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Selective Escape from CD8^{+} T-Cell Responses Represents a Major Driving Force of Human Immunodeficiency Virus Type 1 (HIV-1) Sequence Diversity and Reveals Constraints on HIV-1 Evolution†

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The sequence diversity of human immunodeficiency virus type 1 (HIV-1) represents a major obstacle to the development of an effective vaccine, yet the forces impacting the evolution of this pathogen remain unclear. To address this issue we assessed the relationship between genome-wide viral evolution and adaptive CD8^{+} T-cell responses in four clade B virus-infected patients studied longitudinally for as long as 5 years after acute infection. Of the 98 amino acid mutations identified in nonenvelope antigens, 53% were associated with detectable CD8^{+} T-cell responses, indicative of positive selective immune pressures. An additional 18% of amino acid mutations represented substitutions toward common clade B consensus sequence residues, nine of which were strongly associated with HLA class I alleles not expressed by the subjects and thus indicative of reversions of transmitted CD8 escape mutations. Thus, nearly two-thirds of all mutations were attributable to CD8^{+} T-cell selective pressures. A closer examination of CD8 escape mutations in additional persons with chronic disease indicated that not only did immune pressures frequently result in selection of identical amino acid substitutions in mutating epitopes, but mutating residues also correlated with highly polymorphic sites in both clade B and C viruses. These data indicate a dominant role for cellular immune selective pressures in driving both individual and global HIV-1 evolution. The stereotypic nature of acquired mutations provides support for biochemical constraints limiting HIV-1 evolution and for the impact of CD8 escape mutations on viral fitness.

One of the greatest challenges facing the design of an effective human immunodeficiency virus type 1 (HIV-1) vaccine is the extensive global sequence diversity of this pathogen. Numerous clades of HIV-1 predominate worldwide (22, 41), and even within individual clades there is sufficient sequence diversity to make selection of optimal antigens for vaccine design extremely difficult. These issues are compounded by the increasing emergence of recombinant viruses, especially in regions of Southeast Asia and Central Africa (24, 52, 53), likely arising through dual infection or superinfection of individuals with different strains (8, 18). This sequence diversity of HIV-1 is thought to result from random errors introduced during reverse transcription (47) as well as host immune selection pressures (44).

Equally challenging to vaccine development is the ability of HIV-1 to evolve within an individual during the course of infection. Viral escape from both CD8^{+} T-cell responses (4, 10, 11, 27, 39, 44, 45) and neutralizing antibodies (2, 9, 12, 36, 46, 55) in HIV-1 and simian immunodeficiency virus (SIV) infection is well documented. However, while evolution within the envelope protein is clearly associated with strong autologous neutralizing antibody responses (46, 55), the contribution of immune selection pressure to viral evolution in nonenvelope proteins of HIV-1 is less clear. With few exceptions (39, 40), most studies have focused on examining viral evolution within only a single or a few epitopes, although there is now strong evidence for adaptation of HIV-1 to host CD8^{+} T-cell responses at the population level (37), suggesting a role for escape from immune responses in driving global HIV-1 sequence diversity. Therefore, the extent to which HIV-1 evolves over the course of infection remains unclear, as does the degree to which these changes are specifically driven by CD8^{+} T-cell-associated selective pressures.

Conversely, recent reports of reversion of CD8 escape mutations upon transmission of HIV-1 and SIV to a new host (3, 20, 30), and of the impact of some SIV CD8 escape mutations on viral fitness (20, 21, 43), imply structural or functional constraints on evolving mutations (20, 21, 32, 51). However, little is known still regarding the extent to which biochemical sequence constraints impact the accumulation of mutations arising from immune selection pressures, or the relationship

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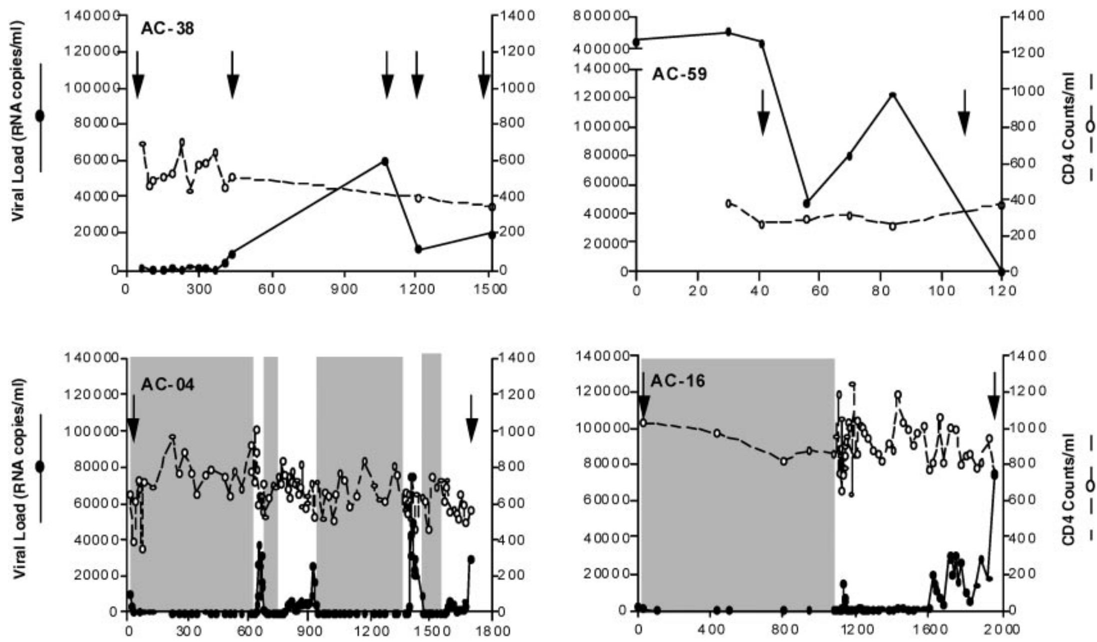

FIG. 1. Clinical course of HIV-1 infection in four study subjects. Viral loads and $CD4^+$ T-cell counts are shown for four subjects identified during acute HIV-1 infection and followed longitudinally. Arrows indicate time points from which viral sequences were derived. Shaded regions, time under treatment with highly active antiretroviral therapy.

between immune selection pressures and global HIV-1 diversity.

