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Virological and Immunological Factors Associated with HIV-1 Differential Disease Progression in HLA-B*58:01-Positive Individuals▽†

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Molecular epidemiology studies have identified HLA-B*58:01 as a protective HIV allele. However, not all B*58:01-expressing persons exhibit slow HIV disease progression. We followed six HLA-B*58:01-positive, HIV subtype C-infected individuals for up to 31 months from the onset of infection and observed substantial variability in their clinical progression despite comparable total breadths of T cell responses. We therefore investigated additional immunological and virological factors that could explain their different disease trajectories. Cytotoxic T-lymphocyte (CTL) responses during acute infection predominantly targeted the TW10 and KF9 epitopes in $p24^{Gag}$ and Nef, respectively. Failure to target the TW10 epitope in one B*58:01-positive individual was associated with low $CD4^+$ counts and rapid disease progression. Among those targeting TW10, escape mutations arose within 2 to 15 weeks of infection. Rapid escape was associated with preexisting compensatory mutations in the transmitted viruses, which were present at a high frequency (69%) in the study population. At 1 year postinfection, B*58:01-positive individuals who targeted and developed escape mutations in the TW10 epitope ($n = 5$) retained significantly higher $CD4^+$ counts ($P = 0.04$), but not lower viral loads, than non-B*58:01-positive individuals ($n = 17$). The high population-level frequency of these compensatory mutations may be limiting the protective effect of the B*58:01 allele.

HLA-B*57 and -B*58:01 are overrepresented among elite and viremic controllers of HIV-1 subtype C infections (8, 18, 24, 26); however, the specific mechanism(s) underlying these protective effects remains incompletely understood. Strong cytotoxic T-lymphocyte (CTL) responses targeting multiple epitopes in the conserved p24 region of Gag during the critical acute/early stage of disease likely contribute to viral control in these individuals (2, 18, 29). Moreover, CTL-driven selection of viral escape mutations associated with substantial costs to viral protein function and replication capacity is also likely a major contributor to better control of viremia in individuals carrying particular HLA alleles (4, 9, 19, 21).

One of the most extensively studied HLA-B*57/B*58:01-restricted escape mutations is the T242N mutation within the immunodominant TW10 epitope in $p24^{Gag}$. In acute subtype B and subtype C infection, TW10 ranks among the earliest targeted epitopes (5, 15); however, the T242N escape mutation is generally rapidly selected. *In vitro* studies have directly shown that the T242N mutation incurs a fitness cost (4, 8, 21); however, compensatory mutations upstream and downstream of the epitope have been reported to partially rescue these defects (4, 19). Recently, it has been observed that HLA-B*57- and -B*58:01-positive elite controllers select for rare CTL escape variants within TW10 that are associated with severe defects in viral replication, supporting the role of immune-driven HIV attenuation as an important correlate of the controller phenotype (24).

Although HLA-B*58:01 is a "protective" allele, not all B*58:01-expressing persons exhibit slow HIV disease progression. Here we investigate the immunological (i.e., CTL responses) and viral (i.e., CTL escape mutations) correlates of differential disease progression in six HIV-1 subtype C-infected HLA-B*58:01-positive individuals.

MATERIALS AND METHODS

Study subjects. Individuals involved in this study were part of the CAPRISA 002 cohort investigating the role of viral and immunological factors in HIV progression during acute/early HIV-1 subtype C infection (7, 32). The cohort includes high-risk HIV-negative women monitored monthly for recent HIV-1 infection using two HIV-1 rapid antibody tests and PCR (Roche Amplicor v1.5). HIV-1 infection was confirmed by enzyme immunoassay (EIA). Women were enrolled within 3 months of infection from both the HIV-negative cohort and other seroincident cohorts in Durban, South Africa. The timing of infection was estimated either to be at the midpoint between the last HIV-1-negative test and the first antibody-positive test or to be 14 days during which individuals were PCR positive but antibody negative. Samples were collected at enrollment, weekly for 3 weeks, fortnightly until 3 months, monthly until a year, and quarterly thereafter. $CD4^+$ T cell counts were assessed using a FACSCalibur flow cytometer, and viral loads were measured using a Cobas Amplicor HIV-1 Monitor Test, v1.5 (Roche Diagnostics). Plasma collected in EDTA was stored at −70°C until use. Written informed consent was obtained from all participants. This study received ethical approval from the University of Kwa-Zulu Natal, the University of the Witwatersrand, and the University of Cape Town. A total of 36

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individuals from the cohort who had reached 12 months postinfection were included in this study, of which 24 were part of a study described previously (7).

RNA isolation, reverse transcription-PCR (RT-PCR), and viral sequencing. Viral sequencing was carried out as previously described (7). RNA isolated from plasma samples using the Magna-Pure Compact Nucleic Acid Extractor (Roche) was reverse transcribed using the Invitrogen Thermoscript reverse transcription kit (Invitrogen) and the primers Gag D reverse (5′-AAT TCC TCC TAT CAT TTT TGG-3′; HXB2 positions 2382 to 2402) for *gag* and Nef O reverse (5′-AGG CAA GCT TTA TTG AGG-3′; HXB2 positions 9608 to 9625) for *nef*. Limiting dilution nested PCR was carried out by serial endpoint dilution of the cDNA (27). The first-round PCR primers for *gag* amplification were Gag D forward (5′-TCT CTA GCA GTG GCG CCC G-3′; HXB positions 626 to 644) and Gag D reverse. The second-round PCR primers were Gag A forward (5′-CTC TCG ACG CAG GAC TCG GCT T-3′; HXB2 positions 683 to 704) and Gag C reverse (5′-TCT TCT AAT ACT GTA TCA TCT GC-3′; HXB2 positions 2334 to 2356). For *nef*, the first-round PCR primers were SQ15FC (5′-GAG AGC GGT GGA ACT TCT-3′; HXB2 positions 8561 to 8578) and Nef O reverse. The second-round PCR primers were Nef forward (5′-CCT AGA AGA ATA AGA CAG GGC TT-3′; HXB2 positions 8754 to 8776) and Nef reverse (5′-CCT GGA ACG CCC CAG TGG-3′; HXB2 positions 9443 to 9461). PCR products were either directly sequenced or cloned using the pGEM-T Easy vector system (Promega). Sequencing was carried out using an ABI Prism dye terminator cycle-sequencing kit (Applied Biosystems) and the primers Gag A forward, Gag A reverse (5′-ACA TGG GTA TCA CTT CTG GGC T-3′; HXB2 positions 1282 to 1303), Gag B forward (5′-CCA TAT CAC CTA GAA CTT TGA AT-3′; HXB2 positions 1226 to 1246), Gag B reverse (5′-CTC CCT GAC ATG CTG TCA TCA T-3′; HXB2 positions 1825 to 1846), Gag C forward (5′-CCT TGT TGG TCC AAA ATG CGA-3′; HXB positions 1748 to 1768), and Gag C reverse for Gag sequencing. The primers Nef forward and Nef reverse were used for Nef sequencing. Sequences were assembled using ChromasPro (Technelysium, Australia) and aligned using ClustalW (with default settings [31]).

