Differential Escape Patterns within the Dominant HLA-B*57:03-Restricted HIV Gag Epitope Reflect Distinct Clade-Specific Functional Constraints

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ABSTRACT

HLA-B*57:01 and HLA-B*57:03, the most prevalent HLA-B*57 subtypes in Caucasian and African populations, respectively, are the HLA alleles most protective against HIV disease progression. Understanding the mechanisms underlying this immune control is of critical importance, yet they remain unclear. Unexplained differences are observed in the impact of the dominant cytotoxic T lymphocyte (CTL) response restricted by HLA-B*57:01 and HLA-B*57:03 in chronic infection on the Gag epitope KAFSP EVIPMF (KF11; Gag 162 to 172). We previously showed that the HLA-B*57:03-KF11 response is associated with a >1-log-lower viral setpoint in C clade virus infection and that this response selects escape mutants within the epitope. We first examined the relationship of KF11 responses in B clade virus-infected subjects with HLA-B*57:01, we observed strong selection pressure driven by the HLA-B*57:01-KF11 response for B clade virus-infected subjects expressing HLA-B*57:01, we observed strong selection pressure driven by the HLA-B*57:01-KF11 response for the escape mutation S173T. This mutation reduces recognition of virus-infected cells by HLA-B*57:01-KF11 CTLs and is associated with a >1-log increase in viral load in HLA-B*57:03-positive subjects (P = 0.009). We demonstrate functional constraints imposed by HIV clade relating to the residue at Gag 173 that explain the differential clade-specific escape patterns in HLA-B*57:03 subjects. Further studies are needed to evaluate the role of the KF11 response in HLA-B*57:01-associated HIV disease protection.

IMPORTANCE

HLA-B*57 is the HLA class I molecule that affords the greatest protection against disease progression in HIV infection. Understanding the key mechanism(s) underlying immunosuppression of HIV is of importance in guiding therapeutic and vaccine-related approaches to improve the levels of HIV control occurring in nature. Numerous mechanisms have been proposed to explain the HLA associations with differential HIV disease outcome, but no consensus exists. These studies focus on two subtypes of HLA-B*57 prevalent in Caucasian and African populations, HLA-B*57:01 and HLA-B*57:03, respectively. These alleles appear equally protective against HIV disease progression. The CTL epitopes presented are in many cases identical, and the dominant response in chronic infection in each case is to the Gag epitope KF11. However, there the similarity ends. This study sought to better understand the reasons for these differences and what they teach us about which immune responses contribute to immune control of HIV infection.
HIV-1 clade B virus-infected subjects expressing HLA-B*57:01 were studied from antiretroviral therapy (ART)-naive cohorts in Oxford, United Kingdom (the Thames Valley Cohort, as previously described [20]), and in Barcelona, Spain [21]. Additional study cohorts for evaluation of HLA-B*57:03 in the context of B clade and C clade virus infection in Barbados and Botswana, respectively, were (i) Bridgetown, Barbados (B clade; n = 246; median age, 38 years [interquartile range, 31 to 47]; female/male ratio, 60:40; samples collected between 2008 and 2010), where study subjects were attendees at the Ladymeade Reference Clinic, and (ii) Gaborone, Botswana (C clade; n = 514; median age, 27 years [interquartile range, 23 to 32]; female/male ratio, 100:6; samples collected between 2007 and 2008), where study subjects were antenatal women from the Mma Bana Study, as previously described (8, 13, 22, 23).

Ethics approval was given by the Health Research Development Committee, Botswana Ministry of Health, by the Barbados Ministry of Health, the Hospital Germans Trias i Pujol Ethics Committee, and by the Oxford Research Ethics Committee. Subjects received voluntary testing and counseling, and written informed consent was obtained from all individuals. Viral load in chronic infection was measured using the Roche Amplicor, version 1.5, assay; CD4+ T-cell counts were measured by flow cytometry. Viral load and absolute CD4 count measurements were obtained at study entry (baseline) for all individuals. All study subjects were ART naive.

Four-digit HLA typing of the class I locus was performed from genomic DNA as previously described [24] by sequence-based typing at the American Society for Histocompatibility and Immunogenetics (ASHI)-accredited HLA typing laboratory, University of Oklahoma.