MATERIALS AND METHODS

Subjects. The four acutely HIV-1 infected subjects were enrolled from the Boston Acute Infection Cohort and identified during primary HIV-1 infection. Subjects AC-04 and AC-16 were enrolled in a structured treatment interruption protocol described previously (48). Chronically infected subjects were enrolled from the Massachusetts General and Shattuck Hospitals in Boston, Queen Elisabeth Hospital in Bridgetown, Barbados (19), Praxis Heiko Jessen, Berlin, Germany, and Department of Internal Medicine, University of Bonn, Germany. HLA class I typing of the four acute-phase study subjects was as follows: for AC-38, A01, A03, B07, B57, C06, and C07; for AC-59, A02, B07, B44, C05, and C07; for AC-04, A02, A11, B18, B44, C05, and C12; and for AC-16, A28, A29, B14, B44, and C08. HLA class II typing was as follows: for AC-38, DR7/15, DRw51/53, and DQ6/9; for AC-59, DR8/13, DRw52, and DQ4/6; for AC-04, DR4/15, DRw51/53, and DQ1/7; and for AC-16, DR7/13, DRw52/53, and DQ2/7. Time points for each subject, defined from the day of presentation with symptomatic acute HIV-1 infection, are as follows: for AC-38, days 64, 408, 1,073, 1,213, and 1,510; for AC-59, days 41 and 111; for AC-04, days 0 and 1,650; and for AC-16, days 0 and 1,934.

IFN-γ ELISPOT assay. HIV-1-specific $CD8^+$ T-cell responses were quantified by a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay as described previously (57), using overlapping peptides (15- to 18-mer peptides overlapping by 10 amino acids) spanning the entire expressed HIV-1 clade B 2001 consensus sequence, as well as peptides corresponding to optimal described clade B cytotoxic T-lymphocyte (CTL) epitopes (13) and autologous virus sequences. IFN-γ-secreting T cells were counted by an automated reader and expressed as spot-forming cells (SFC) per 10^6 input cells after subtraction of the negative control. A response was considered positive if there were ≥50 SFC per 10^6 cells and this level was least 3 times greater than mean background activity.

Sequencing of autologous virus. Viral DNA was isolated from peripheral blood mononuclear cells (5×10^6 cells), and viral RNA was extracted from plasma samples and reverse transcribed as described previously (8). A set of 49 primary and nested PCR primer pairs amplified the entire HIV-1 genome as described previously (8). When necessary, PCR fragments were gel purified, and PCR products were then population sequenced bidirectionally on an ABI 3100 PRISM automated sequencer. Sequencher (Gene Codes Corp., Ann Arbor, MI) and MacVector 4.1 (Oxford Molecular) software were used to edit and align sequences.

Identification of HLA-associated sequence polymorphisms. HLA-associated sequence polymorphisms were identified using the customized software program Epipop, as described previously (37). The Epipop analysis utilized partial or whole-genome sequences (>75% from clade B) derived from 230 subjects HLA typed to 4 digits by using high-resolution HLA-A, -B, and -C typing.

Statistical analysis. Wilcoxon rank sum tests and Fisher exact tests were conducted using Prism 4.0 (GraphPad, San Diego, CA).

Nucleotide sequence accession numbers. Sequence data for autologous virus are available from GenBank under accession no. DQ127534 to DQ127551. Data for HLA-associated sequence polymorphisms are listed under GenBank accession no. AY856956 to AY857186.

RESULTS

In vivo sequence evolution is strongly associated with virus-specific $CD8^+$ T-cell responses. To determine the relationship between immune selection pressure and intrahost viral evolution, we performed whole-genome sequencing together with assessment of $CD8^+$ T-cell responses for four HIV-1-infected subjects identified during acute infection, two of whom were transiently treated with antiretroviral drugs (Fig. 1). For this analysis, we excluded the envelope gene, which is known to be under dominant humoral immune selection pressure (46, 55). Population sequencing of virus circulating in plasma, performed at the time of acute infection, showed a relatively homogeneous virus population in each person, with the infecting clade B viruses differing from one another by 8.7% to 10.8% (data not shown).

Longitudinal viral sequence data indicated a progressive accumulation of amino acid mutations that occurred in a stepwise fashion. Representative data are presented in Fig. 2A for subject AC-38, in whom a total of 52 amino acid mutations arose sequentially over the 4 years of follow-up. Nearly half of