HLA typing. High-resolution (four-digit) HLA typing was performed on all participants. DNA was extracted from either peripheral blood mononuclear cells (PBMCs) or granulocytes using the Pel-Freez DNA isolation kit (Pel-Freez). HLA-A, -B, and -C typing was performed by sequencing of exons 2, 3, and 4 using Atria HLA-AlleleSeqr kits (Abbott) or HLA-SBTexcellerator kits (Qiagen) and Assign-SBT v3.5 software (Conexio Genomics). Any ambiguities resulting from either polymorphisms outside the sequenced exons or identical heterozygote combinations were resolved using sequence-specific primers or resolved statistically using the algorithm developed for this purpose (20).

IFN-γ ELISpot assay. PBMCs were prepared and screened for HIV-1-specific T cell responses across the entire HIV-1 clade C proteome by gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assay (22). For our analyses, we focused on Gag and Nef because these proteins have been a key feature in candidate CTL-based vaccines and are the most dominant targeted proteins in HIV. T cell responses were either determined using a deconvoluted pool matrix approach or confirmed using individual peptides. The epitopes within peptides showing responses were delineated using published epitope sequences in the Los Alamos HIV database (www.hiv.lanl.gov).

Statistical analyses. Wilcoxon rank-sum tests were used to compare viral loads and $CD4^+$ counts. Fisher's exact test was used to compare differences in frequencies of compensatory mutations between sequences from the study cohort and reference sequences. Statistical tests were implemented in GraphPad Prism 5.0 (GraphPad Software, Inc.).

Nucleotide sequence accession numbers. Nucleotide sequences determined in this work were submitted to GenBank under accession numbers EU347404 to EU347714 and JF704235 to JF705197.

RESULTS

The CAPRISA 002 study follows high-risk HIV-negative individuals who are subsequently enrolled into an acute cohort upon HIV infection. At the time of this study, 36 enrollees had reached 1 year postinfection. Of these, six possessed the HLA-B*58:01 allele while none had the closely related HLA-B*57 allele (14, 18). To control for fluctuations, viral load and $CD4^+$ counts at set point (12 months postinfection) were calculated as the mean of three measurements taken at time points closest to 12 months postinfection (range, 9 to 16 months). The median log viral load and $CD4^+$ count for the 36 participants at 12 months postinfection were 4.60 copies ml^{-1} and 408 cells μl^{-1}, respectively (see Table S1 in the supplemental material). There was no significant difference in viral load between the B*58:01-positive participants ($n = 6$) and non-B*58:01-positive participants ($n = 30$) at 12 months postinfection (median log viral load, 4.77 versus 4.65 copies ml^{-1}, respectively; $P = 0.43$). However, B*58:01-positive participants tended to have higher $CD4^+$ T cell counts than non-B*58:01-positive participants (median, 600 versus 430 cells μl^{-1}, respectively; $P = 0.13$; data not shown).

Early evolution in $p24^{Gag}$ is characterized by escape mutations in the B*58:01-restricted TW10 epitope. Several studies have attributed viremia control in HLA-B*57- and B*58:01-expressing persons to their ability to target conserved epitopes such as ISW9, KF11, and TW10 in $p24^{Gag}$ (1, 2). Escape mutations in these epitopes have been shown to impair viral replication (3, 4, 9, 30). In order to determine the impact of immune pressure on the $p24^{Gag}$ region, we monitored viral *gag* and *nef* evolution in the six HLA-B*58:01-positive participants from infection until up to 31.4 months postinfection.

Escape mutations arose in the TW10 epitope in four of the six individuals within 3.4 months postinfection, with the earliest variation in the epitope being detected only 2 weeks postinfection (CAP239; Fig. 1). At this time point, this individual (CAP239) was antibody negative but HIV RNA positive. Emergence of the T242N mutation was often preceded by variation at other sites within the epitope. This variation occurred at six sites within the epitope at positions 3, 4, 5, 7, 8, and 9 (T242, L243, Q244, Q246, I247, and A248, respectively). Whereas in four individuals (CAP217, CAP229, CAP268, and CAP270) the earliest sampled viruses had only the wild-type TW10 sequence (TSTLQEQIAW), in the other two individuals (CAP239 and CAP274) the earliest sampled viruses carried a mixture of wild-type and mutant TW10 sequences (Fig. 1). The presence of variation at this earliest time point suggests that escape from TW10-specific CTL responses occurred very early during infection, although transmission of the escape variant cannot be ruled out. Following its emergence, T242N rapidly took over the population within the infected host: time to fixation ranged from 1.2 (in individual CAP239) to 6.2 (in individual CAP217) months from its first detection.

IFN-γ production by T cells in response to the TW10 epitope was investigated in the six B*58:01-positive participants using ELISpot assays. Three of four individuals harboring viruses that evolved escape mutations in the TW10 epitope displayed a reduction in TW10-specific ELISpot responses following escape (Fig. 1). The fourth individual (CAP274), whose virus harbored a 22%/78% mixture of the T242 and N242 polymorphisms at the earliest time point (2.4 months postinfection), had no detectable responses to TW10, suggesting that an earlier transient CTL response to TW10 may have already waned by the time the ELISpot assay was performed. In the two individuals where CTL escape was not identified, CAP268 and CAP270, only CAP268 had detectable ELISpot responses to TW10. In this individual, responses were 518 spot-forming units (SFU)/10^{-6} PBMCs at 1.8 months postinfection, which strengthened to 2,523 SFU/10^{-6} PBMCs by 5.5 months postinfection. Interestingly, the virus infecting CAP268 carried the HLA-B*58:01-associated M250I mutation previously associated with reduced frequency of escape in TW10 (21), suggesting that