MATERIALS AND METHODS
Study cohorts and subjects. HIV-1 B clade virus-infected subjects expressing HLA-B*57:01 were studied from antiretroviral therapy (ART)-naive cohorts in Oxford, United Kingdom (the Thames Valley Cohort, as previously described [20]), and in Barcelona, Spain [21]. Additional study cohorts for evaluation of HLA-B*57:03 in the context of B clade and C clade virus infection in Barbados and Botswana, respectively, were (i) Bridgetown, Barbados (B clade; n = 246; median age, 38 years [interquartile range, 31 to 47]; female/male ratio, 60:40; samples collected between 2008 and 2010), where study subjects were attendees at the Ladymeade Reference Clinic, and (ii) Gaborone, Botswana (C clade; n = 514; median age, 27 years [interquartile range, 23 to 32]; female/male ratio, 100:6; samples collected between 2007 and 2008), where study subjects were antenatal women from the Mma Bana Study, as previously described (8, 13, 22, 23).

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Health Sciences Center, USA. Exons 2 and 3 of HLA class I were amplified by locus-specific PCR and then sequenced. Resolution of ambiguities was undertaken according to the ASHI committee recommendations.

Additional viral sequence analyses were performed on two previously described multicenter cohorts: (i) the international HIV Adaptation Collaborative (IHAC), consisting of 1,443 clad B Gag sequences (12), and (ii) 1,470 African clad C Gag sequences from cohorts based in Durban (8), Bloemfontein (25), and Kimberley (20), South Africa, Zambia, and the Thames Valley area of the United Kingdom (20). Where high-resolution HLA typing was unavailable, we employed a published machine learning algorithm trained on a data set of high-resolution HLA class I types from >13,000 individuals with known ethnicity to complete these data to high resolution (26).

IFN-γ ELISPOT assays. IFN-γ enzyme-linked immunospot (ELISPOT) assays were performed as previously described (13, 27), using optimally defined epitopes and 18-mer overlapping peptides (OLP) with input cells/ well ranging from 30,000 to 100,000. The number of specific spot-forming cells (SPC) was calculated by subtracting the mean number of spots in the negative-control wells from the number of spots counted in each well. The magnitude of epitope-specific responses was calculated as SPC per million cells.

Site-directed mutagenesis of NL43. The mutation S173T (serine to threonine at Gag HXB2 position 173) was introduced by site-directed mutagenesis (QuikChange II; Stratagene, United Kingdom) into wild-type NL43 plasmid DNA, as well as NL43 containing the mutations A163G and/or S165N (14). Whole plasmid DNA was PCR-amplified from two overlapping primers containing the target mutation. Primers used for the mutagenesis reaction were F 5′-CCCCAGAATATCCGCGTATATCACGAGGACG-3′ and R 5′-GCTGCTCTCGATAATGCCGTAAACATGGGTATTACTTCTGGG-3′ (the mutagenesis site is underlined). The presence of mutations was verified by DNA Gag sequencing in newly generated plasmid clones. The DNA fragment ranging from the Spl to Apal restriction sites was then cloned into a new plasmid vector to avoid potential carryover of additional mutations during the mutagenesis, and the coding region sequence was verified again as previously described (28).

Virus production and replication kinetics. Viral stocks were produced by cotransfection of the different site-directed mutant plasmids (5′-half strain HIV-1NL43) with p83-10EGFP (3′-half strain HIV-1NL43) into MT4 cells (29). Viral stocks were harvested and viral RNA was extracted (Qiagen, United Kingdom). The gag p24/p17 coding region was PCR amplified and sequenced to confirm the presence of the mutations contained in the plasmid DNA. Primers used for the mutagenesis reaction were F 5′-CCCAGAATATCCGCGTATATCACGAGGACG-3′ and R 5′-GCTGCTCTCGATAATGCCGTAAACATGGGTATTACTTCTGGG-3′ (the mutagenesis site is underlined). The presence of mutations was verified by DNA Gag sequencing in newly generated plasmid clones. The DNA fragment ranging from the Spl to Apal restriction sites was then cloned into a new plasmid vector to avoid potential carryover of additional mutations during the mutagenesis, and the coding region sequence was verified again as previously described (28).