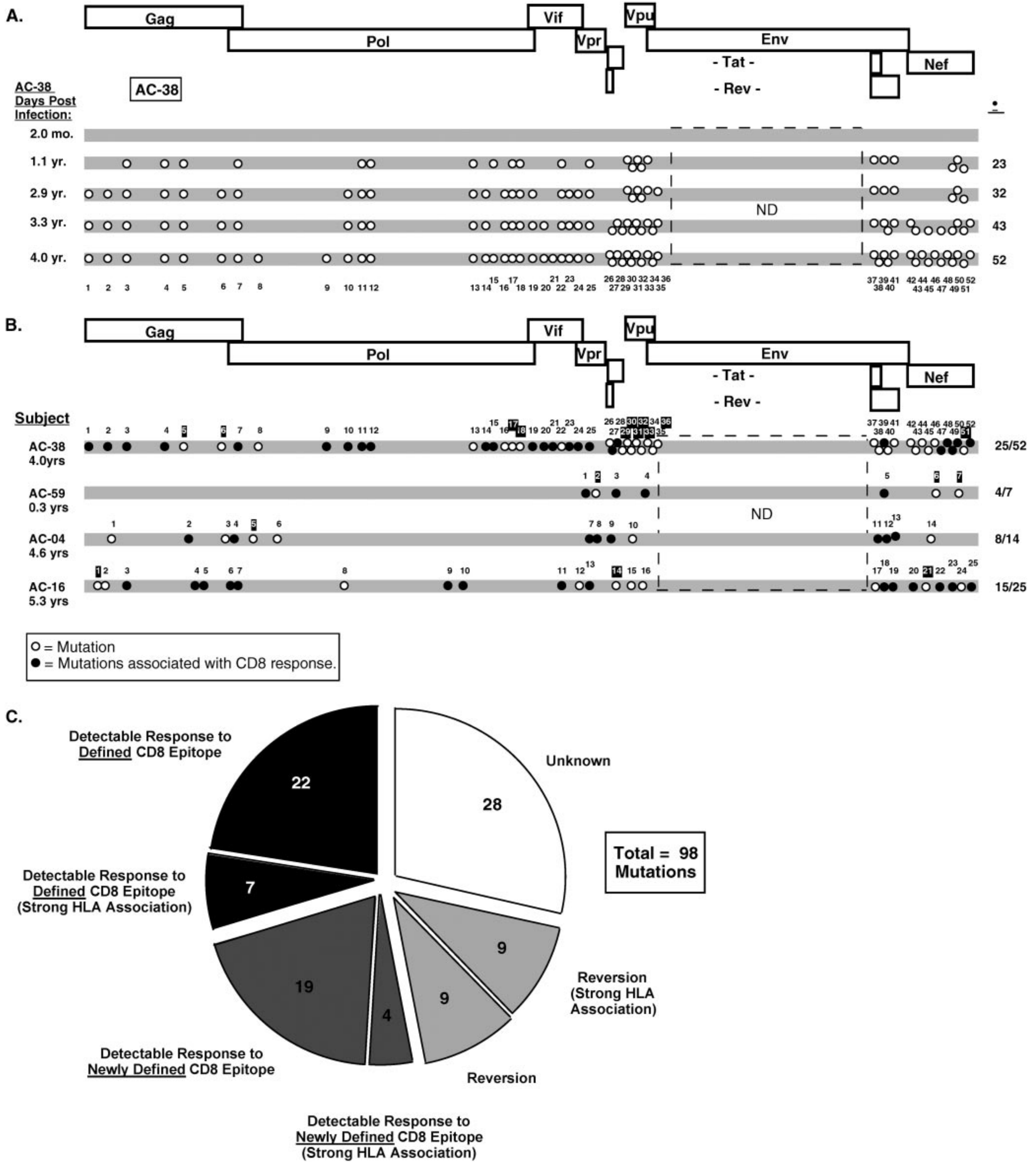

FIG. 2. Sites of sequence variation and associated CD8 T-cell responses. Longitudinal whole-genome sequencing, excluding Env, was conducted on four subjects. (A) Mutations accumulating in subject AC-38 over 4 years of follow-up (○). (B) Mutations developing in each subject over 0.3 to 5.3 years of follow-up (○). ●, mutations associated with detectable IFN-γ ELISPOT $CD8^+$ T-cell responses. Numbers corresponding to mutations associated with reversions are boxed in black. (C) Summary of mutations associated with detectable $CD8^+$ T-cell responses in either defined or newly identified epitopes or with reversions. Mutations strongly associated with HLA alleles at the population level are indicated separately.

these mutations occurred in the first year after infection, before viral loads rose above 5,000 copies/ml; all but one mutation remained stable subsequently. Similar analyses were performed for all four persons, ranging from 0.3 to 5.3 years of follow-up, and showed evolution of 7 to 52 mutations per individual (Fig. 2B). Of note, there were an additional 82 silent nucleotide mutations in these subjects which did not code for amino acid substitutions (data not shown).

Peripheral blood mononuclear cells from subjects were also screened by an IFN-γ ELISPOT assay to determine the specificity of the $CD8^+$ T-cell response against all nonenvelope proteins (8, 57). Where indicated, peptides corresponding to autologous virus sequences were also tested when the autologous virus differed from the clade B consensus sequence in a region undergoing evolution (see Table S1 in the supplemental material). Of the 98 amino acid mutations defined in these subjects, 29 resided within previously defined CD8 epitopes restricted by HLA alleles expressed in the individual subjects, and in each case a $CD8^+$ T-cell response was detected against the optimally defined CD8 epitope or the autologous variant (Fig. 2B). An additional 23 mutations were also targeted by circulating $CD8^+$ T cells in these individuals, representing previously undescribed novel epitopes (Fig. 2B; see also Fig. S1 and Table S1 in the supplemental material). Thus, 52 of the 98 mutations (53% overall; range, 48% to 60%) arising from the time of acute infection were linked to $CD8^+$ T-cell selection pressures documented to be present in vivo.

We next compared these evolving mutations to an extensive HIV-1 clade B sequence database in which allele-specific polymorphisms have been defined for the most frequently expressed alleles and statistically linked to immune selection pressure, but for which direct immunologic evidence has been lacking (37). Allele-specific polymorphisms were identified using the customized software program Epipop, as previously described (37). Eleven mutations were strongly associated at the population level in subjects expressing the respective restricting HLA class I alleles (Table 1), providing direct experimental support for the notion that these population sequence polymorphisms are linked to host $CD8^+$ T-cell responses.