PID	Variant TTSTLQEQIAWM*	Mo. PI[a]	Frequency	Elispot response (SFU/10^6 PBMC)
CAP217	.....	2.2	6/6[b]	218
	.....	2.8	3/3	2038
	.....	3.4	13/18	2008
	.....P.....	3.4	4/18	
	.....E.....	3.4	1/18	
	.....	3.8	2/12	1360
	...N.....	3.8	6/12	
	.....P.....	3.8	4/12	
	...N.....	4.3	12/12	485
	.....	5.2	1/9	195
	...N.....	5.2	7/9	
	.....T.....	5.2	1/9	
	...N.....	6.2	10/10[b]	ND[c]
	...N.....	13.8	14/14	ND
CAP229	.....	1.6	12/12[b]	1188
	.....	1.8	6/10	105
	.....T..	1.8	2/10	
	...N.....T..	1.8	2/10	
	...N.....T..	2.1	6/6b	88
	...N.....T..	4.8	3/3	ND
	...N.....T..	5.8	23/23[b]	ND
	...N.....T..	18.4	15/15	ND
	...N.....T..	21.2	24/24	ND
	...N.....T..	24.2	15/15	ND
	...N.....T..	28.1	16/16	ND
CAP239	.....V...	0.5	20/21[b]	ND
	....I...VT..	0.5	1/21	
	...N....VT..	1.2	27/27	380
	...N....VT..	1.4	19/19[b]	80
	...N....V...	5.1	9/9[b]	113
	...N....V...	18.2	9/9	ND
CAP268	I	1.8	6/6[b]	518
	I	5.5	8/8[b]	2523
	I	12.3	12/12[b]	ND
	I	19.8	8/8	ND
	I	31.4	10/10	ND
CAP270	.....	1.8	9/9	NR[d]
	.....	2.9	7/7	NR
	.....	6.6	9/9	ND
	.....	13.3	29/29	ND
CAP274	.....H..VQ..	2.4	2/9	NR
	...N....VQ..	2.4	7/9	
	...N....VQ..	5.1	7/7	ND
	...N....VQ..	13.9	19/19	ND

[a]Mo. PI – months postinfection
[b]Pop –same sequence as population sequencing
[c]ND - Not done
[d]NR - No response
*Consensus subtype C TW10 epitope underlined

FIG. 1. Alignment of TW10 epitope sequences (sequence underlined) and their flanking residues (one on either side) showing changes in the six HLA-B*58:01-positive study participants over time. The relative frequencies of variants carrying the respective mutations are indicated along with the ELISpot responses of the study participants from which the sequences were derived. Gray highlighting indicates different sampling time points.

mutational antagonism by the M250I mutation prevented escape within TW10. CAP270, the other individual whose virus did not exhibit escape in TW10, did not respond to this epitope.

Disease progression in HLA-B*58:01-positive individuals is influenced by immune targeting of TW10 and escape mutations at T242. Viral load and $CD4^+$ cell count trajectories of the six HLA-B*58:01-positive study participants were analyzed to determine the impact of (i) TW10 targeting and CTL escape through mutation at T242, (ii) TW10 targeting in the absence of CTL escape at T242, and (iii) an absence of both TW10 targeting and CTL escape at T242. The four HLA-B*58:01-positive individuals who targeted the TW10 epitope and whose virus subsequently escaped this response (CAP217, CAP229, CAP239, and CAP274) displayed moderate viral loads (Fig. 2A). CAP268, the individual who displayed TW10-specific ELISpot responses but who did not develop T242N, had the lowest viral load (Fig. 2A). CAP270, the individual who did not respond to TW10 (and consequently did select

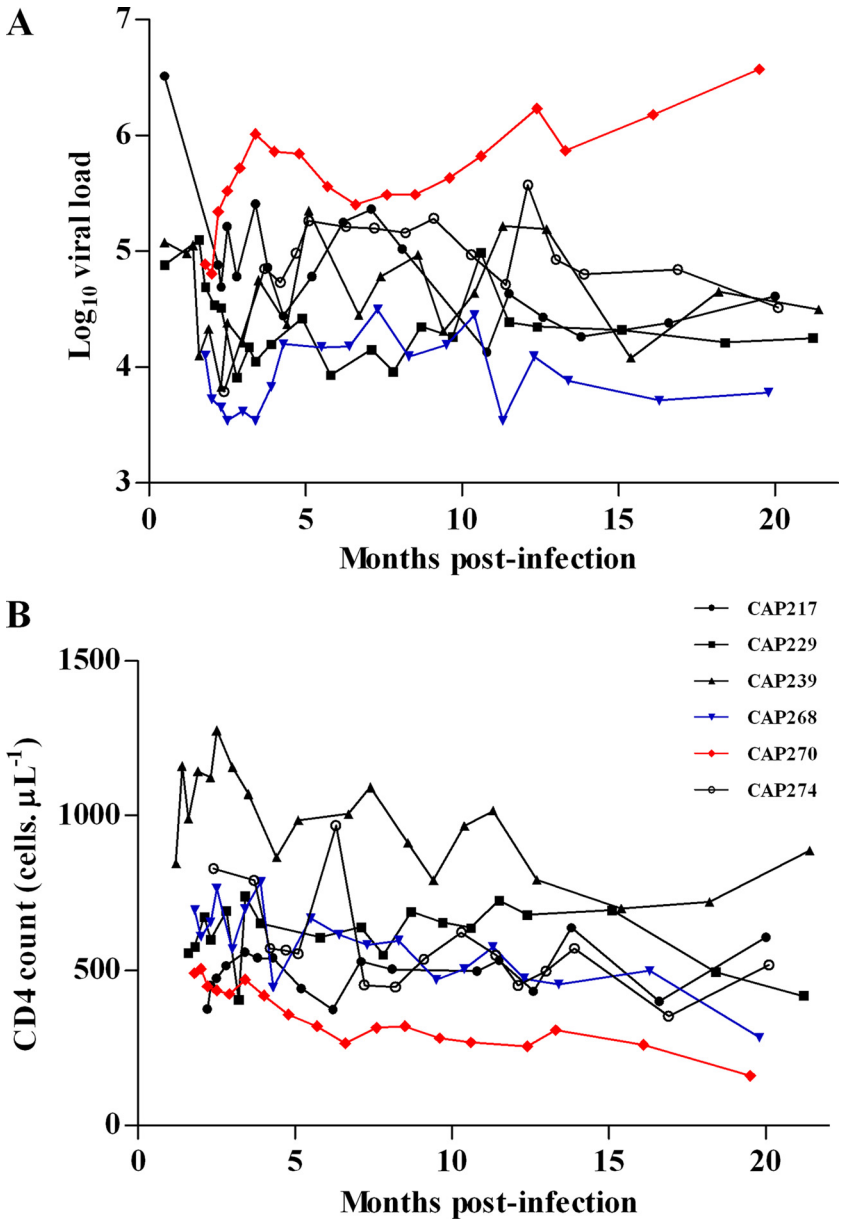


FIG. 2. Viral load (A) and CD4$^+$ count (B) trajectories of the six HLA-B*58:01-positive study participants, including those infected with viruses that escaped an active TW10-targeted CTL response via the T242N mutation (black), the study participant infected with viruses carrying the wild-type TW10 sequence and who did not mount a detectable response to the epitope (red), and the study participant who responded to TW10 but whose infecting viruses did not accumulate escape mutations in the epitope (blue).

T242N), had a high viral load (Fig. 2A) and the lowest CD4$^+$ counts overall (Fig. 2B). CAP268, the individual who responded to TW10 but whose infecting viruses did not develop the T242N mutation, had moderate CD4$^+$ T cell counts (Fig. 2B), while the four individuals (CAP217, CAP229, CAP239, and CAP274) who responded to the epitope and harbored viruses that developed the T242N mutation had moderate to high CD4$^+$ counts (Fig. 2B).