Amplification and sequencing of proviral DNA. Gag p17/p24 sequences (from the Barbados cohort, n = 125; from the Botswana cohort, n = 322) were generated from genomic DNA extracted from peripheral blood mononuclear cells (PBMCs) where available and amplified by nested PCR using previously published primers to obtain population sequences, as previously described (31). Sequencing was undertaken using the BigDye Ready Reaction Terminator Mix (v3.1) (Applied Biosystems, United Kingdom). Sequences were analyzed using Sequencher v4.8 (Gene Codes Corporation) and aligned by Secl to the HXB2 B clade reference strain.

Identification of HLA-associated viral polymorphisms from proviral DNA. HLA-associated viral polymorphisms were identified from proviral DNA using a previously described method that corrects for phylogeny, HLA linkage disequilibrium, and codon covariation (8, 32). A Q value statistic, representing the P value analogue of the false discovery rate (FDR), was computed for each association. The FDR is the expected proportion of false positives among the associations identified at a given P value threshold; for example, among associations for which the Q value is ≤0.2, we expect 20% to be false positives. The phylogenetically corrected methods rely on an inferred phylogeny. We constructed two phylogenies for this study: (i) a phylogeny consisting of clad B and C sequences from Barbados and Botswana constructed using Phyml v2.4.5, under the general time reversible (GTR) model (33), and (ii) a phylogeny consisting of 3,298 p17/p24 sequences from all cohorts described for this study. This phylogeny was too large for Phyml, so we employed a 3-stage process to infer the phylogeny. In the first stage, a combined alignment was created, then sites with >10% missing data were removed, after which sequences with missing data in >10% of remaining sites were removed (resulting in the above-noted total). In the second stage, a phylogeny was inferred separately for clad B and C alignments, using Phyml v2.4.5 under the GTR model. Finally, in the third stage, the resulting phylogenies were joined by adding a single common ancestor to the two clad trees, and the branch lengths were optimized using hyphy, under the GTR model (34).

Phylogenetically corrected odds ratio. Identification of HLA-associated polymorphisms and assessment of differential escape between viral clades and/or closely related HLA alleles were performed as previously described (12, 32, 35). Briefly, a maximum likelihood phylogenetic tree was constructed for each gene, and a model of conditional adaptation was inferred for each observed amino acid at each codon (32). In this model, the amino acid is assumed to evolve independently along the phylogeny until it reaches the observed hosts (tree tips). In each host, the HLA-mediated selection pressure is modeled using a weighted logistic regression, in which the individual’s HLA repertoire is used as a predictor and the bias is determined by the transmitted sequence (35). Because the transmitted sequence is not observed, we average over the possible transmitted sequences, and all possible phylogenetic histories, as inferred from the phylogeny. Similarly, where high-resolution HLA types are not available, we perform a weighted average over possible completions (12).

To test for differential escape between HLA-B*57:01 and B*57:03, or to test for clade-specific effects on selection, interaction variables were added to the phylogenetically corrected logistic regression model and significance was determined via a likelihood ratio test, as previously described (35).

Effect of S173T mutation on epitope recognition by KF11-specific CD8+ T cells. CD8+ T cells were enriched from PBMCs from healthy donors expressing HLA-B*57:03 using negative selection (Dynabeads) and activated for 3 to 6 days using interleukin 2 (IL-2) (50 U/ml; Roche) and phytohemagglutinin (PHA) (3 µg/ml). KF11-specific CD8+ T cells (<98% specificity) were enriched from PBMCs from HIV-infected donors using tetramers as previously described (36). B*57:03-positive CD8+ T cells were infected with NL43EGFP or NL43EGFP containing the S173T mutation as described above. To test for epitope recognition, epitope-specific CD8+ T cells (<98% specificity) were cocultured with the HIV-infected CD4+ T cells in the presence of CD107a antibodies (phycoerythrin [PE]-Cy5), 10 µg/ml of brefeldin A, Golgi stop (BD), CD49d, and CD28 for 6.5 h at 37°C in a 5% CO2 incubator. Cells were stained for surface and intracellular antibodies against CD4 (allophycocyanin [APC]), CD8 (Alexa Fluor 700), MIP1B (fluorescein isothiocyanate [FITC]), p24 (PE), gamma interferon (IFN-γ) (PE-Cy7), and LIVE/DEAD marker (Pacific blue) and then immediately acquired by FACS (BD LSRII).