We also addressed the proportion of $CD8^+$ T-cell epitopes that remained invariant despite documented selection pressure in the three subjects monitored for more than 1 year. In addition to the 21, 6, and 13 $CD8^+$ T-cell responses against evolving regions in subjects AC-38, AC-04, and AC-16, another 22, 16, and 16 responses, respectively, that never varied despite the persistence of responses were detected in each subject (see Table S2 in the supplemental material). Thus, a majority of the $CD8^+$ T-cell responses in these subjects (51% to 73%) targeted regions that remained invariant over time. Including those epitopes that were associated with variation and those that remained stable, 9% to 18% of the non-Env residues in the viral genomes of these subjects were exposed to $CD8^+$ T-cell selective pressures, and mutations were >7-fold more likely to occur within $CD8^+$ T-cell-targeted regions of the virus than in nontargeted regions ($P < 0.0001$), indicating that viral evolution was not random but rather was due to specific selection pressures.

HIV evolution following acute infection is also influenced by reversion of transmitted CD8-associated mutations. In addition to mutations arising under $CD8^+$ T-cell selection pressure, other mutations arose in regions with no detectable $CD8^+$ T-cell response. A possible cause of these sequence changes is reversion of transmitted mutations selected by $CD8^+$ T-cell responses in the prior host, which occurs in the absence of the original selection pressure (3, 20, 30). Of the 46 mutations not associated with $CD8^+$ T-cell responses, 18 (39%) represented evolution toward the more common HIV-1 clade B consensus sequence, and most of these represented highly conserved residues in the database, consistent with mutations that are intrinsically unstable in the absence of selection pressure (see Table S1 in the supplemental material). Notably, in Fig. 2A, of the 11 mutations representing reversions in subject AC-38, 8 (72%) were evident at the earliest time point, while only 15/41 (36%) of forward mutations had arisen within a similar time frame. This observation may suggest that reversions preferentially occur early after infection, perhaps representing a driving force for early evolution of HIV. Again, by use of the Epipop program (37), 9 of the 18 mutations were found to be associated with allele-specific polymorphisms at the population level, and in each case the associated allele was not expressed by the respective acutely infected subject (Table 1). Therefore, in addition to the 52 mutations associated with detectable $CD8^+$ T-cell responses in the host, at least 9 and as many as 18 additional mutations are consistent with adaptations due to previous $CD8^+$ T-cell selective pressures. These data suggest that reversion of HLA class I-associated mutations following transmission is a common event. Thus, together with mutations associated with detectable CD8 responses, 62% to 71% of all nonenvelope mutations in these subjects were associated with cellular immune pressures (Fig. 2C).

Evidence of selective escape in CD8 T-cell epitopes. The above data suggest that the majority of mutations arising during HIV-1 infection do not simply reflect random variation but rather arise in response to specific selection pressures. To further address the predictability of mutations, we examined whether precise sites in evolving epitopes are reproducibly selected in the context of specific class I alleles. We focused on HLA-B57, an allele known to be associated with enhanced immune control following acute infection (6, 35) and therefore likely exerting substantial selective pressures (34). Each of the 12 previously defined HLA-B57-restricted CD8 epitopes (13) was targeted in the untreated subject AC-38, and viral evolution was observed in 8 of these (see Table S1 in the supplemental material). We focused on the seven HLA-B57 epitopes residing in Gag and Pol, since these conserved proteins were most likely to reveal clear evolutionary patterns. Viral evolution within these seven Gag and Pol epitopes was examined in 10 additional HLA-B57 subjects exhibiting high levels of viral replication, which are clinically linked to disease progression (33). Four of these epitopes exhibited strikingly similar mutations at seven residues, with six out of seven residues significantly more variable in persons expressing HLA-B57 than in the Los Alamos National Laboratory (LANL) database overall (Table 2; range, $P < 0.05$ to $P < 0.0001$). A fifth epitope, QW9, exhibited only a low frequency of mutations at positions 3 and 5 (P3 and P5), whereas the remaining two epitopes, KF11 and KF9, were rarely observed to exhibit any sequence variation and were similarly highly conserved in the LANL database (Table 2). For some of these CD8 epitopes in Gag, similar patterns of viral evolution have also been observed previously

TABLE 1. HIV-1 mutations

Subject and mutation[a]	Protein	Site	Sequence	Polymorphism	HLA association	OR[b]	P
Mutations associated with HLA alleles expressed by current host							
AC-38							
1	Gag	28	RLRPGGKKK	K→Q	A*0301	8.44	<0.0001
2	Gag	79	GSEELRSLY	Y→F	A*0101	2.71	0.0048
3	Gag	147	ISPRTLNAW	I→L	C*0602	6.41	0.0001
5[c]	Gag	310	YKTLRAEQATQEVKNWMTE	T→S	B*0702	10.16	0.0034
37	Rev	30	LLYQSDPPPSPEGTRQARR	S→N	C*0702	2.63	0.0182
48	Nef	114	RQDILDLWVY	V→I	C*0701	5.88	0.0001
49	Nef	116	HTQGYFPDWQ	H→N	B*5701	5.30	<0.0001
AC-04							
2	Gag	357	ACQGVGGPGHK	G→S	A*1101	2.99	0.0153
9	Tat	32	CCFHCQVC	F→X	C*1203	23.15	0.0043
					A*0207	8.92	0.0486
AC-16							
10	Pol	726	QEEHEKYHSNW	E→A	B*4402	17.76	<0.0001
10	Pol	726	QEEHEKYHSNW	E→A	B*4403	5.44	<0.0018
13a	Vif	183	WNKPQKTKGYRGSHTMSGH	Y→H	B*4402	2.09	<0.0001
Transmitted HLA-associated mutations reverting to consensus							
AC-38							
5	Gag	310	YKTLRAEQATQEVKNWMTE	T→S	A*3001	0	<0.0001
					B*4201	0	<0.0001
					B*1503	0.07	0.0493
6	Gag	403	NCGKEGHIAKNCRAPRKKG	K→R	A*3101	0.04	0.0028
					A*3303	0.05	0.0066
					B*1801	0.28	0.0317
					B*5301	0.08	0.0328
29b	Tat	67	APQDsQTHQAsiSKQPTSQ	A→V	C*1502	0	<0.0001
32	Vpu	44	RQRKIDRLIERIRERAEDS	E→D	C*0102	0.25	0.0276
33	Vpu	65	ESDGDQEEL--LVEMGHLA	_→S	C*0202	0.21	0.0413
51	Nef	152	FKLVPVDPDEVEEANEGEN	E→K	C*0501	0.18	0.0213
AC-59							
2	Vpr	48	LHGLGQQIYGTYGDTWEGV	G→E	B*4601	0.05	0.0008
					A*3001	0.04	0.0102
					B*4901	0.13	0.0107
					B*1503	0.13	0.0311
AC-16							
14	Tat	71	SQTHQVPLPEQPNSQPRGD	E→K	A*0101	0.19	0.0230
					C*0304	0.21	0.0379
21	Nef	71	EDEEVGFPVKPQVPLRPMT	K→R	C*0702	0.07	0.0000
					B*4002	0.13	0.0491