These data suggest that CTL targeting of TW10 and the subsequent accumulation of escape mutations in the epitope may play an important role in disease progression. We show here that events during acute and early subtype C infections, such as the T242N escape mutation, are associated with the preservation of CD4$^+$ counts, an association which may have long term-benefits for the infected individuals.

Immune responses targeting p24 influence disease progression. We used IFN-γ ELISpot assays to assess whether differences in disease progression observed in the six HLA-B*58:01-positive individuals correlated with the breadth and specificity of their T cell responses. All six HLA-B*58:01-positive individuals responded to at least six epitopes (range, 6 to 10) across the HIV-1 proteome (Table 1); between 2 and 4 of these represented previously described B*58:01-restricted immunodominant epitopes (2) (see Table S2 in the supplemental material).

TABLE 1. IFN-γ ELISpot responses of the six HLA-B*58:01-positive study participants[a]

PID	Protein	Peptide Sequence	Restricting HLA
CAP217	Gag (p24)	SDIAGTTSTLQEQIAWMTSNPPVPV	B*58:01
	Gag (p2p7p1p6)	GRPGNFLQSRPEPTAPPAESFRF	B*58:01
	Pol (Int)	GSNFTSAAVKAACWWAGIQQEFGIPYNPQSQGVV	B*15:03
	Pol (Int)	HLKTAVQMAVFIHNFKRKGGIGGYSA	B*58:01
	Vif	VRKLVEDRWNKPQKTRGRR	B*58:01
	Nef	RPMTYKAAFDLLSFFLKEKG	B*58:01
CAP229	Gag (p24)	SDIAGTTSTLQEQIAWMTSNPPVPV	B*58:01
	Pol (RT)	YELHPDKWTVQPIQLPEKDSWTVNDI	B*58:01
	Tat	MEPIDPNLEPWNHPGSQPNTPCNNCYCKHCSYH	B*58:01
	Nef	RPMTYKAAFDLLSFFLKEKG	B*58:01
	Nef	FPDWQNYTPGPGVRYPLTF	B*58:01
	Nef	DREVLKWVFDSSLAR	A*01:23
CAP239	Gag (p17)	EKIRLRPGGKKHYMLKHL	B*42:01
	Gag (p24)	SDIAGTTSTLQEQIAWMTSNPPVPV	B*58:01
	Pol (RT)	KWTVQPIVLPEKESWTVN	B*58:01
	Vpr	HFPRPWLHGLGQHIYATY	B*42:01
	Env	RAIYSIPRRIRQGFEAAL	B*42:01
	Nef	RPMTYKAAFDLLSFFLKEKG	B*58:01
CAP268	Gag (p24)	SDIAGTTSTLQEQIAWMTSNPPVPV	B*58:01
	Gag (p24)	WVKVIEEKAFSPEVIPMFTALSEGA	B*58:01
	Pol (Int)	ELKKIIGQVRDQAEHLKTAVQMAVF	B*58:01
	Vpr	ELKQEAVRHFPRPWLHGL	B*58:01
	Nef	RTEPAAEGVGAASQDLDKHGALT	A*02:05
	Nef	EVGFPVRPQVPLRPMTYKA	B*07:02
	Nef	IHSKRRQDILDLWVYHTQG	Cw*07:01/07:02
CAP270	Gag (p17)	RSLYNTVATLYCVHAGIEV	A*30:02
	Gag (p24)	NANPDCKTILRALGPGA	B*08:01
	Pol (Int)	QSNFTSAAVKAACWWAGIQQEFIPY	B*58:01
	Pol (Int)	GQVRDQAEHLKTAVQMAVFIHNFKRK	B*58:01
	Tat	PKPEGTRQARKNRRRRWR*	
	Env	SAISLLDTIAIAVAEGTDR	B*58:01
	Nef	GGKWSKSSIVGWPAVRERI	B*08:01
	Nef	RPMTYKAAFDLSFFLKEKG	B*58:01
CAP274	Gag (p17)	EKIRLRPGGKKHYMLKHL	B*42:01
	Gag (p24)	CFNCGKEGHIARNCRAPR	A*30:01
	Pol (RT)	IYPGIKVRQLCKLLRGTK	B*42:01
	Pol (RT)	HKYAKRRTTHTNDVKQL	A*30:01
	Pol (Int)	QSNFTSAAVKAACWWAGIQQEFIPY	B*58:01
	Pol (Int)	KVVPRRKVKIIRDYGKQMAGADCVAGRQDED	B*42:01
	Tat	PKPEGTRQARKNRRRWR	A*30:01
	Env	SAISLLDTIAIAVAEGTDR	A*02:01
	Nef	DKHGALTSSNTAHNN	A*02:01
	Nef	RPMTYKAAFDLSFFLKEKG	B*58:01

[a] Shown in blue are the confirmed single peptide responses. In black are the unconfirmed responsive peptides containing known epitopes. In green are predicted epitopes based on HLA binding motifs. The epitopes are underlined (bold). The unconfirmed peptides were predicted by pool matrix deconvolution. *, no known or predicted epitope.

Overall, 23 of the 43 (53%) responses detected by ELISpot assay were likely restricted by HLA-B*58:01. Besides TW10-specific responses and escape mutations within this epitope, no other common responses or mutational patterns that could explain the observed differences in disease progression were observed (data not shown). However, it is noteworthy that the individual displaying the best control of virus replication (CAP268) responded to both the TW10 and KF11 epitopes in $p24^{Gag}$. No potential escape mutations were observed in KF11 in this participant. None of the other HLA-B*58:01-positive individuals recognized KF11. Also interesting is the fact that CAP270, the individual who exhibited the least favorable clinical profile, completely lacked HLA-B*58:01-restricted responses in p24. The sole anti-p24 response detected in this individual was directed at the HLA-B*08:01-restricted DL9 (DCKTILRAL) epitope. In chronic subtype C infection, a weak association has been reported between HLA-B*08:01 and higher viremia (17).