Nucleotide sequence accession numbers. Sequences obtained in this study were submitted to GenBank under accession numbers FJ497801 to
RESULTS

B clade HLA-B*57:01-KF11 responders have higher viral setpoints than nonresponders. Previous studies of B clade virus-infected individuals expressing HLA-B*57:01 have suggested that a detectable HLA-B*57:01 response is more frequently observed in progressors (including those with viral loads of $>90,000$) than in elite controllers or long-term nonprogressors ($2, 11$). These studies, however, were not sufficiently powered to demonstrate a statistically significant result. We therefore started by comparing responses to KF11 in B clade virus-infected, ART-naive individuals expressing HLA-B*57:01 whose viral setpoints ranged from undetectable to 500,000 copies/ml (Fig. 1). Here the association between KF11 responders and high viral setpoint reaches statistical significance ($P = 0.02$, Mann-Whitney test). These findings are consistent with the earlier studies cited of B clade virus-infected subjects expressing HLA-B*57:01 and provide a result opposite that obtained in HLA-B*57:03-positive individuals infected with C clade virus ($13$), using the identical approach of measuring IFN-γ ELISPOT responses to KF11, where a response was associated with a $>10$-fold-lower viral setpoint. Equivalent studies of KF11 responses in 17 HLA-B*57:03-positive subjects infected with B clade virus similarly showed substantially lower median viral loads in KF11 responders than in nonresponders (median viral loads, 1,629 and 6,127 copies/ml, respectively), although here this difference did not reach statistical significance ($P = 0.28$ [data not shown]).

Differential escape in the B*57:03-KF11 epitope in B clade versus C clade virus infection. In order to evaluate further the potential differences between HLA-B*57:01 and HLA-B*57:03, we investigated a B clade virus-infected, ART-naive study cohort in Barbados, where HLA-B*57:03 is highly prevalent. It has been noted in several other studies that HLA-B*57:03 is associated with immune control of HIV in B clade and C clade virus infection ($1–5$). Consistent with these studies, HLA-B*57:03-positive subjects in Barbados exhibited significantly lower median viral loads than HLA-B*57:03-negative subjects (median, 3,450 versus 13,350 [$P = 0.015$, Mann-Whitney test]) and significantly higher CD4$^+$ counts (median, 565 versus 398 [$P = 0.003$, Mann-Whitney test]) (Fig. 2).

To determine the nature of any selection pressure imposed on the B clade virus through the HLA-B*57:03 KF11 response, we analyzed viral sequences in gag in the Barbados cohort in order to identify associations between HLA-B*57:03 and viral polymorphisms in the region of the KF11 epitope. This revealed that HLA-B*57:03 expression was associated with the previously described escape mutations T242N, in epitope TW10 (TSTLQEIQGW; Gag HXB2 240 to 249) ($7, 37$), and I147X, in the epitope ISW9 (ISPR TLNAW; Gag HXB2 147 to 155) (Table 1) ($38, 39$). However, the intraepitope escape mutations within KF11 (KAFSPEVIPMF; Gag HXB2 162 to 172), namely, A163G and S165N, selected in approximately 70% of C clade virus-infected HLA-B*57:03-positive sub-

![Median viral load of KF11 responders versus nonresponders in B clade virus-infected individuals expressing HLA-B*57:01. PBMCs from B clade virus-infected, ART-naive individuals expressing HLA-B*57:01 were analyzed by IFN-γ ELISPOT assay for responses to the KF11 epitope. Viral loads of responders and nonresponders were compared. Mann-Whitney U tests were performed.](https://jvi.asm.org/content/88/9/4671)