[a] Mutation numbers correspond to Fig. 2A and to Fig. S1 and Table S1 in the supplemental material.
[b] OR, odds ratio.
[c] Associations observed for both forward and reverse mutations.

(30, 34). These data provide support for the concept of predictable viral evolutionary dynamics in persons sharing the same HLA allele, and they reveal that viral evolution within CD8 epitopes may be stereotypic in nature.

The reproducibility of mutations in the HLA-B57 epitopes suggested that a relationship may exist between the mutations induced by immune pressures and highly polymorphic residues of HIV-1 at the population level. We therefore determined the degree to which mutating and nonmutating residues within each CD8 epitope were associated with sequence polymorphisms at the population level. Using sequences reported in the LANL HIV Sequence Database (www.hiv.lanl.gov), normalized Shannon entropy scores (50) were determined for each residue of HIV-1 clade B, providing an overall assessment of the sequence diversity present at each residue in circulating HIV-1 clade B strains (58). Figure 3A illustrates the relative conservation (1/entropy) of each residue within the seven HLA-B57-restricted CD8 epitopes studied and the position where mutations reproducibly arose. This analysis showed not only that the residues commonly mutating in a subject reflected the most polymorphic residues in each epitope at the population level but also that the majority of nonmutating residues within these CD8 epitopes remained highly conserved at the population level.

The analysis was then broadened to examine the additional CD8 epitopes defined in the four longitudinally followed subjects as well as published epitopes with documented CD8 escape mutations (3, 17, 23, 25, 26, 28, 30, 34), again focusing on responses to the more conserved structural proteins Gag and Pol. Here, 18 of the 28 mutations (64%) in these epitopes were found to lie within the most variable residue in the epitope (Fig. 3). Therefore, within the epitopes demonstrating escape,

TABLE 2. Selective CD8 escape mutations observed in chronic Gag and Pol HLA-B57 epitopes

Subject or group	Sequence for the following epitope:						
	Gag B57-TW10[a]	Gag B57-IW9[b]	Pol B57-SW10[c]	Pol B57-IW9[d]	Gag B57-QW9[e]	Gag B57-KF11[e]	Pol B57-KF9[e]
Consensus	TSTLQEQYGW	AISPRTLNAW[f]	STTVKAACWW	IVLPEKDSW	QASQEVKNW	KAFSPEVIPMF	KTAVQMAVF
AC-38	--N-----A-	-L--------	-N--------	-M-------	---------	-----------	---------
PRLS22	--N-----T-	-L--------	----------	-E-------	---------	-----------	---------
PRLS12	--N-----A-	-L--------	-NAL------	-E-------	---------	-----------	---------
MS	--N-----A-	P---------	-N--------	---------	--T------	-----------	---------
CN 641 114	--N-----A-	PL--------	-NA-------	----D----	--T------	-----------	---------
F719	--N-------	PL--------	-N--------	---------	----D----	-N---------	R--------
EB 570 825	--N-----A-	PL--------	-AA-------	-M----E--	---------	------I----	---------
RRM	--N-----A-	----------	-N--------	-M--N----	--T------	-----------	---------
7780	--N-------	P---------	-N-------	-	---------	-----------	---------
696	----------	P---------	-N--------	-E-------	---------	-----------	---------
AC-57	--N-----A-	----------	-NV-------	-K-------	-	-----------	--------L
LANL clade B[g]	**---------- (52/61)**	**---------- (35/59)**	**---------- (25/39)**	**--------- (31/40)**	**--------- (35/59)**	**----------- (57/62)**	**--------- (38/41)**
	--------N- (5/61)	P--------- (11/59)	-A-------- (5/39)	-M------- (5/40)	----D---- (16/59)	-----K----- (2/62)	----H---- (1/41)
	--------A- (3/61)	-L-------- (8/59)	--A------- (3/39)	-T------- (2/40)	--T------ (4/59)	------I---- (1/62)	-R------- (1/41)
	--N-----A- (1/61)	P--------R (1/59)	GA--R----- (2/39)	----H---- (1/40)	-CT-D---- (2/59)	------M---- (1/62)	--------L (1/41)
	--------R- (1/61)	P--A--F--- (1/59)	-N-------- (1/39)	-K----E-- (1/40)	--------R (1/59)	------G---- (1/62)	
	------H--- (1/61)	------F--- (1/59)	-NA------- (1/39)		--T-D---- (1/59)		
		---A------ (1/59)	-NS------- (1/39)				
		S--------- (1/59)	-AA------- (1/39)				

[a] For T242N and G248A, $P < 0.0001$.
[b] For A146P, $P < 0.05$; for I147L, $P < 0.01$.
[c] For T840N, $P < 0.0005$; T841A is not statistically significant.
[d] For V401K, $P < 0.005$.
[e] Mutations at this epitope are not statistically significant.
[f] A CD8 escape processing mutation (A→P) immediately 5′ of B57-IW9 (underlined) has been described previously (17).
[g] The predominant form of each epitope in the LANL HIV Sequence Database is boldfaced. The number of viruses with a particular sequence/total viruses is given in parentheses.