No HLA enrichment associated with differential disease progression. Certain HLA alleles, particularly those encoded by HLA-B, are associated with rates of HIV disease progression (12, 17). We sought to determine whether other HLA alleles besides HLA-B*58:01 could have contributed to the disease progression patterns observed in the B*58:01-positive

TABLE 2. HLA alleles for the six HLA-B*58:01-positive individuals

PID[a]	Allele pair		
	HLA-A	HLA-B	HLA-C
CAP217	02:02, 29:01	15:03, 58:01	02:10, 06:02
CAP229	01:23, 01:23	58:01, 58:01	06:02, 06:02
CAP239	01:23, 29:02	42:01, 58:01	06:02, 17:01
CAP268	02:05, 26:01	07:05, 58:01	07:01, 07:02
CAP270	03:01, 30:02	08:01, 58:01	07:01, 07:01
CAP274	02:01, 30:01	42:01, 58:01	03:02, 17:01

[a] PID, participant identification.

individuals. In addition to HLA-B*58:01, two of the six individuals (CAP239 and CAP274) also expressed B*42:01, an allele associated with modest protective effects in subtype C infection (17) (Table 2). The remaining individuals did not express protective HLA-B alleles other than B*58:01. The B*15:03 allele expressed by CAP217 (an individual displaying intermediate viremia) and the B*08:01 allele expressed by CAP270 (who was the most viremic of the HLA-B*58:01-positive individuals) are both associated with poorer-than-average control of virus replication during HIV infection (17). It is conceivable, therefore, that the HLA-B*42:01, -B*15:03, or -B*08:01 allele occurring in tandem with the HLA-B*58:01 allele may have contributed at least partially to various patterns of disease progression observed within the HLA-B*58:01-positive individuals. HLA homozygosity has previously been associated with rapid HIV disease progression (6). One B*58:01-positive patient in this cohort (CAP229) was homozygous at the HLA-A, -B, and -C loci (Table 2) but did not show significantly higher viral loads or lower $CD4^+$ counts than other B*58:01-positive individuals (Fig. 2), suggesting that in this case at least, HLA homozygosity did not have a substantial impact on disease progression.

Additional CTL escape predominantly occurred in the Nef KF9 epitope. Next, we analyzed evolution within other immunoreactive epitopes in Gag and Nef. Four of six studied individuals (CAP217, CAP229, CAP239, and CAP270) also exhibited escape in the Nef KF9 epitope (Fig. 3). Viruses from the remaining two individuals already harbored KF9 escape mutations at the time of enrollment (data not shown), indicating very early escape or transmission of the escape variant. Furthermore, potential escape mutations were also detected in the HLA-B*42:01-restricted p17 Gag RY10 epitope (in both CAP239 and CAP274), in the HLA-B*07:02-restricted Nef RM9 epitope (in CAP268), in the HLA-A*30:02-restricted p17 Gag SL9 epitope (in CAP270), and in the HLA-B*08:01-restricted Nef WI8 epitope (in CAP270). These data suggest that other CTL escape mutations besides those occurring in the Gag p27 TW10 epitope may have additionally contributed to differences in disease progression observed in the six HLA-B*58:01-positive individuals.

HLA-B*58:01 immune responses targeting TW10 and those targeting T242N escape are both associated with improved disease progression. We next wished to investigate the impact of T cell responses to TW10, and escape within this epitope, on markers of disease progression in HLA-B*58:01-negative compared to B*5801-negative individuals. Previously published data indicate that HLA-B*57/B*58:01-negative individuals acquiring HIV harboring "signature" HLA-B*57- or -B*58:01-associated escape mutations A146X and T242N exhibit lower viral loads and higher $CD4^+$ counts than do individuals infected with viruses not harboring these substitutions (7, 13, 30); therefore, we excluded 13 HLA-B*57- and -B*58:01-negative study participants infected with viruses carrying either A146X (X = P or S) or T242X (X = N or S) mutations from our subsequent analyses.

A comparison of $CD4^+$ counts and viral loads of the B*58:01-positive ($n = 6$) and -negative ($n = 17$) individuals at 12 months postinfection revealed no significant differences between these two groups (median $CD4^+$ counts, 600 versus 382 [$P = 0.09$], respectively, and median viral load, 4.77 versus 4.69 [$P = 0.81$], respectively). However, the B*58:01-positive individuals whose viruses developed the T242N mutation ($n = 4$) had significantly higher $CD4^+$ counts than did B*58:01-negative participants whose viruses did not carry the A146X and T242X ($n = 17$) mutations ($P = 0.04$) (Fig. 4A). The viral loads of these four individuals, however, did not significantly differ from those of the control group ($P = 0.50$) (Fig. 4B). Comparison of $CD4^+$ counts between the five B*58:01-positive individuals with detectable TW10 responses and the 17 HLA-B*58:01-negative individuals revealed significantly higher $CD4^+$ counts ($P = 0.04$) (Fig. 4C) but no differences in viral load ($P = 0.88$) (Fig. 4D) between the former and latter groups, respectively. We also analyzed clinical markers of disease progression at 3 years postinfection. At this time, six participants (one B*58:01-positive participant, CAP270, and five B*58:01-negative participants) had left the study due to initiation of antiretroviral treatment. We observed a similar trend of higher $CD4^+$ counts in HLA-B*58:01-positive individuals targeting TW10 ($n = 5$) compared to HLA-B*58:01-negative participants infected with viruses not carrying the B*57/B*58:01-associated A146X and T242X mutations ($n = 12$; 510 versus 316 cells μl^{-1}; $P = 0.04$) (see Fig. S1A in the supplemental material), but no significant differences in plasma viral loads (median log viral load, 4.54 versus 4.38 copies ml^{-1}; $P = 0.96$) (see Fig. S1B). Taken together, these findings suggest that, despite relatively rapid escape, the ability to target TW10 may be associated with slower disease progression.

Early escape may be facilitated by circulating compensatory mutations. Secondary mutations upstream and downstream of TW10 have been reported to partially restore viral fitness (4, 19, 21). Analysis of Gag sequences from the six HLA-B*58:01-positive individuals revealed that at enrollment, five individuals already carried viral variants containing at least one mutation that is either known, or strongly suspected, to compensate for T242N-associated replicative fitness costs (Fig. 5). Previous reports have detected a high frequency of the compensatory mutation H219X in the absence of T242N, suggesting that the compensatory mutation does not readily revert following transmission to HLA-B*57/B*58:01-negative individuals (19). All four of the study participants who developed T242N (CAP217, CAP229, CAP239, and CAP274) had at least one previously described compensatory mutation upstream of the epitope (Fig. 5). Interestingly, CAP268, the one participant who exhibited TW10-specific ELISpot responses but who did not develop escape mutations within the epitope, had no upstream compensatory mutations at enrollment (1.8 months postinfection). In this individual, the compensatory mutation