![Median viral load and CD4 count of B*57:03-positive versus B*57:03-negative subjects in the Barbados cohort (B clade). B*57:03-positive subjects were compared to B*57:03-negative subjects for viral load (A) and CD4 count (B). Medians and 5th to 95th percentiles are shown. Mann-Whitney U tests were performed.](https://jvi.asm.org/content/88/9/4671)
TABLE 1 HLA-B*57:03-associated polymorphisms in Gag p17 and p24 (Barbados cohort)

<table>
<thead>
<tr>
<th>Protein</th>
<th>HLA</th>
<th>Polymorphism location</th>
<th>HXB2</th>
<th>P value</th>
<th>Q value</th>
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<tr>
<td>p17</td>
<td>B*5703</td>
<td>ASVLSGGEELDWEKIRLRPGG</td>
<td>15</td>
<td>0.00211</td>
<td>0.115</td>
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<tr>
<td></td>
<td>B*5703</td>
<td>QPSLQVGEESLSKSLNTVATL</td>
<td>75</td>
<td>0.00388</td>
<td>0.14</td>
</tr>
<tr>
<td>p24</td>
<td>B*5703</td>
<td>NLOGQMVHAASPRTLNAVYK</td>
<td>147</td>
<td>0.00544</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>B*5703</td>
<td>AESPEVPMPSALSQGATPOD</td>
<td>173</td>
<td>0.000301</td>
<td>0.0218</td>
</tr>
<tr>
<td></td>
<td>B*5703</td>
<td>RGSIDAITVTSQEOIHMGN</td>
<td>242</td>
<td>3.02E-11</td>
<td>6.56E-09</td>
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TABLE 2 HXB2 sequences in B*5703-positive and -negative subjects (Barbados cohort)

<table>
<thead>
<tr>
<th>HXB2 position</th>
<th>B*5703 positive (n=15)</th>
<th>B*5703 negative (n=110)</th>
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<tr>
<td></td>
<td>n</td>
<td>%</td>
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<tr>
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Impact of S173T on recognition of virus-infected target cells and on viral setpoint. The location of the HLA-B*57:03-associated mutation immediately downstream of the KF11 epitope suggests that the S173T mutation reduces processing of the epitope. To test whether the HLA-B*57:03-associated S173T polymorphism reduces recognition of virus-infected target cells, CD4+ T cells from HLA-B*57:03+ healthy subjects were infected with NL43 HIV that was either wild type, expressing Ser-173, or engineered to express the S173T viral polymorphism. Infected cells were incubated with HLA-B*57:03-KF11-specific CD8+ T cells (>98% specific), and the level of CD8+ T-cell activation was monitored by CD107a and MIP1β expression. We observed that the S173T mutant indeed significantly reduced recognition by the KF11-specific CD8+ T cells (Fig. 3) (P = 0.0038, Student’s t test). In the same assay, using CD8+ T cells specific for the HLA-B*57:03-restricted Pol-specific epitope IATESIWI (IAW9), no difference were observed in the level of stimulation by the two viruses on the HLA-B*57:03-restricted IAW9-specific CD8+ T cells (Fig. 3). These data support the hypothesis that S173T specifically reduces presentation of the KF11 epitope by HLA-B*57:03. Furthermore, mismatched CD4+ T cells induced consistently low levels of stimulation, confirming that activation of the KF11- and IAW9-specific CD8+ T cells was HLA-B*57:03 dependent.

We next examined the viral setpoints and CD4 counts in HLA-B*57:03-positive subjects with and without the S173T mutation. Viral loads in HLA-B*57:03-positive subjects with the B clade wild type, serine at Gag 173, were more than 10-fold lower than in B*57:03-positive subjects with the S173T polymorphism (median viral loads, 520 and 6,905, respectively [P = 0.009, Mann-Whitney test]). Furthermore, Ser-173 was associated with a substantially higher CD4 count in HLA-B*57:03-positive subjects with the S173T mutation (median CD4 counts, 878 and 375, respectively [P = 0.036, Mann-Whitney test]) (Fig. 4). However, no differences in median viral load or CD4 counts were observed in B*57:03-negative subjects with serine versus threonine at Gag 173 (median viral loads, 14,450 and 10,600, respectively [P = 0.949], and median CD4 counts, 358 and 374, respectively [P = 0.522, Mann-Whitney test]). These data together support the conclusion that HLA-B*57:03-KF11 responses drive the selection of the S173T mutation in B clade virus-infected individuals expressing HLA-B*57:03 and that this is an escape mutation in that it reduces recognition of virally infected targets. These findings are consistent with the hypothesis that this response contributes to HLA-B*57:03-associated control
of HIV, since viral loads are significantly higher in those with the S173T escape mutation.

