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Clade-Specific Evolution Mediated by HLA-B*57/5801 in Human Immunodeficiency Virus Type 1 Clade A1 p24[▽]

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HLA-B*57-mediated selection pressure leads to a typical escape pathway in human immunodeficiency virus type 1 (HIV-1) CD8 epitopes such as TW10. Whether this T242N pathway is shared by all clades remains unknown. We therefore assessed the nature of HLA-B*57 selection in a large, observational Kenyan cohort where clades A1 and D predominate. While T242N was ubiquitous in clade D HLA-B*57$^+$ subjects, this mutation was rare (15%) in clade A1. Instead, P243T and I247L were selected by clade A1-infected HLA-B*57 subjects but not by HLA-B*5801$^+$ subjects. Our data suggest that clade A1 consensus proline at Gag residue 243 might represent an inherent block to T242N escape in clade A1. We confirmed immunologically that P243T and I247L likely represent escape mutations. HLA-B*57 evolution also differed between clades in the KF11 and IW9 epitopes. A better understanding of clade-specific evolution is important for the development of HIV vaccines in regions with multiple clades.

Human immunodeficiency virus type 1 (HIV-1) displays extreme genetic diversity, with nine clades (subtypes) described in group M, and frequent genomic recombination among and within the clades (7, 44). HIV is also capable of rapid evolution, which can lead to mutational escape from immune control (43). Escape from CD8$^+$ T-cell responses occurs frequently in HIV-1 infection through mutations that affect epitope processing, HLA class I binding, and/or T-cell receptor recognition (23). In early HIV-1 infection, the majority of amino acid substitutions are associated HLA class I alleles (1). The timing and consequences of mutational escape from CD8$^+$ T-cell responses vary considerably (8, 22).

HLA-B*57, and to a lesser extent HLA-B*5801, has been associated with slower progression to AIDS in several studies (18, 27, 39), and HLA-B*5701 was associated with a lower viremia set point in a genome-wide association study (16). Several attributes of HLA-B*57-restricted CD8$^+$ T-cell responses may contribute to their protectiveness, including dominant responses in acute infection (2), recognition of protective epitopes in HIV-1 p24 (33), better recognition of epitope variation (45), and retention of proliferative capability in chronic infection (24).

HLA-B*57/5801 also exert powerful selection pressure on HIV to avoid CD8$^+$ T-cell recognition. This was first demonstrated in the HLA-B*57-restricted TW10 epitope (TSTLQE QIGW [$Gag_{240-249}$]), which accounts for >30% of overall HIV-specific CD8$^+$ T-cell responses in acutely infected HLA-B*57$^+$ subjects (3). Escape in this epitope usually occurs early in infection, which coincidently is when HLA-B*57 is most protective (18). In clade B and C infections, >75 to 100% of HLA-B*57/5801$^+$ subjects develop the T242N escape mutation, while HLA-B*57/5701-negative subjects rarely display polymorphism at this residue (5, 9, 10, 15, 32, 35, 38, 41). When T242N is transmitted to HLA-B*57/5801-negative subjects, it rapidly reverts to the consensus, suggesting that T242N is associated with a fitness defect (32, 35).

While CD8$^+$ T-cell cross-clade recognition has been tested extensively (6, 11, 19, 36, 48), few studies have addressed the possibility of clade-specific escape from CD8$^+$ T-cell responses. This may be especially relevant where clade consensus sequences differ in immunologically relevant epitopes. Here we demonstrate in a large Kenyan cohort substantial differences in HLA-B*57/B*5801-mediated selection among HIV clades.

Participants were enrolled from a Nairobi, Kenya-based cohort, and the relevant ethical review boards approved the study. HLA typing was performed as described previously (34). CD4 counts were measured longitudinally at biannual visits. Multiple and other clade infections were excluded. The HIV-1 p24 gene was amplified from proviral HIV DNA or RNA using a nested PCR approach and sequenced, and viral subtyping was carried out as described previously (42). Previously described HLA-B*57 epitopes IW9 (ISPRTLNAW), KF11 (K AFSPEVIPMF), and TW10 (TSTLQEQIGW) and selected variants were tested in immunological assays and described where relevant. Gamma interferon enzyme-linked immunospot (ELISPOT) assays were performed as described previously (37) using blood samples from HLA-B*57$^+$ and -B*5801$^+$