PID	Epitope	Mo. PI[a]	Frequency	Protein	Restricting HLA
	YKAAFDLSFFL			Nef	B*58:01
CAP217	....	2.2	20/24[b]		
	....V.....	2.2	2/24		
	------.....	2.2	1/24		
	..E.......	2.2	1/24		
	....	5.2	10/24		
	..E.......	5.2	13/24[b]		
	..G.......	5.2	1/24		
	..E.......	13.8	22/22		
	YKAAFDLSFFL			Nef	B*58:01
CAP229	....	1.6	23/23[b]		
	....	5.8	23/24[b]		
	.R........	5.8	1/24		
	..G.......	18.4	24/24[b]		
	IRLRPGGKKHYM			p17 Gag	B*42:01
CAP239	....	0.5	21/21		
	....	1.2	27/27		
	....	1.4	19/19		
	.........R..	18.2	4/9		
	.........Q..	18.2	5/9		
	YKAAFDLSFFL			Nef	B*58:01
	....V.....	0.5	24/24[b]		
	....V.....	1.2	23/23[b]		
	..G.V.....	5.1	22/23[b]		
	....	5.1	1/23		
	..G.V.....	18.2	17/23[b]		
	..G.......	18.2	6/23		
	VRPQVPLRPMT			Nef	B*07:02
CAP268	....	1.8	P[c]		
	....	3.4	P		
	.K........	5.5	P		
CAP270	LRSLYNTVATLYC			p17 Gag	A*30:02
	.K...........	1.8	6/6		
	.K...........	2.9	7/7		
	.K...........	6.6	9/9		
	.K...........	13.3	17/29		
	.K.......V...	13.3	12/29		
	YKAAFDLSFFL			Nef	B*58:01
	F..........	1.8	24/24[b]		
	F..........	2.9	23/24[b]		
	F......G...	2.9	1/24		
	F......G...	13.3	22/22[b]		
	GWPAVRERIR			Nef	B*08:01
	...N....M.	1.8	24/24		
	...N....M.	2.9	11/24		
	...D....M.	2.9	11/24		
	...N...GM.	2.9	1/24		
	...D.G..M.	2.9	1/24		
	...D...KM.	13.3	1/22		
	...E....M.	13.3	21/22		
	IRLRPGGKKHYM			p17 Gag	B*42:01
CAP274	.K..........	2.4	9/9		
	.K..........	5.1	7/7		
	.K.......R..	13.9	19/19		

[a]Mo.PI – Months post-infection
[b]Same sequence as population sequencing
[c]P – Population sequencing

FIG. 3. Alignment of Gag and Nef epitope sequences where additional CTL escape was detected and their flanking residues (one on either side) showing amino acid changes over time. The relative frequencies of variants and the restricting HLA alleles are indicated.

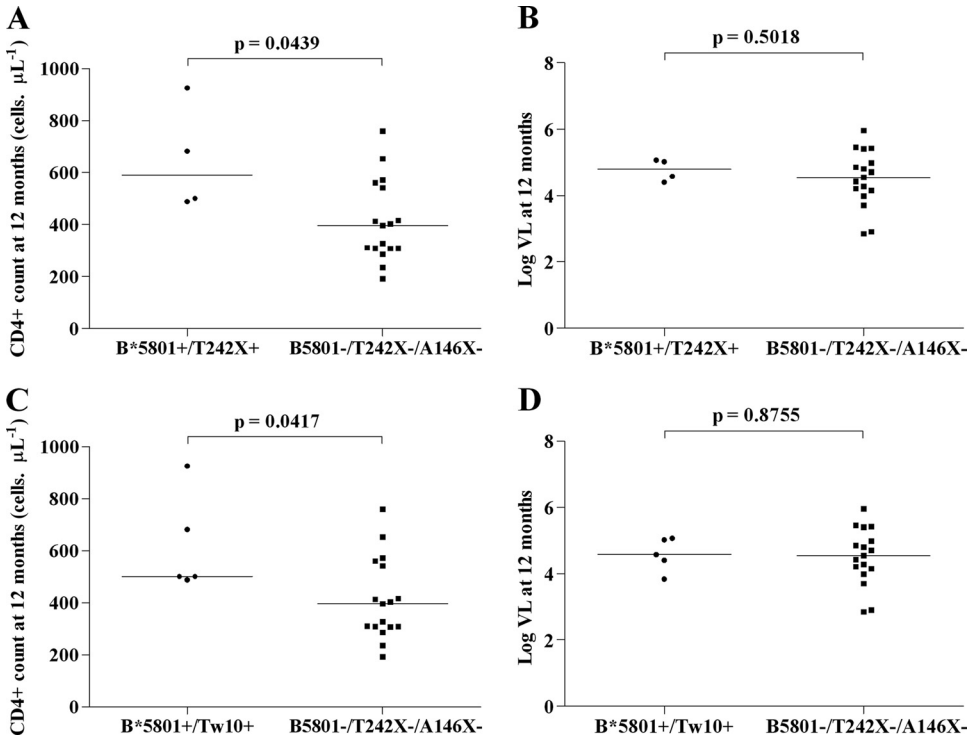


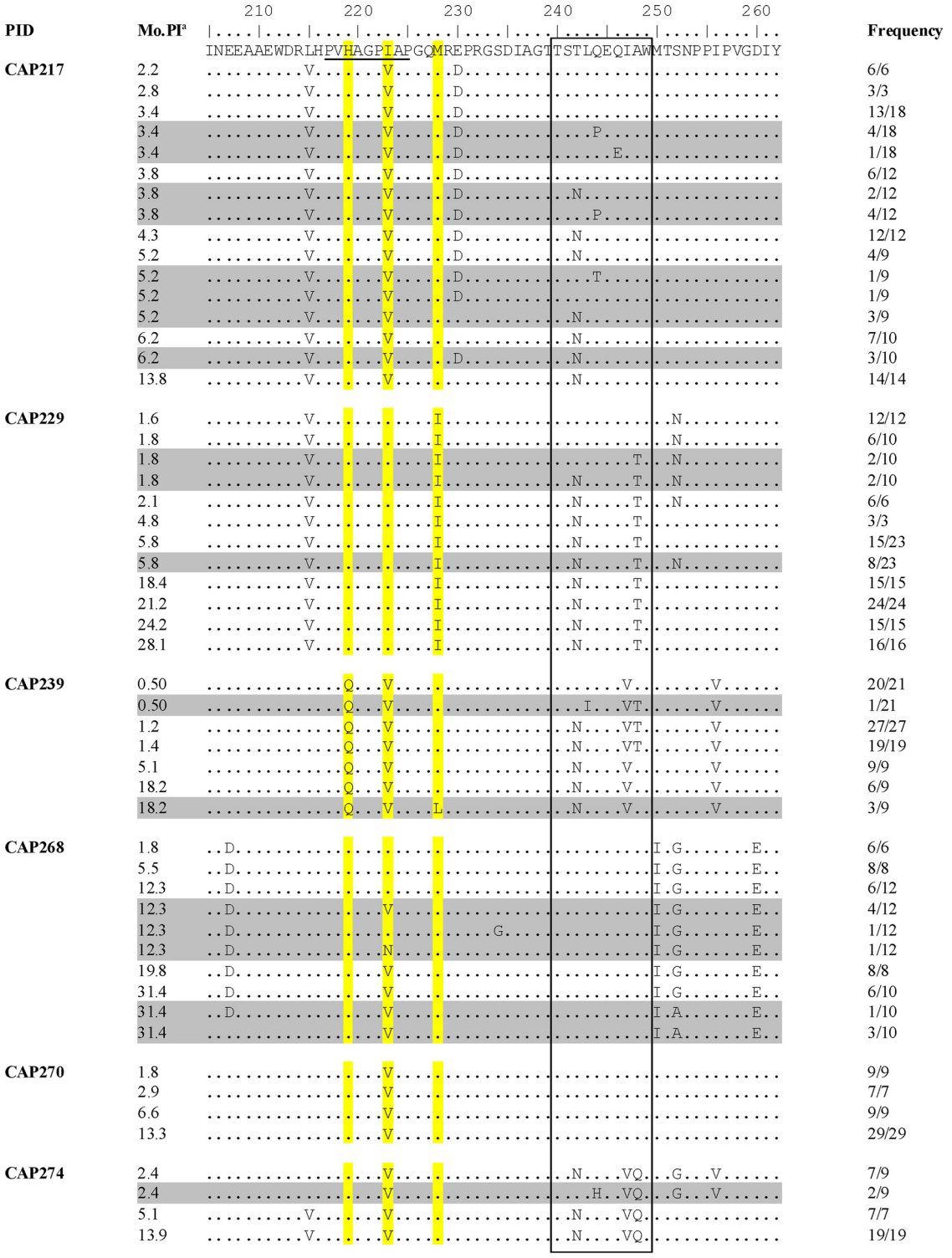
FIG. 4. $CD4^+$ count and viral load comparisons between B*58:01-positive and B*58:01-negative individuals. (A and B) $CD4^+$ cell count (A) and viral load (B) comparisons between HLA-B*58:01-positive study participants who developed the T242N mutation (B*58:01+/T242X+; $n = 4$) and the HLA-B*58:01-negative individuals who were not infected with viruses carrying B*57B*58:01-associated A146X and T242X mutations (B*58:01−/T242X−/A146X−; $n = 17$) at 12 months postinfection. (C and D) $CD4^+$ cell count (C) and viral load (D) comparisons between HLA-B*58:01-positive study participants who had ELISpot responses to the TW10 epitope (B*58:01+/TW10+; $n = 5$) and HLA-B*58:01-negative individuals who were not infected with viruses carrying B*57B*58:01-associated A146X and T242X mutations (B*58:01−/T242X−/A146X−; $n = 17$) at 12 months postinfection. The $CD4^+$ counts and viral loads were calculated as the mean of three measurements taken at time points closest to 12 months postinfection (range, 9 to 16 months).