S173T with A163G and S165N significantly reduces viral replicative capacity. The observations described above prompted the following question: if A163G and S165N are escape mutations frequently selected in HLA-B*57:03-positive subjects infected with C clade virus, why are they not selected in HLA-B*57:03-positive subjects with B clade virus infection? To assess the functional significance of the HLA-B*57:03-associated S173T mutation and the possible impact of this polymorphism on the selection of A163G and S165N, the viral polymorphisms S173T, A163G, and S165N were introduced by site-directed mutagenesis into the B clade virus backbone of NL43GFP. Infectious viral stocks were generated by transfecting MT4 T cells with the relevant DNA constructs. H9, MT4, or Jurkat T cells were then infected, and the rate of viral growth was determined by monitoring the percentage of NL43GFP-infected cells over 14 days.

Analysis of the rate of viral growth in MT4, H9, and Jurkat T

<table>
<thead>
<tr>
<th>X (HXB2)</th>
<th>Country</th>
<th>Phylogenetically corrected odds ratio</th>
<th>P value</th>
<th>Difference in effect</th>
<th>P value</th>
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<tr>
<td>Asn-242</td>
<td>Botswana</td>
<td>4.9</td>
<td>3.26E−07</td>
<td>N</td>
<td>0.815</td>
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<td>Barbados</td>
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<td>Barbados</td>
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<td>Gly-163</td>
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<td>Thr-173</td>
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<td></td>
<td>Barbados</td>
<td>13.52</td>
<td>0.0033</td>
<td></td>
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</tbody>
</table>

*Presence of difference in B*5703’s effect on X in Botswana versus Barbados. N, no; y, yes.

FIG 3 Effect of viral mutation S173T on epitope recognition of HIV-infected cells by KF11-specific CD8⁺ T cells. Ex vivo CD4⁺ T cells from B*57:03⁻ and B*57:03⁺ donors were infected with wild-type NL43 virus or NL43 virus harboring the S173T viral mutation. Infected CD4⁺ T cells were then cultured with KF11-specific CD8⁺ T cells (A) or IAW9-specific CD8⁺ T cells (B), and the level of CD8⁺ T-cell activation was monitored by expression of CD107 and Mip1B. Data from both experiments were standardized relative to percent recognition by wild-type virus (C). Experiments were performed in triplicate; means and standard deviations are shown. Student’s t-test was performed, *, P < 0.01; **, P < 0.001; ***, P < 0.0001. NS, not significant.
cells showed, first, that the S173T polymorphism had no significant effect on viral fitness in this *in vitro* system in any of the three cell lines used (Fig. 5 and data not shown). We previously showed that the introduction of A163G or A163G/S165N into the NL43 backbone significantly reduced viral replicative capacity, with S165N acting as a partial compensatory mutant for A163G that also completely abrogated recognition of KF11 (14, 15). In this study, we observed that the introduction of either A163G or S165N into the NL43 backbone in combination with S173T also significantly reduces viral spread but substantially more than in the absence of S173T. Furthermore, the combination of S173T and both of the KF11 mutations, A163G and S165N, dramatically reduced viral spread even further, indicating a significant cost to viral fitness of this combination of viral mutations in a B clade virus (Fig. 5). These data together suggest that the KF11 escape mutant S173T is more commonly selected in B clade virus-infected subjects expressing HLA-B*57:03 because the cost to replicative capacity is negligible, less than that resulting from A163G or S165N. Subsequent mutations in addition to S173T result in such a substantial reduction in replicative capacity, without any apparent amelioration from S165N to reduce these fitness costs, that these arise very rarely (Table 2).