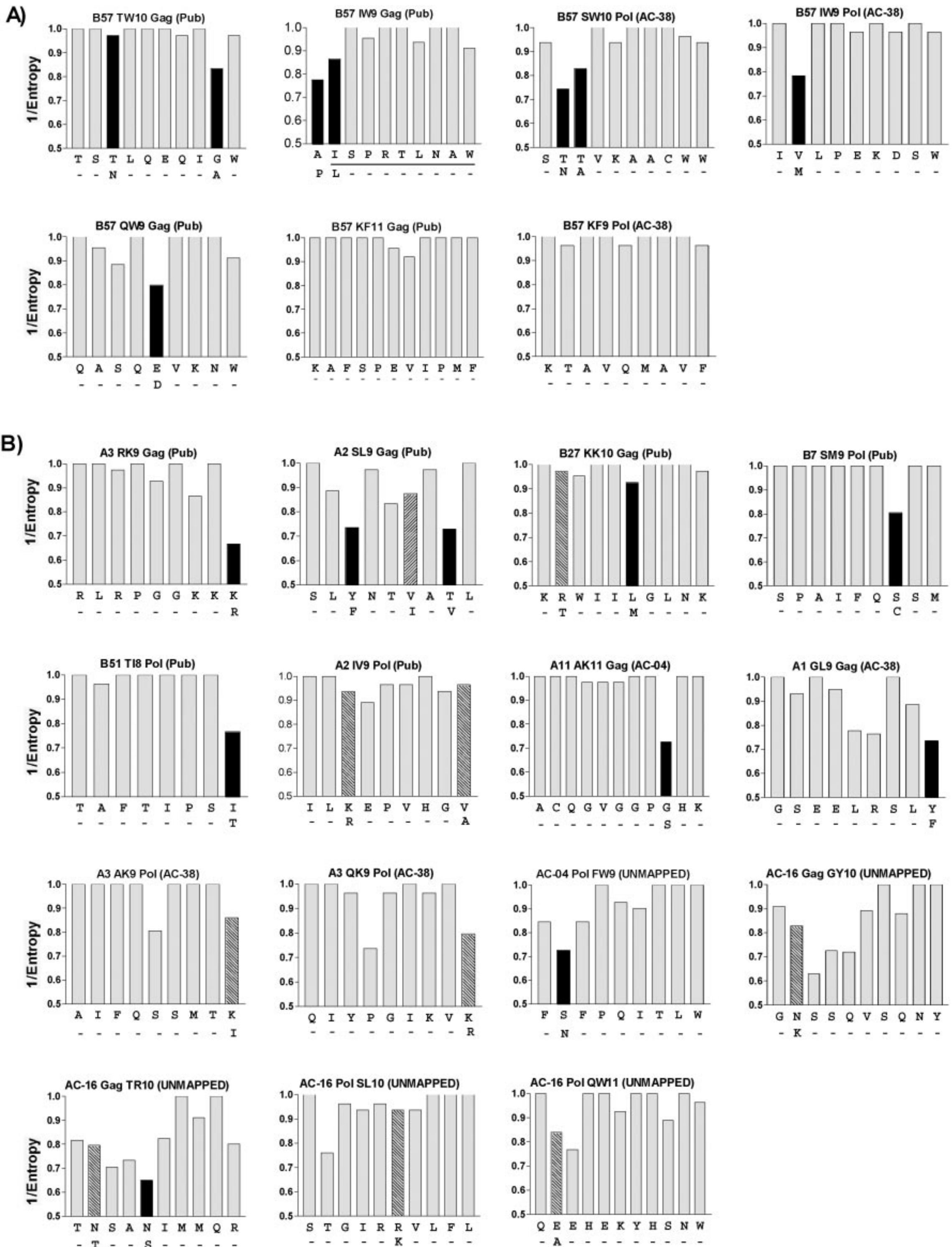

FIG. 3. Escape mutations in Gag and Pol occur at highly variable residues in the population. The degrees of variability of Gag and Pol residues were assessed by plotting normalized Shannon entropy scores. The residue observed to arise during sequence evolution in a subject is shown below each epitope. Solid bars, escape mutations corresponding to the most polymorphic residue in each epitope; hatched bars, escaping residues not corresponding to the most polymorphic residue in each epitope. CD8-targeted regions include (A) B57 epitopes and (B) published epitopes (Pub), epitopes in study subjects (with subject designation given above the bar graph, e.g., AC-38), and partially mapped epitopes in study subjects (UNMAPPED).

the most variable residue was significantly more likely to undergo an amino acid change in a subject than all other residues ($P < 0.00005$). One exception was the A3-AK9 Pol epitope (AIFQSSMTK) (Fig. 3B), in which viral escape was observed at P9 rather than at the most polymorphic residue (P5). Notably, a second epitope, B7-SM9 (SPAIFQSSM) (Fig. 3B), with an escape mutation at the same residue, overlaps A3-AK9 and may account for this increased variability at P5 in the A3-AK9 epitope. Therefore, within these highly conserved proteins of HIV-1, the same residues are repeatedly selected for sequence variation, both in an individual and at the population level. These data suggest that selection against deleterious mutations (purifying selection) (38, 51) may be functioning to conserve important residues in clade B while allowing sequence variation only at precise residues to facilitate evasion of host immune pressures.

As a result of these strong relationships, we conducted a similar analysis of the remaining more variable accessory and regulatory proteins of HIV-1. By combining previously described CD8 escape mutations in Tat and Nef (14, 45), as well as evolving targeted CD8 epitopes in our study subjects, a total of 23 epitopes were examined. In this analysis, however, only 8/26 mutations correlated with the most variable residue in each epitope (see Fig. S2 in the supplemental material). The inability to draw similar correlations between host CD8 escape mutations and sequence diversity in these proteins is likely due to the greater overall sequence variability within these proteins. This increased variability may reflect either a more intense targeting of these regions of HIV-1 by host $CD8^+$ T-cell responses (1, 5, 7, 31, 58) or a reduced requirement to maintain such sequence conservation in these nonstructural proteins (20).