at I223 developed at 12.3 months postinfection and reached fixation by 19.8 months postinfection (Fig. 5). However, the T242N mutation had still not been detected by 31.4 months postinfection. The appearance of the compensatory mutation before the escape mutation may suggest an alternative escape route whereby the fitness costs of escape are minimized by the preceding compensatory mutation. In CAP239, an additional potentially compensatory mutation (M228L) was detected at 18.2 months postinfection (Fig. 5).

TW10-associated compensatory mutations (H219X, I223X, and M228X) were present at surprisingly high background frequencies in our cohort (data not shown). Viruses infecting 25 out of the 36 individuals (69%) had at least one of the three compensatory mutations (4 with H219X [11%], 23 with I223X [64%], and 9 with M228X [25%]). Nine participants (25%) harbored more than one compensatory mutation. To determine if the frequency of compensatory mutations was increasing over time in subtype C infection, we compared transmitted sequences from our study (which were sampled between 2004 and 2007; $n = 36$) to sequences sampled from the same region (South Africa) in chronic infections between 1997 and 2000 (www.hiv.lanl.gov; $n = 136$). The total number of sequences with any compensatory mutations in our study cohort was higher than that in the reference data set, although this did not reach statistical significance (69% versus 57%, respectively; $P = 0.25$) (see Table S3 in the supplemental material). Similarly, there was no significant difference between the numbers of sequences with variations at individual compensatory sites (see Tables S3 and S4). However, there was a trend toward a higher frequency of variation at position 223 in our study cohort with respect to the background data set (64% versus 46%, respectively; $P = 0.09$). We also examined reversion of the compensatory mutations within the first 6 months in the 25 study participants who were infected with viruses carrying these mutations at enrollment (see Table S5). Reversion was observed at position 223 in only two individuals (CAP084 and CAP256), indicating that compensatory mutations do not revert readily upon transmission to new hosts (19). Interestingly, both these individuals were dually infected. CAP084 was found to be dually infected at the first available time point, while CAP256 was superinfected with a second strain which was detected at 3 months postinfection (25, 33). The high background prevalence of TW10-associated compensatory mutations in the study population has implications for the South African HIV epidemic. Specifically, these mutations may increase the rate at which viruses are able to escape TW10-targeted CTL responses following their transmission to HLA-B*58:01-positive individuals.

DISCUSSION

The favorable disease outcome associated with HLA-B*58:01 has been attributed to strong CTL responses targeting the



PID	Mo.PI[a]	Frequency
CAP217	2.2	6/6
	2.8	3/3
	3.4	13/18
	3.4	4/18
	3.4	1/18
	3.8	6/12
	3.8	2/12
	3.8	4/12
	4.3	12/12
	5.2	4/9
	5.2	1/9
	5.2	1/9
	5.2	3/9
	6.2	7/10
	6.2	3/10
	13.8	14/14
CAP229	1.6	12/12
	1.8	6/10
	1.8	2/10
	1.8	2/10
	2.1	6/6
	4.8	3/3
	5.8	15/23
	5.8	8/23
	18.4	15/15
	21.2	24/24
	24.2	15/15
	28.1	16/16
CAP239	0.50	20/21
	0.50	1/21
	1.2	27/27
	1.4	19/19
	5.1	9/9
	18.2	6/9
	18.2	3/9
CAP268	1.8	6/6
	5.5	8/8
	12.3	6/12
	12.3	4/12
	12.3	1/12
	12.3	1/12
	19.8	8/8
	31.4	6/10
	31.4	1/10
	31.4	3/10
CAP270	1.8	9/9
	2.9	7/7
	6.6	9/9
	13.3	29/29
CAP274	2.4	7/9
	2.4	2/9
	5.1	7/7
	13.9	19/19

[a]Mo.PI – Months post-infection

FIG. 5. Alignment of Gag p24 sequences surrounding the TW10 epitope (boxed) determined from clones sampled from the six HLA-B*58:01-positive study participants. Indicated in yellow are the sites of previously described T242N compensatory mutations upstream of the TW10 epitope. The gray highlighting indicates minority variants. The Cyp A binding loop is underlined. The sequences are aligned to the subtype C consensus p24 sequence, and numbering is according to the HXB reference sequence.

functionally and structurally constrained $p24^{Gag}$ protein and to mutational escape in this region resulting in viral variants with reduced replicative capacity (9, 19, 21). However, possession of HLA-B*58:01 does not guarantee slower disease progression, and we were therefore interested in identifying immunological and virological correlates of these differences.