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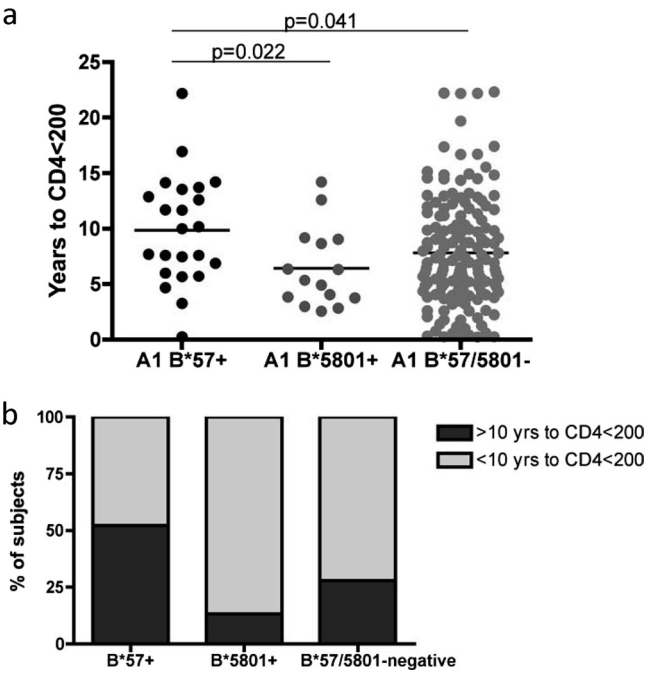

FIG. 1. HLA-B*57, but not HLA-B*5801, is associated with a lower rate of disease progression in clade A1-infected subjects than that of the overall cohort. (a) Number of years from cohort entry until sequential CD4 counts fell below 200/μl. (b) Slow progressors (>10 years with CD4 counts of >200) were also more common in HLA-B*57$^+$ clade A1-infected subjects than in those expressing HLA-B*5801 or neither.

subjects. All peptides were tested at concentrations of 10 μg, 1 μg, 0.1 μg, and 0.01 μg/ml. Responses were considered positive if they were more than two times higher than that of the negative control and were measured at ≥100 spot-forming units ml^{-1}. Fisher's exact test and chi-square analyses were used to determine differences among groups in categorical analyses. Mann-Whitney U tests were used to compare response magnitudes and disease progression between groups.

We confirmed the protective effects of HLA-B*57 in clade A1 infection (mean of 9.9 years versus 7.8 years until CD4 counts were <200, $P = 0.041$) (Fig. 1). Slow progressors were overrepresented in HLA-B*57$^+$ clade A1$^+$ subjects (52.2%) compared to both HLA-B*5801$^+$ clade A1$^+$ (13.3%, $P = 0.02$) and HLA-B*57/5801-negative clade A1$^+$ (27.8%, $P = 0.028$) subjects (Fig. 1b). In contrast to what has been shown for other clades (2, 27), protection was not observed for clade A1-infected HLA-B*5801$^+$ subjects (mean of 6.5 years versus 7.8 years until CD4 counts were <200, $P > 0.3$) (Fig. 1).

Stratification of TW10 ($Gag_{240-249}$) proviral sequences on the basis of HLA allele and clade revealed several differences in selection between clades A1 and D (Fig. 2a). We observed the expected T242N substitution in 100% of HLA-B*57$^+$ clade D-infected subjects (7/7), compared to only 14.7% variability at Gag residue 242 in HLA-B*57/5801-negative subjects (13/88, $P = 3.26 \times 10^{-9}$) (Fig. 2b). In contrast, T242N was found infrequently in clade A1-infected HLA-B*57$^+$ subjects (15%, 5/33, $P = 0.0004$). Instead, variants containing the mutations P243T and I247L were more frequently observed (both observed in 11/33 subjects). Overall, variation at residues 243 and 247 was more common in HLA-B*57$^+$ subjects (51% and 15%, respectively; $P = 2.92 \times 10^{-6}$) than in HLA-B*57/5801-negative clade A1$^+$ subjects (33% and 9%, respectively; $P = 0.0008$). Selection at both residues 243 and 247 was observed only in 2/33 HLA-B*57$^+$ subjects, suggesting that these mutations are independent. Selection at residue 248, observed in clade B infection (32), was not evident in either clade A1 or D. While I247X selection has been described in other clades at low frequencies and in elite controllers (21, 40), HLA-B*57-mediated selection at Gag residue 243 has not yet been described. In summary, the T242N mutation, which is typical of other clades, does not appear to be the primary escape mutant in clade A1.