In order to determine whether the inherent variability of a region of HIV-1 containing a CD8 epitope was predictive of epitopes undergoing sequence variation in a subject, we compared the average entropy scores of mutating epitopes with those of epitopes refractory to sequence changes, again using sequences from the LANL database. A comprehensive analysis of the 57 evolving and nonevolving minimally defined CD8 epitopes in this study, including those from the regulatory and accessory genes, demonstrated that epitopes exhibiting evolution in a subject were significantly more variable in the population than epitopes that remained unchanged in the subjects ($P < 0.0005$). Evolving epitopes exhibited average entropy scores of 90.5% per residue, versus 94.8% per residue for nonevolving epitopes. However, this finding may have been influenced by a trend for mutating epitopes to reside within the more variable nonstructural proteins while nonmutating epitopes reside within the more conserved structural proteins ($P = 0.0531$). Indeed, those proteins that accumulated the most mutations in these subjects, after adjustment for protein length (Rev > Tat > Vpr > Vpu > Nef > others), closely reflect the relative variability of each of the HIV-1 proteins (Vpu > Tat > Rev > Nef > others) (58). Therefore, the inherent variability of a targeted CD8 epitope, or protein, appears to influence whether sequence variation is likely to occur in response to $CD8^+$ T-cell selective pressures.

Polymorphic residues are shared across different HIV-1 clades. The above data suggest that within HIV-1 there are restrictions influencing which residues within an immune-targeted region are able to mutate. If purifying selective pressures are indeed playing a role in dictating these restrictions, then similar patterns of conserved and variable residues would be expected to exist within other clades of HIV-1. Seventeen of the 19 mutations in the clade B CD8 epitopes in Gag and Pol which correlated with the most polymorphic residue at the population level (Fig. 3) also reflected the most polymorphic residue in clade C (Fig. 4). In fact, for the majority of residues in these epitopes, very similar patterns of conserved and variable residues were observed between clades B and C, even at highly polymorphic residues (Fig. 4).

An analysis of $CD8^+$ T-cell-associated mutations in the accessory and regulatory proteins again did not exhibit any strong associations, although similar patterns of conserved and variable residues were still evident. Therefore, we broadened the analysis to all 2,299 amino acid residues of HIV-1 (excluding Env). In total, 84% of residues exhibited similar entropy scores (difference within 10%) between clades B and C, indicating similar conserved and variable residues between these clades. This was despite the fact that as many as one-third of all residues exhibited some degree of variability (>10%) in one clade or the other (data not shown). These data suggest that purifying selective pressures are at work to maintain the overall structure and function of HIV-1 proteins across multiple clades, while certain residues preferentially support sequence variation in the presence of host immune selective pressures.

DISCUSSION

These data assessing the impact of $CD8^+$ T-cell selective pressures on HIV-1 evolution suggest that nearly two-thirds of all mutations arising in nonenvelope proteins during the transition from acute to chronic infection can be attributed to either $CD8^+$ T-cell-associated selective pressures in the infected individual or reversion of HLA class I-associated mutations selected in a previous host. These data are in surprisingly close agreement with data recently derived from the SIV-infected rhesus macaque model (40), in which >60% of sequence variation outside of the envelope occurs within recognized CD8 T-cell epitopes. Similarly, a recent study by Jones et al. analyzing responses to Gag, Env, and Tat in four HIV-infected subjects observed similar extensive escape from CD8 T-cell responses very soon after infection (27). Viral evolution following acute HIV-1 infection is thus not a random process but rather is substantially influenced by adaptive $CD8^+$ T-cell selection pressures. The preferential selection of individual residues for mutation and the repeated selection for identical mutations suggest that there are significant biochemical constraints on viral evolution and explain the ability to repeatedly link certain sequence polymorphisms in HIV-1 with various HLA alleles (37). Moreover, these data, showing selective mutation of specific residues within epitopes in concordance with highly variable sites among different clades, provide support for the idea that immune selection pressures contribute to global HIV-1 sequence diversity.

These studies also indicate that while a substantial proportion of the HIV proteome is under immune selection pressure, with as many as 20% of all residues being targeted in a given individual, a majority of $CD8^+$ T-cell responses do not result in sequence evolution. Since the nonmutating residues are more