Our data provide evidence as to how TW10-specific CTL responses influence disease progression in HLA-B*58:01-positive individuals. Whereas the absence of TW10-specific responses was associated with high viral loads, the presence of sustained TW10-specific responses in the absence of escape was associated with better control of viremia. Individuals with TW10-specific responses and who harbored viruses with known escape mutations had typical viral loads. From the perspective of $CD4^+$ counts, our study also provides some insight. CAP270, who did not exhibit TW10-specific responses, had low $CD4^+$ counts at 12 months postinfection (Fig. 2B). The absence of escape in TW10 despite consistent targeting may have resulted in intermediate $CD4^+$ counts in this individual, whereas individuals whose viruses escaped in TW10 under consistent selection pressure had higher $CD4^+$ counts. Although it is difficult to draw broad conclusions, the data support an important role for TW10 targeting in B*58:01-mediated control of subtype C infection. Even though the HLA-B*58:01 allele was not broadly associated with lower viral loads, its association with better preservation of $CD4^+$ cell counts may provide long-term clinical advantages to infected individuals (11, 23, 28). HLA-B*58:01-positive individuals who acquired the T242N mutation within TW10 maintained higher $CD4^+$ cell counts up to 3 years postinfection.

The current study had the advantage of monitoring infected individuals very close to their time of infection, enabling the determination of mutational dynamics within immunodominant epitopes. Escape from the immunodominant TW10 epitope was rapid, with escape mutations emerging as early as 2 weeks postinfection, confirming strong selection pressures on the virus exerted by HLA-B*58:01. In all cases, emergence of T242N was preceded by another mutation within the epitope (Fig. 1). This may suggest that (i) the route to escape is via intermediate mutations; (ii) the T242N mutation, being at the HLA binding site, provides the best selective advantage to the virus; and/or (iii) the T242N mutation incurs a lower replicative fitness cost than do the other mutations observed in the epitope. The fact that all the variants detected prior to the emergence of the T242N mutation are only rarely found in circulating subtype C sequences (I243, 0%; H244, 0.24%; P244, 0%; T244, 0%; E246, 0%; V247, 7.26%; T248, 10.65%; Q248, 1.21%: www.hiv.lanl.gov) is consistent with the notion that they are escape intermediates and that, compared to the TW10 mutation, they provide inferior selective advantages with respect to balancing the demands of CTL escape fitness costs.

In CAP268, despite strong TW10-specific CTL responses and selection of an upstream compensatory mutation at Gag codon 223, no escape mutations occurred within TW10 up to 31.4 months postinfection. Although the lack of escape in CAP268 could have been due to the lack of preexisting compensatory mutations, it is possible that the M250I mutation may have prevented TW10 escape due to fitness constraints, as it tends to be mutually exclusive with the T242N mutation (21). It is unlikely that the M250I mutation is a processing escape mutation, as previously suggested (21), as CAP268 displayed a strong ELISpot response to the TW10 epitope in the presence of the mutation. If the autologous virus had escaped through the M250I processing mutation, then TW10-specific CTL responses would have waned.

The other individual who did not develop escape mutations within TW10, CAP270, also did not show any detectable responses to this epitope. This individual had a very high viral load and a low $CD4^+$ count at 12 months postinfection (1,680,000 RNA copies ml^{-1} and 255 cells μl^{-1}, respectively) and was classified as a rapid progressor. This supports previous reports suggesting that control of viral replication by HLA-B*57/B*58:01-positive individuals is primarily through immune targeting of the functionally and structurally constrained TW10 epitope (14, 18). The reason for the lack of TW10 responses in CAP270 is unclear. However, it is possible that host factors associated with epitope processing or presentation may influence epitope targeting and disease progression. Further characterization of the differences between HLA-B*57/B*5801-positive individuals to distinguish TW10 responders from nonresponders may unravel the mechanisms behind effective control of viremia. A recent study by Dinges et al. reported that HLA-B*57/B*58:01 individuals who lacked responses to the conserved epitopes had higher plasma viral loads (10), suggesting that it is the precise genomic region targeted rather than the allele *per se* that is predictive of disease progression. Here we show that specific targeting of the highly conserved TW10 epitope in B*58:01-positive individuals contributes to this association. Our study further illustrates that escape patterns may contribute to disease progression.

Escape mutations arising in other B*58:01-restricted Gag and Nef epitopes (other than TW10) were not obviously associated with differences in disease progression, although responses to these epitopes may still have had subtle influences. It is noteworthy that only one HLA-B*58:01-positive subject (CAP268) targeted an additional B*57/B*58:01-restricted immunodominant epitope in $p24^{Gag}$ (KF11) with no evidence of escape in this epitope, suggesting that additional targeting of a p24 epitope may have contributed to the low viral load observed in this individual. The absence of responses targeting other p24 immunodominant epitopes (namely, ISW9, KF11, and QW9) in the remaining B*58:01-positive individuals may be due to the observed hierarchy of B*58:01 responses in subtype C infection, where TW10 represents the primary target during early infection and while other epitopes are targeted only later (15).

Early CTL escape in the TW10 was likely facilitated by the preexistence of compensatory mutations in the transmitted viruses. The early escape, due to the preexistence of compensatory mutations, enables HIV to evade immune recognition at a low fitness cost. This may result in high viral loads at set point, causing rapid disease progression. Notably, the high frequency of detection of compensatory mutations in the circulating viruses compared to viruses sampled historically from the same region suggests that their background frequencies are increasing, possibly foreshadowing a diminishing protective effect of the B*57/B*58:01 alleles in this population over time.

Although our study identifies associations between disease progression and virological and immunological factors, it has several limitations. Other factors such as host genetics which

may have influenced disease progression were not examined here. While we examined immune responses to all HIV-1 proteins, we examined sequences only from Gag and Nef. It is conceivable that unstudied escape mutations elsewhere in the genome could have contributed to disease progression. Our reason for focusing on Gag and Nef was their immunodominance and the fact that responses targeting Gag have in particular been associated with better disease outcomes (16, 18). In addition, we considered only IFN-γ secretion, measured via ELISpot assay, as an indicator of HIV-1-specific responses. Not all activated HIV-1-responsive $CD8^+$ T cells produce IFN-γ, however; thus, the HIV-specific responses measured here are most likely an underestimation of the total responses observed *in vivo*. Also, consensus subtype C peptides were used in the ELISpot assays, possibly resulting in a further underestimation of responses due to mismatches between the autologous and consensus peptide sequences. Lastly, we examined only six B*58:01-expressing individuals, and it would therefore be of interested to confirm these findings in a larger data set.

Our study shows that the B*58:01 allele plays a key role in disease progression and that this influence is largely attributable to the targeting of constrained epitopes, TW10 in this case. Our results also suggest that the presence of high frequencies of compensatory mutations at the population level may limit the protective value of B*58:01. Finally, our study further emphasizes the notion that the design of HIV vaccine immunogens should take into consideration predictable patterns of immune evasion.

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