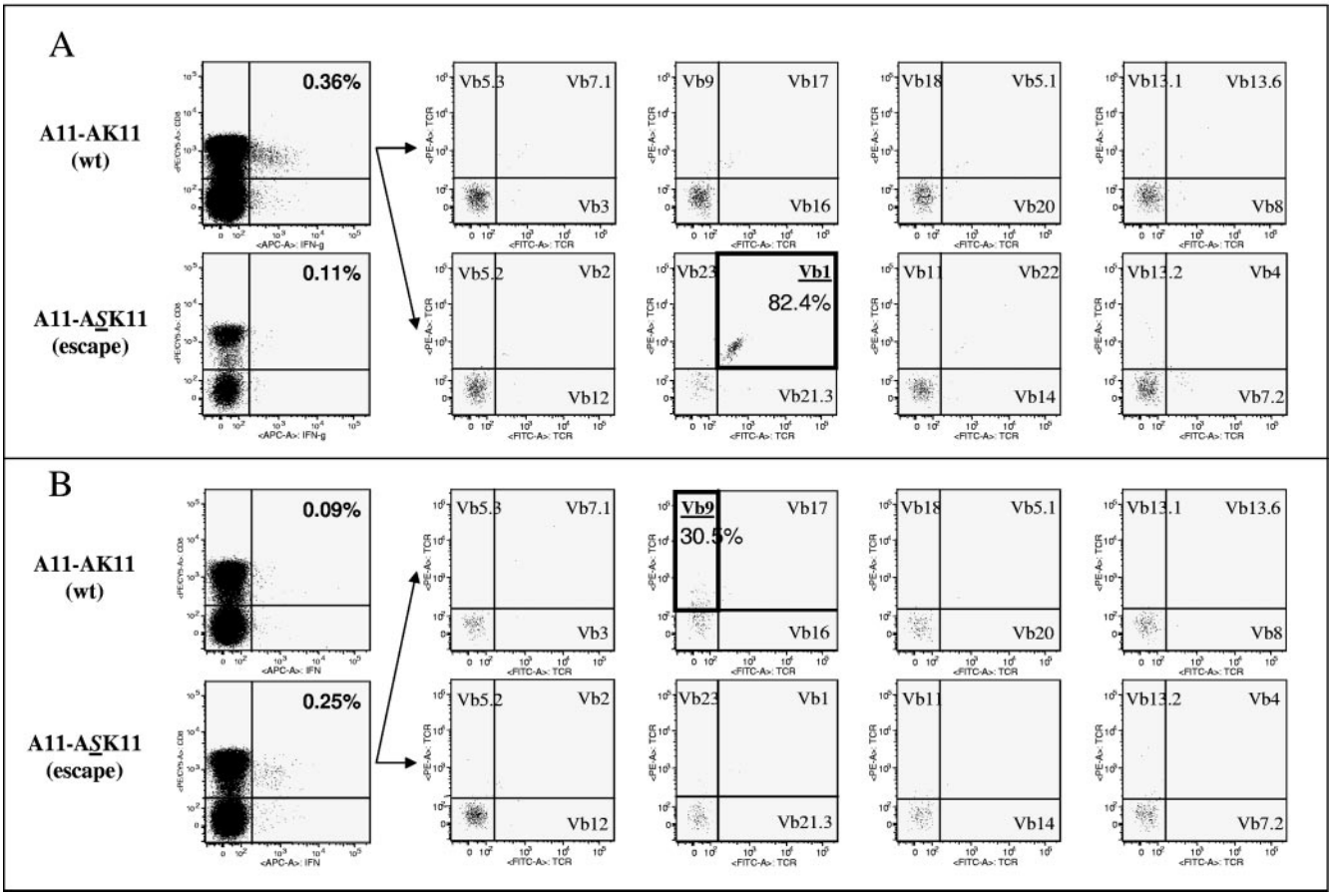

FIG. 4. Usage of different TCR Vβ repertoires by A11-AK11-specific and A11-AK11$_{G357S}$-specific CD8$^+$ T cells. A11-AK11- and A11-AK11$_{G357S}$-specific CD8$^+$ T cells were characterized by intracellular IFN-γ staining following stimulation with the respective peptide at day 1,427 pp (A) and day 2,220 pp (B). TCR Vβ usage was determined using Vβ-specific antibodies. At day 1,427 pp (A), only A11-AK11 (wild type [wt])-specific CD8$^+$ T cells were detectable, while at day 2,220 pp (B), only A11-AK11$_{G357S}$ (escape variant)-specific CD8$^+$ T-cell responses were detectable. A11-AK11-specific CD8$^+$ T cells utilized TCR Vβ1 (82.4%), while A11-AK11$_{G357S}$-specific CD8$^+$ T cells did not utilize TCR Vβ1 (1.7%) but rather TCR Vβ9 (30.5%). FITC, fluorescein isothiocyanate.