Previous studies have suggested that HLA-B*5801 places selection pressure on TW10, similar to that of HLA-B*57 (35). Similar to clades B and C, selection of T242N was evident in HLA-B*5801$^+$ clade D-infected subjects (TW10 variation in 8/11 HLA-B*5801$^+$ subjects versus 13/88 HLA-B*57/5801-negative subjects; $P = 0.0069$) (Fig. 2c). Limited T242N selection was observed in clade A1-infected HLA-B*5801$^+$ subjects, and in contrast to HLA-B*57, there were no HLA-B*5801-associated substitutions at residues 243 and 247 in clade A1 (P values of 0.75 and 0.29, respectively) (Fig. 2c). In summary, these data suggest that in addition to HLA-B*5801 not being associated with protection in clade A1 (Fig. 1), HLA-B*5801 does not select the HLA-B*57-associated clade A1 TW10 escape mutations.

Inclusion of all clade A1 sequences with T242X substitutions (regardless of the HLA allele) reveals that in every case (10/10), there is an accompanying residue 243 mutation. Polymorphisms at these sites correlate very strongly ($P = 3.71 \times 10^{-8}$) (Fig. 2d). Together, these data suggest that residue 242 polymorphism in clade A1 is incompatible with proline at residue 243, which is the clade A1 consensus.

We next assessed the immunological implications of novel clade A1 variants in HLA-B*57$^+$ ($n = 12$) and -B*5801$^+$ ($n = 6$) subjects infected primarily by clade A1. Clade A1-infected subjects commonly made anamnestic, low-avidity responses to TW10. The majority of HLA-B*57$^+$ subjects who recognized clade A1 TW10 did not respond to P243T or I247L in ELISPOT assays (Fig. 3), supporting the hypothesis that these represent escape mutations. Those who did recognize P243T and I247L had lower magnitude responses than those who recognized clade A1 TW10 at the 10-μg/ml peptide concentration (P of 0.0005 for both) (Fig. 3a). Similarly, these variants were not well recognized by CD8$^+$ T cells from HLA-B*5801$^+$ subjects (Fig. 3b). For two HLA-B*57$^+$ subjects, P243T and I247L responses had lower avidity than clade A1 TW10 responses (Fig. 3c). These data support the hypothesis that P243T and I247L likely represent escape mutations.

Recognition of the clade B/D consensus (TSTLQEQIGW) was diminished compared to that of clade A1 TW10. However, despite the presumed absence of this variant in these subjects' autologous sequences, the clade B/D escape variant (TSNLQEQIGW [T242N]) was recognized at a magnitude similar to that of the consensus clade A1 TW10 ($r = 0.71$, $P = 0.0099$) (Fig. 3d). No responses to T242N/G248A were observed (not shown), as described previously (32). These data suggest that clade A1 and B TW10, and their escape variants, are immunologically distinct from one another.

a

TSTLQEQIGW (TW10)	Clade A1 (n=283)	Clade D (n=106)
HLA-B*57	---P------ (9)	--N------- (3)
	---T------ (7)	--N-----A- (1)
	---P---LQ- (3)	--N-----R- (1)
	---S---LQ- (3)	--N----VA- (1)
	---P----A- (3)	--N----V-- (1)
	---P---L-- (2)	n=7
	--NI------ (1)	
	--N------- (1)	
	--NT------ (1)	
	--NT---LA- (1)	
	--NV------ (1)	
	---P---LA- (1)	
	---ts---l-- (1)	
	---T---L-- (1)	
	n=33	
HLA-B*5801	---P------ (13)	--N-----A- (3)
	---P---LA- (2)	--N-A---A- (2)
	---P---L-- (2)	--N------- (2)
	--NI------ (1)	--N-----Q- (1)
	--N------- (1)	--------A- (1)
	--nti------ (1)	---------- (1)
	---I------ (1)	---T----A- (1)
	---P----A- (1)	n=11
	---P----r- (1)	
	---P----T- (1)	
	n=24	
Alleles other than HLA-B*57/5801	---P------ (148)	---------- (32)
	---P----A- (13)	--------A- (29)
	---P---LQ- (9)	--S------- (10)
	---I------ (8)	--------T- (6)
	---T------ (8)	--N-----A- (2)
	---P----R- (5)	--------ta- (1)
	---P---V-- (3)	--------T- (1)
	---------- (3)	-------VT- (1)
	---A------ (3)	--S-----R- (1)
	---P---L-- (3)	----E---A- (1)
	---P---LA- (2)	--------D- (1)
	---S----A- (2)	--------S- (1)
	---T----A- (2)	-------VA- (1)
	---I----A- (2)	-------V-- (1)
	Additional 15 variants found in 1 subject only n=226	n=88