As mentioned above, Gag Thr-173, the consensus in C clade viruses, arises at a significantly lower frequency in HLA-B*57:03-positive than in HLA-B*57:03-negative subjects in Botswana (P = 0.0062). A larger analysis of the KF11 epitope region of 1,899 C clade sequences confirmed that the presence of A163G, S165N, or both in combination was significantly associated with serine at position 173 and that this was the case for both HLA-B*57:03-positive and HLA-B*57:03-negative individuals (Fig. 6). Thus, although Thr-173 is the consensus in C clade viruses, it appears unfavorable in the context of the KF11 intraepitope escape mutations, supporting the findings in B clade viruses suggesting that this combination of mutations has a detrimental impact on viral fitness.

**DISCUSSION**

HLA-B*57:01 and HLA-B*57:03 are the two most protective HLA molecules against HIV disease progression in both B and C clade

![FIG 4](image-url) Median viral load and CD4 count of B*57:03-positive HIV-infected subjects with viral polymorphisms Ser-173 and Thr-173. Proviral DNA sequences from B*57:03-positive subjects from the Barbados cohort (B clade) were analyzed for the presence of the viral polymorphisms Ser-173 and Thr-173. Viral loads (A) and CD4 counts (B) were compared. Medians and 5th to 95th percentiles are shown. Mann-Whitney t tests were performed.

![FIG 5](image-url) Viral replication capacity of NL43GFP virus with multiple B*57:03-associated viral mutations. NL43GFP virus was engineered to contain combinations of the viral mutations Thr-173, Gly-163, and Asn-165. MT4 cells were infected and monitored for GFP-positive cells over 14 days (A). The slope of the curve was calculated from the exponential growth phase using the LOGEST function and converted to natural logs (B). Experiments were performed in triplicate; mean and standard deviations are shown. Dunnett’s multiple-comparison tests were performed. *, P < 0.01; **, P < 0.001; ***, P < 0.0001.
virus infection (5). These molecules differ by only two amino acids (D114N and S116Y), and the peptide binding motifs are almost indistinguishable (42, 43). In chronic infection, the dominant HIV-specific CD8+ T-cell response in subjects expressing HLA-B*57:01 or HLA-B*57:03 is to the Gag epitope KAFSPEVIPMF (KF11; Gag HXB2 162 to 172) (2, 10, 13). Studies of HLA-B*57:03-positive subjects infected with C clade virus indicate that this KF11 response makes an important contribution to immune control of HIV infection. The clade-specific differences in the selection of KF11-driven escape mutants observed in Barbados (B clade virus-infected cohort) and Botswana (C clade virus-infected cohort) were corroborated in analyses of larger data sets.

The position of S173T one residue downstream (P1’) of the KF11 epitope suggests that it may be a processing mutation, since this residue would be involved in the cleavage site of the proteasome (44). Previous studies of peptide cleavage motifs have suggested that the constitutive proteasome and immunoproteasome have a strong preference for alanine at P1’, but prefer serine over threonine; thus, the mutation S173T could affect efficient cleavage of the C-terminal end of the KF11 epitope by the proteasome (44).

We show that the HLA-B*57:03-associated S173T mutation effectively precludes further selection of the KF11 intraepitope viral mutations, A163G and S165N, since the combination of these three mutations in a B clade virus backbone results in a virus with a severely reduced replicative capacity. Indeed, the close proximity of the amino acid positions 173, 163, and 165 between helix 1 and helix 2 of the Gag p24 structure suggests that structural constraints prevent selection of A163G and S165N if S173T has already been selected. Previous work has shown that, using a B clade virus backbone, and in the presence of S173, the mutation A163G reduces replicative capacity but that the further addition of S165N, as observed in vivo, partially restores replicative capacity (14, 45). This fits with the order of selection of A163G and S165N, with S165N apparently always arising subsequent to A163G (14). However, in B clade virus infection, it appears that the selection of S173T prevents the selection of further mutants within the epitope because the fitness cost is too high. S173T appears to be the preferred choice of viral escape from the KF11-specific response, since it has minimal effect on viral fitness.