was present in five subjects and a glycine-to-alanine substitution was present in position p24 Gag$_{357}$ in the remaining two subjects. Three out of the five subjects with the A11-AK11$_{G357S}$ escape variant had mounted a detectable A11-AK11$_{G357S}$-specific CD8$^+$ T-cell response. Interestingly, in the two subjects exhibiting the rare A11-AK11$_{G357A}$ variant (Table 3), no or only very weak (100 SFC/10^6 PBMC) CD8$^+$ T-cell responses were detectable against the A11-AK11 wild-type and A11-AK11$_{G357S}$ escape variant peptides, potentially suggesting further evolution of the virus away from the variant-specific response. Taken together, these data suggest that the G$_{357}$S mutation occurs frequently in HIV-1-infected individuals expressing HLA-A11, resulting in significantly more frequent recognition of the A11-AK11$_{G357S}$ escape variant than the wild-type sequence in individuals with chronic HIV-1 infection.

TABLE 3. Sequence variability within HIV-1 clade B Gag$_{349–359}$

Sequence	Frequency	Percentage
ACQGVGGPGHK	71/101	70
--------S--	24/101	24
--------A--	2/101	2
---E----S--	1/101	<1
--------H--	1/101	<1
----G------	1/101	<1
-----E--A--	1/101	<1

DISCUSSION

A number of recent studies have demonstrated that viral escape from CTL-mediated immune pressure occurs frequently in HIV-1 and SIV infections (1, 2, 6, 10–12, 16, 20, 21, 26, 37, 40, 42) and can be associated with the loss of vaccine-induced protection from disease progression (6). However, little is known about the ability of the immune system to respond to viral escape by developing novel CD8$^+$ T-cell responses against these CTL escape variants. Here we demonstrate that de novo CD8$^+$ T-cell responses restricted by the same HLA class I allele can be generated against HIV-1 CTL escape variants harboring sequence variations within residues that determine TCR binding. This observation is relevant for vaccine design, as immunization with both the wild-type se-

quence and the predictable CTL escape variant of the respective epitopes may be able to restrict the ability of the virus to evade virus-specific $CD8^+$ T-cell responses through sequence variation.

Here we demonstrate that the immune system is capable of mounting novel $CD8^+$ T-cell responses against CTL escape variants that affect TCR recognition, using epitope-specific $CD8^+$ T cells expressing a different TCR repertoire. Our data are in line with previous reports that HIV-1-specific $CD8^+$ T-cell responses can adapt to viral evolution (25) but demonstrate for the first time convincingly, providing data on the TCR repertoire used, development of de novo $CD8^+$ T-cell responses specific for the escape variant epitope. These data demonstrating the ability to generate de novo $CD8^+$ T-cell responses to escape variants are in partial contrast to a recently published observation in the SIV macaque model describing the poor recognition of $CD8^+$ T-cell escape mutations by $CD8^+$ T-cell responses in naive hosts (18). In this study, however, the three SIV CTL epitopes investigated exhibited sequence variations within positions important for the binding of the epitope to the respective MHC class I molecules (C-terminal anchor in one epitope and secondary anchors in the remaining two epitopes), and each of the mutant peptides showed at least a 90% reduction in binding to MHC class I (18). These mutations in residues impacting the presentation by MHC class I most likely explain the inability of these three epitope variants to prime de novo responses in a naive host. In contrast, the HIV-1 p24 Gag $G_{357}S$ mutation studied here represented a mutation in a residue responsible for the interaction with the TCR (34) and was able to recruit novel $CD8^+$ T cells utilizing a different TCR Vβ chain. These data demonstrate that the impact of a CTL escape mutation on recognition by $CD8^+$ T cells depends on the position of the mutated residue within the epitope and that mutations within residues responsible for the interaction with the TCR may enable the immune system to mount novel $CD8^+$ T-cell responses against these variant epitopes.