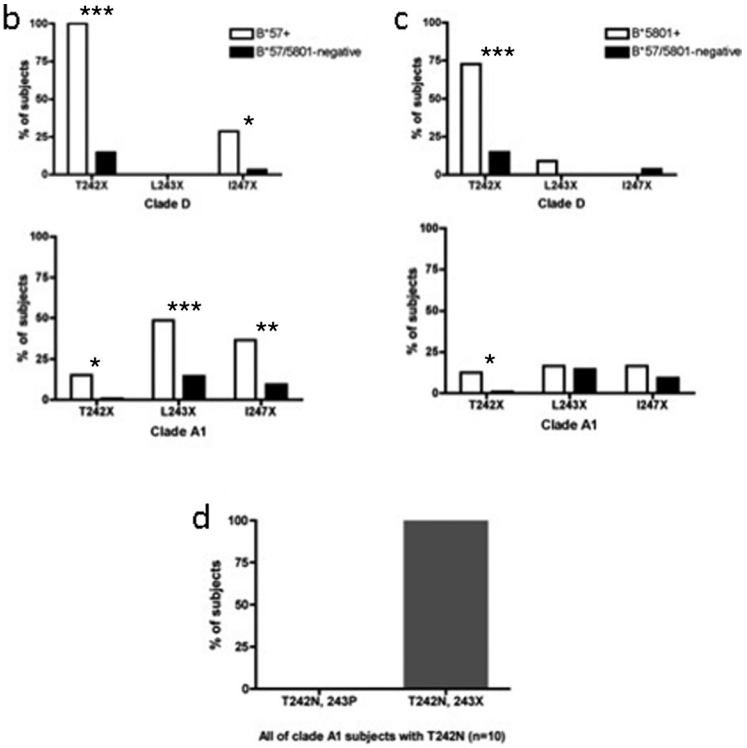

FIG. 2. HLA-B*57-mediated selection in TW10 differs between clade A1 and clade D. (a) TW10 sequences were stratified by HLA-B*57, HLA-B*5801, or other alleles (HLA-B*57/5801$^-$) and compared between clades A1 and D, based on the clade B consensus TW10 sequence. Each subject is represented by one sequence, and the numbers of subjects with a given sequence are shown in parentheses. A summary of variation from the TW10 consensus at Gag residues 242, 243, 247, and 248 is shown for HLA-B*57$^+$ (b) and -B*5801$^+$ (c) subjects. (b and c) Clade D is shown at the top, and clade A1 is shown at the bottom. Variation is shown in dark gray, and consensus is shown in light gray. (d) The proportions of clade A1-infected subjects with selection at Gag residue 242 only, and those with selection at residues 242 and 243 in combination, are shown. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

We next assessed whether clade-specific selection was evident in other immunodominant HLA-B*57 p24 epitopes that are commonly targeted in chronic clade B infection (2). In clade D IW9 (ISPRTLNAW [$Gag_{147-155}$]), variants containing the escape variant I147L (14) were more common in HLA-B*57$^+$ subjects than in HLA-B*57/5801-negative subjects (86% and 30%, respectively; $P = 0.0055$) (Table 1). However, this variant was not selected in clade A1 (variation in 30% versus 21% subjects; P value was not significant), where leucine is the consensus. Interestingly, ELISPOT data indicated substantial cross-reactivity between 147L and 147I in clade A1-infected subjects (10 μg/ml, $r = 0.987$, $P < 0.0001$) (data not shown), suggesting that infection with an escape variant from one clade (clade D) does not necessarily preclude recognition of this epitope in another one (clade A1). Other amino acids (F, M, and P) were common in HLA-B*57$^+$ subjects at residue 147 (>30% versus 3% in HLA-B*57/5801-negative subjects, $P = 3.85 \times 10^{-6}$). Although the immunological consequences are unknown, these HLA-associated substitutions could represent novel escape variants.