Our inference from the data described above and from previous studies (23) is that the HLA-B*57:03-KF11 response contributes to immune control of B and C clade HIV infection. The reduced recognition of S173T virus-infected cells by KF11-specific CTLs together with the lack of cost to viral replicative capacity resulting from S173T is consistent with the observation that viral loads are higher and CD4 counts lower in B clade virus-infected subjects expressing HLA-B*57:03.

In view of the substantial reduction in viral replicative capacity resulting from the A163G/S165N/S173T combination in B clade virus infection, it is perhaps surprising to observe the selection of A163G/S165N at high frequency in HLA-B*57:03-positive subjects infected with C clade virus in which the vast majority of sequences carry Thr at Gag 173. It may be inferred from this that the presence of consensus Thr-173 in the context of C clade virus Gag does not have the same prohibitive effect on viral fitness, as it does not prevent the selection of A163G and S165N. Nevertheless, in C clade virus infection, both in HLA-B*57:03-positive and in HLA-B*57:03-negative individuals, A163G/S165N are significantly associated with Ser at Gag 173 (Fig. 6), as opposed to the consensus Thr at this position, suggesting that the combination of
A163G/S165N/S173T is not favored in either B or C clade virus infection.

Gag 173 has been well studied in relation to HLA-B*27, another protective HLA molecule, because of the S173T mutation that accompanies the R264K escape variant within the dominant HLA-B*27-restricted epitope KRWIILGLNK (KK10) (46–48). It is noteworthy that in C clade virus infection, R264K escape in HLA-B*27-positive subjects is typically accompanied by compensatory mutations not at Gag 173 but at Gag 165 (Brener et al., unpublished data). These data underline the tight constraints on amino acid substitutions in the capsid protein, the interdependence of residues at certain key positions in the structure, including Gag 163, Gag 165, and Gag 173, and therefore the impact that clade can have on the escape options for the virus.

These data help to explain why HLA-B*57:03 is not associated with the “usual” KF11 intraepitope mutations A163G/S165N in clade B, but they do not explain why HLA-B*57:01 is not associated either with the S173T flanking mutation or with any of KF11 intraepitope mutations. Previous studies have suggested that TCR usage for the HLA-B*57:01-KF11 response allows recognition of the KF11 variants (15), but these initial TCR studies indicating conservation of a “public” HLA-B*57:01-KF11 TCR have not been borne out by subsequent studies (11, 18). One possible explanation is that the potency of the HLA-B*57:01-KF11 response is so great that a moderate reduction in processed epitope would not affect killing sufficiently to be selected; however, preliminary data suggest that the HLA-B*57:03 response is, if anything, the more potent. Further studies with a large number of KF11-specific clones would be needed to establish whether clear-cut differences between the responses restricted by HLA-B*57:03 and HLA-B*57:01 exist in terms of potency and the relevance of this to viral escape patterns. A recent study comparing the impact of individual HLA class I molecules on immune control (viral load < 2,000 copies/ml) versus noncontrol (viral load > 10,000 copies/ml) of B clade virus infection showed the identical odds ratio for protection via HLA-B*57:01 in a European American cohort and for that via HLA-B*57:03 in an African American cohort (6).

These studies therefore provide an explanation for the distinct clade-specific selection of escape mutants by the HLA-B*57:03-KF11 response but do not resolve the question of why the HLA-B*57:01-KF11 response does not select escape mutants. Insufficient studies have been undertaken with C clade virus-infected subjects who express HLA-B*57:01 to be certain of whether this response selects no escape mutants in C clade as well as in B clade virus infection. The absence of the KF11 response in elite controllers with HLA-B*57:01 does not necessarily mean that these responses have not contributed to immune control in these subjects, since it is possible that the period of detectability may be transient. It is clear that many responses that are undetectable in elite controllers can become detectable after peptide stimulation (49). However, if the KF11-specific CTL response contributes to immune control of HIV in HLA-B*57:01-positive subjects in B clade virus infection, it would be unique in failing to select escape mutants in the process and the mechanism would be invaluable for directing successful vaccine targets.

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