Why is the virus not mutating exclusively at residues that entirely abolish the presentation of the targeted epitope, as this would provide a stronger selection advantage? A number of recent studies have demonstrated that the variability of HIV-1 is not unlimited but restricted by functional or structural constraints (7, 16, 17, 28, 39, 41). Indeed, the analysis of HIV-1 clade B sequences published in the Los Alamos HIV-1 Database demonstrated that most of the positions within the HLA-A11-restricted p24 Gag epitope AK11 are highly (99%) conserved, and only position 9 (HIV-1 Gag_{357}) exhibited some degree of sequence variability that was predominantly restricted to two amino acids, glycine and serine. This observation suggests that viral sequence evolution within the p24 Gag epitope A11-AK11 is restricted, forcing the virus to evolve toward residues that may not represent the most effective substitution for immune escape. Such $CD8^+$ T-cell epitopes, which are limited in their capacity to escape and to which the immune system is reproducibly capable of recognizing the escaped form of the virus, may represent attractive targets for vaccines.

The ability of the escape variant containing the $G_{357}S$ mutation to induce strong epitope-specific $CD8^+$ T-cell responses also indicates that original antigenic sin, that has been proposed to limit the ability of the immune system to generate new responses to escape variants (33), can be overcome in the setting of this $CD8^+$ T-cell epitope. It has been demonstrated that some variant $CD8^+$ T-cell epitopes can function as TCR antagonists, limiting the capacity of the immune system to generate novel responses to the escaped epitope (30, 31, 43). The ability of the immune system to generate de novo responses against the $G_{357}S$ escape variant epitope may be largely due to the apparent lack of cross-recognition between $CD8^+$ T cells specific for the wild-type and $G_{357}S$ escape variant epitopes, limiting the ability of the escape variant to continuously stimulate and expand $CD8^+$ T cells specific for the wild-type epitope and allowing for the recruitment of variant epitope-specific $CD8^+$ T cells utilizing distinct TCRs.

The analysis of statistical associations between sequence polymorphisms within the p24 $Gag_{349–359}$ A11-AK11 epitope and HLA-A11 using data from the Western Australian HIV Cohort Study, a cohort mainly (>75%) composed of HIV-1 clade B-infected individuals (35), showed that the $G_{357}S$ mutation occurs frequently on the population level in individuals expressing this allele. Indeed, the HLA-A11-$AK11_{G357S}$ escape variant was significantly more frequently recognized in HLA-A11-expressing individuals during chronic infection than the wild-type variant, and the autologous virus in five out of these eight chronically infected study subjects for whom viral sequences were available harbored the HLA-A11-$AK11_{G357S}$ escape variant. This is in striking contrast to the significantly lower frequency of the $G_{357}S$ variant in the sequences published in the Los Alamos HIV-1 Database (25% $G_{357}S$ versus 71% G_{357} wild type; $P = 0.001$). These data suggest that individuals expressing HLA-A11 frequently mount $CD8^+$ T-cell responses against a common HLA-A11-restricted escape variant in HIV-1 Gag. Further studies are needed to determine whether this ability of HLA-A11-restricted $CD8^+$ T cells to have a "second chance" to target the evolving virus may at least partially account for the association of the HLA-A11 allele with slower disease progression in HIV-1 (36), the only HLA-A allele that has been shown to be associated with a protective effect on HIV-1 disease progression to date.

Reports on both HIV-1 and SIV infections suggest that viral escape from virus-specific $CD8^+$ T-cell responses can dramatically compromise immune control (6, 8, 14, 21). Current HIV-1 vaccine strategies do not adequately address this important limitation. However, recent data illustrate that the simultaneous delivery of both wild-type and variant forms of an immunodominant $CD8^+$ T-cell epitope in mice was able to avoid both T-cell antagonism and original antigenic sin (46). Together with the data presented here, this suggests that simultaneous delivery of wild-type sequences and TCR escape variant sequences of HIV-1-specific $CD8^+$ T-cell epitopes may represent an effective approach to inducing broadly reactive $CD8^+$ T-cell responses against multiple variant forms of a $CD8^+$ T-cell epitope to prevent viral escape.

Taken together, these studies demonstrate the ability of the immune system to generate de novo $CD8^+$ T-cell responses to CTL escape variants in the context of chronic HIV-1 infection. These data are of relevance for vaccine design, as priming of both wild-type- and variant-specific $CD8^+$ T-cell responses will at the same time provide broad coverage against potential circulating variants and protect against the emergence of es-

cape variants that could be associated with the failure of vaccine-mediated protection.

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