In addition, a substitution at Gag residue 146 (A146P) represents an IW9 processing escape mutation in clades B and C (14), and this mutation was also selected by HLA-B*57 in both clades A1 and D (Table 1). In clade A1, substitutions at Gag residue 146 (primarily P and T) were more frequent in HLA-B*57$^+$ subjects than in HLA-B*57/5801-negative subjects (13/33 and 10/221, respectively; $P = 1.42 \times 10^{-7}$) (Table 1). Therefore, although the consensus at residue 146 differs among clades, here escape at residue 146 occurs in HLA-B*57$^+$ subjects infected by clades A1, B, C, and D.

For KF11 (KAFSPEVIPMF [$Gag_{162-172}$]), HLA-B*57-associated variation from the consensus was more common in clade A1 (67% versus 21%, $P = 2.44 \times 10^{-7}$) than in clade D (43% versus 17%, $P = 0.012$). Previously described A163G and A163G/S165N variants (13, 20) were most common in clade A1 (Table 1). In addition, the novel K162X substitution was present in clade A1. HLA-B*5703 and -B*5701 have previously been shown to display differences in KF11 selection (20, 47), and our data indicate that HLA-B*5702 also differs from HLA-B*5703 in terms of KF11 selection. While the KF11 consensus is present in the majority of HLA-B*5702$^+$ subjects, it is rare in HLA-B*5703$^+$ clade A1 infection (8/9 versus 2/22, $P = 4.74 \times 10^{-5}$).