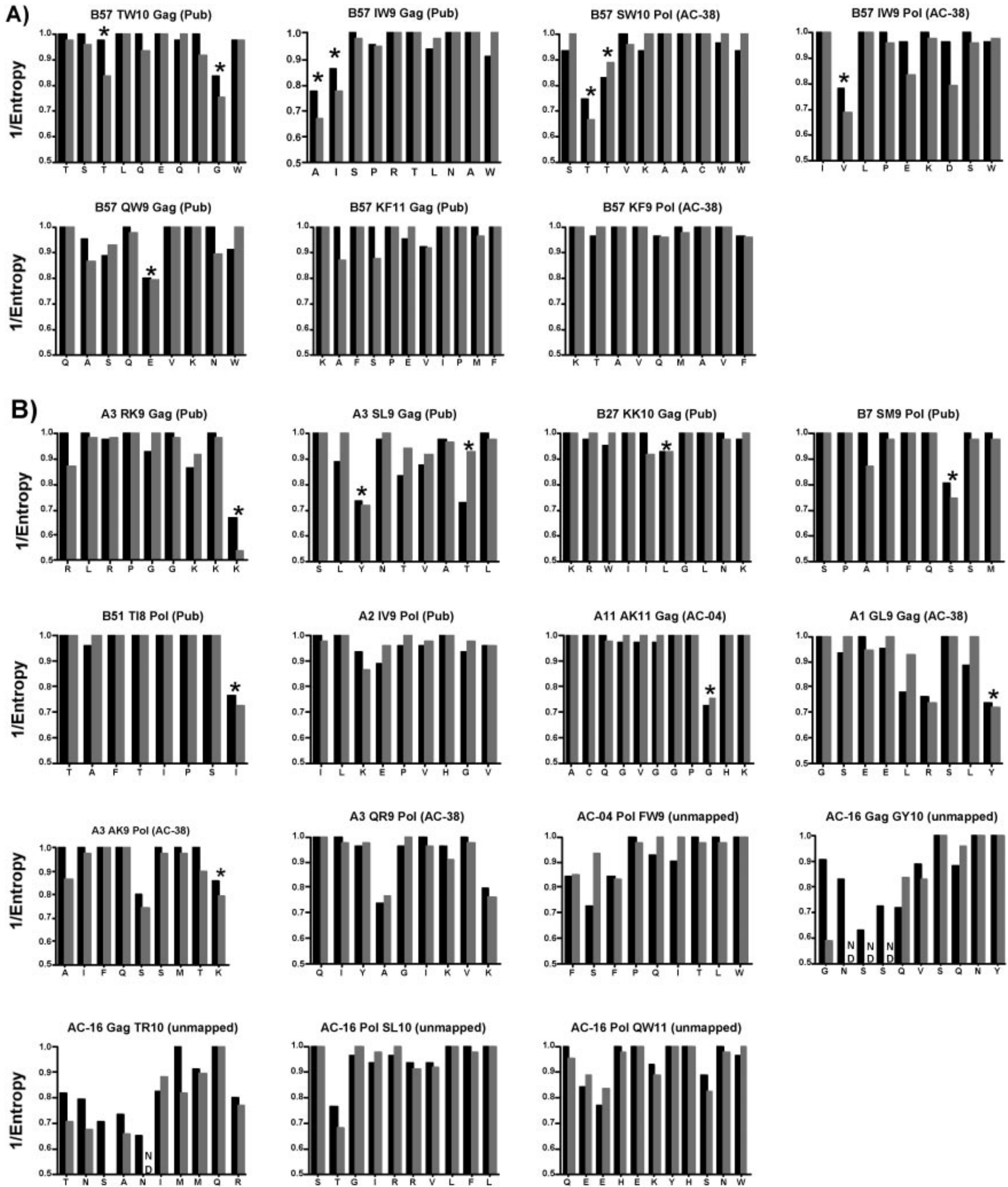

FIG. 4. Shared polymorphic residues in Gag and Pol in clades B and C. The degrees of variability of clade C sequences are plotted against clade B Gag and Pol epitopes shown in Fig. 3. Black bars, clade B sequences; gray bars, clade C sequences. Asterisks indicate highly polymorphic residues shown to mutate in Fig. 3 which are shared across clade B and clade C. (A) B57 epitopes; (B) published epitopes (Pub), optimal epitopes in study subjects (with subject designation given above the bar graph, e.g., AC-38), and partially mapped epitopes in study subjects (unmapped).

highly conserved at a population level, these data indicate substantial constraints on HIV evolution. Indeed, comparison of the entropy values of clade B and clade C sequences indicated very similar patterns of conserved and variable residues, suggesting that viral escape in different HIV-1 clades may occur at similar residues despite unique distributions of HLA alleles present in infected populations. This implies that the same purifying selection pressures contribute to maintaining specific residues and preserving the basic structure of HIV-1 proteins in different HIV-1 clades, while routinely allowing other residues to fluctuate. Indeed, the same residues observed to mutate in CD8 epitopes in clades B and C reflect those consistently capable of supporting alternative amino acids in many other HIV-1 clades, including clades O and CPZ (data not shown), whereas conserved residues are maintained across different HIV-1 clades. Similar findings have recently been reported in the analysis of HIV-1 Env sequences (15, 22, 54).

Although the majority of viral mutations in these subjects

were associated with adaptive immunity to HIV-1, a substantial number of mutations remain to be explained. The methods used to detect $CD8^+$ T-cell responses relied on measuring IFN-γ production and did not include in vitro expansion or alternative measurements of $CD8^+$ T-cell immune function such as cytolytic capacity, production of other cytokines, or HLA class I tetramers (29, 49). It is noteworthy that four of the mutations not associated with $CD8^+$ T-cell responses are within regions exhibiting $CD4^+$ T-cell proliferative responses (data not shown), and viral escape from CD4 responses has been described in murine models of viral infection (16). Finally, compensatory mutations accommodating CD8 escape mutations (21, 28, 30, 42) and antigen processing mutations (3, 17) are likely to account for additional sequence polymorphisms. Although our findings are in close agreement with data recently derived from the SIV-infected rhesus macaque model (40), they contrast with those of Yang et al., who previously reported a strong association between sites undergoing positive selection in HIV and CD4 T-helper epitopes, but not CD8 T-cell epitopes (56). This discrepancy is likely due to different approaches used in these studies to address this issue.

Together these data indicate predictability within individual subjects and across multiple clades in the evolution of HIV-1, and linking of CD8 escape mutations in the host with highly variable residues in the HIV-1 population as a whole supports host immune pressures as playing a major role in driving the global diversity of HIV-1. The striking reproducibility of many escape mutations, their predominance at highly polymorphic sites across different HIV-1 clades, and the observed reversion of HLA-associated polymorphisms together suggest that structural or biochemical constraints limit which residues within a targeted HIV-1 CD8 epitope are likely to mutate in response to $CD8^+$ T-cell selective pressures. These results are consistent with the hypothesis that some mutations induced by immune-mediated selective pressures have an impact on viral replication capacity (3, 20, 21, 27, 30, 43), especially mutations developing within more conserved regions of the virus. Targeting of highly conserved regions and identification of ways to exploit the limitations of the sequence evolution of HIV-1 may hold promise for the rational design of vaccines against such highly polymorphic chronic viral pathogens.

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