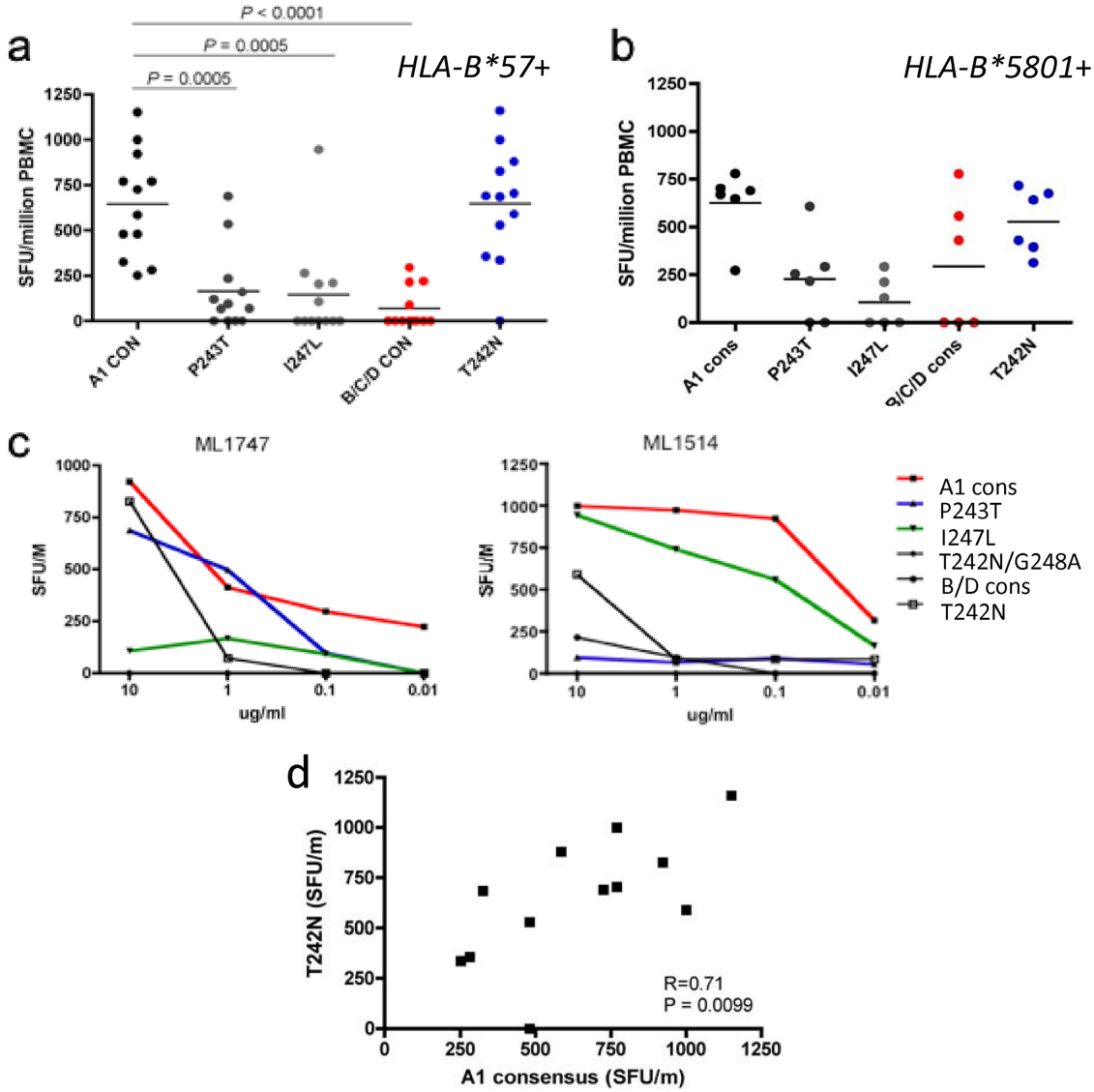

FIG. 3. Peptides with novel TW10 clade A1-selected mutations are poorly recognized in ex vivo gamma interferon ELISPOT avidity assays, suggestive of escape mutations. ELISPOT responses to TW10 and variants at 10 μg/ml peptide by HLA-B*57$^+$ (a) and HLA-B*5801$^+$ (b) subjects are shown. (c) The functional avidity of TW10 and variants for two HLA-B*57$^+$ subjects is shown, suggesting that P243T and I247L are less recognized than TW10, particularly at lower peptide concentrations. Sequence names are described in the text. (d) Elispot responses to A1 TW10 and T242N correlated at 10 μg/ml. SFU/m, spot-forming units/million PBMCs.

Mounting evidence suggests that HLA alleles are a major force in viral evolution (26). We show that in clade A1 p24, HLA-B*57 selection in three epitopes differs from earlier clade B and C data in several important aspects, while clade D selection resembles what has previously been shown. This included a low frequency of T242N in clade A1 TW10, with selection being more common at Gag residues 243 and 247, more extensive KF11 escape, and selection of different amino acids in IW9. Overall, selection was evident in the majority of HLA-B*57$^+$ subjects (>90% of clade A1-infected subjects had selection in more than one epitope, and >75% of them had selection in more than two) (Table 1). Parallel escape in multiple epitopes demonstrates the need to avoid the pressure of CD8$^+$ T-cell responses.

One possible mechanism underlying the differences in TW10 selection is that TSNPQEQIGW (never observed) (Fig. 2d) is not feasible virologically, such that T242N is possible only in conjunction with a preexisting residue 243 mutation (TSNXQ EQIGW, observed in 15% of HLA-B*57$^+$ subjects) (underlining shows mutation). One would expect to observe T242N at a higher frequency, given its dominance in HLA-B*57$^+$ subjects infected by other clades. Therefore, while T242N has been implicated in HLA-B*57-mediated protection, this mutation is rare in clade A1. Because HLA-B*57 remains protective in clade A1, that protection may be mediated by novel mechanisms.

Because TW10 is commonly recognized by 86% of clade A1 subjects, it is evident that clade A1 TW10 can bind HLA-B*57. We therefore hypothesize that TSTPQEQIGW may affect the interaction between epitope and cognate T-cell receptors,

TABLE 1. p24 sequences in HLA-B*57$^+$ subjects infected by clades A1 and D

Clade	HLA-B allele	Subject no.	No. of years infected prior to sample[b]	Polymorphism at residue S_{146}	Epitope sequence[a]		
					IW9	KF11	TW10
					LSPRTLNAW	KAFSPEVIPMF	TSTPQEQIGW
A1	5701	1665	>130	N	F---------	----------	----------
	5702	139	21	N	---------	----------	--NI------
		59	ND	P	---------	----------	--NT---LA-
		41	>122	T	---------	----------	--NV------
		1419	>41	N	---------	----------	----------
		616	>102	T	---------	----------	-------LA-
		1647	18	-	P---------	----------	---S---LQ-
		613	>33	P	---------	----------	---T------
		718	>71	P	M---------	----------	---T---L--
		1315	>58	P	---------	-G--------	---TS---L--
	5703	2125	>0	A	---------	---N-------	--NL------
		561	>78	P	---------	---N-------	----------
		1926	>39	P	---------	---c-------	---T------
		1778	>13	-	---------	----------	----------
		1609	0	T	---------	----------	-------LQ-
		525	>135	-	---------	-G-n-------	-------L--
		1368	>57	-	M---------	-G-n-------	---T------
		509	>150	T	F---------	-G-N-------	----------
		260	>163	T	F---------	-G-N-------	-------L--
		111	>133	-	---------	-G-N-------	-------LQ-
		1111	>34	-	---------	-G-N-------	-------LQ-
		1638	>27	-	---------	-G-N-------	---T------
		2101	>0	P	---------	-G--------	--NT------
		532	>28	-	---------	-G--------	--------A-
		1669	>23	P	P---------	-G--------	--------A-
		703	>71	T	F---------	-G--------	----------
		1741	>11	T	F---------	-G--------	----------
		1452	>37	P	---------	-G--------	----------
		30	>121	P	---------	R---------	--------A-
		1122	>39	P	---------	RG-Q-------	----------
		995	>80	P	F---------	RG-Q-------	---T------
		1564	>27	P	---------	RG--------	---T------
	5707	330	>164	T	---------	-G-N-------	---T------
D	5701	1859		P	---------	----------	--NL------
		1852		P	---------	----------	--NL---VA-
	5703	1756		P	---------	----------	--NL------
		1423		P	-T-------	----------	--NL------
		1188		P	I---------	-G-N-------	--NL----A-
		1894		P	---------	-N--------	--NL----R-
		199		P	-T-------	-S--------	--NL---V--

[a] The first row of epitope sequences shows the consensus sequences.
[b] ND, not done.

which in turn influences which escape mutations are optimal. This hypothesis is supported by our immunological data showing cross-reactivity between clade A1 TW10 and TSNLQE QIGW (underlining shows mutation), which imply that mutation at residue 242 may not lead to effective escape in clade A1 (Fig. 3d).

In contrast to other clades (including clade D), HLA-B*5801 does not appear to place selection pressure on clade A1 TW10. A previous study in Rwanda similarly showed that in clade A1, HLA-B*5703 but not HLA-B*5801 was associated with lower HIV viral loads (30). Therefore, HLA-B*5801 was associated with neither protection nor selection in clade A1 TW10. Our data also show that HLA-B*5702- and -B*5703-mediated KF11 selection differs, despite these alleles differing at only one codon. Similar findings have been published for HLA-B7 supertype alleles (31). These data highlight the differences in immunological pressure within HLA supertype alleles, even though these alleles often present the same epitopes to the immune system.

Previous reports have suggested that HIV evolution can differ among clades for a variety of reasons. HLA-B*1503 differed in its protectiveness in clade B- and clade C-infected cohorts, and the apparent mechanism is broader recognition of subdominant epitopes, which remain intact due to limited selection where HLA-B*1503 is less common (17). Similarly, Yu et al. showed that differences in KF11 evolution between clades B and C were largely the result of differences in immunological features of HLA-B*5701- and -B*5703-restricted responses, with the latter allele being more frequent in clade C-infected populations (47). The temporality of selection can also differ between clades; while TW10 and IW9 selection is similar between clade B and C, the order in which they are selected is opposite (12). Our data show that virological factors (i.e., sequence differences) can also lead to clade-specific es-

cape. Other reports have found few differences in evolution among clades, including no differences in HLA-A2 Gag SL9 escape among clades A, B, and D (25), so the presence of clade-specific evolution will depend on the epitope and allele under study.

Recent reports have suggested that Gag-specific $CD8^+$ T-cell responses are protective in HIV infection (28), possibly because escape in Gag comes at a fitness cost. In support of this, infection by strains containing multiple Gag escape mutations was associated with lower set point viremia independent of HLA alleles in the recipients (21). One of the first demonstrations of Gag escape with fitness cost was T242N selection and reversion (32), and this substitution dominates in clade B- and clade C-infected HLA-B*57^+ subjects in numerous cohorts (5, 9, 10, 15, 32, 35, 38, 41). Our data show that while clade D follows clades B and C, HLA-B*57-mediated evolution in clade A1 differs not only in TW10 but also in other p24 epitopes. Knowledge of clade-specific escape pathways will be important for vaccines that aim to cover multiple clades, particularly where clades differ in immunologically critical epitopes.

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