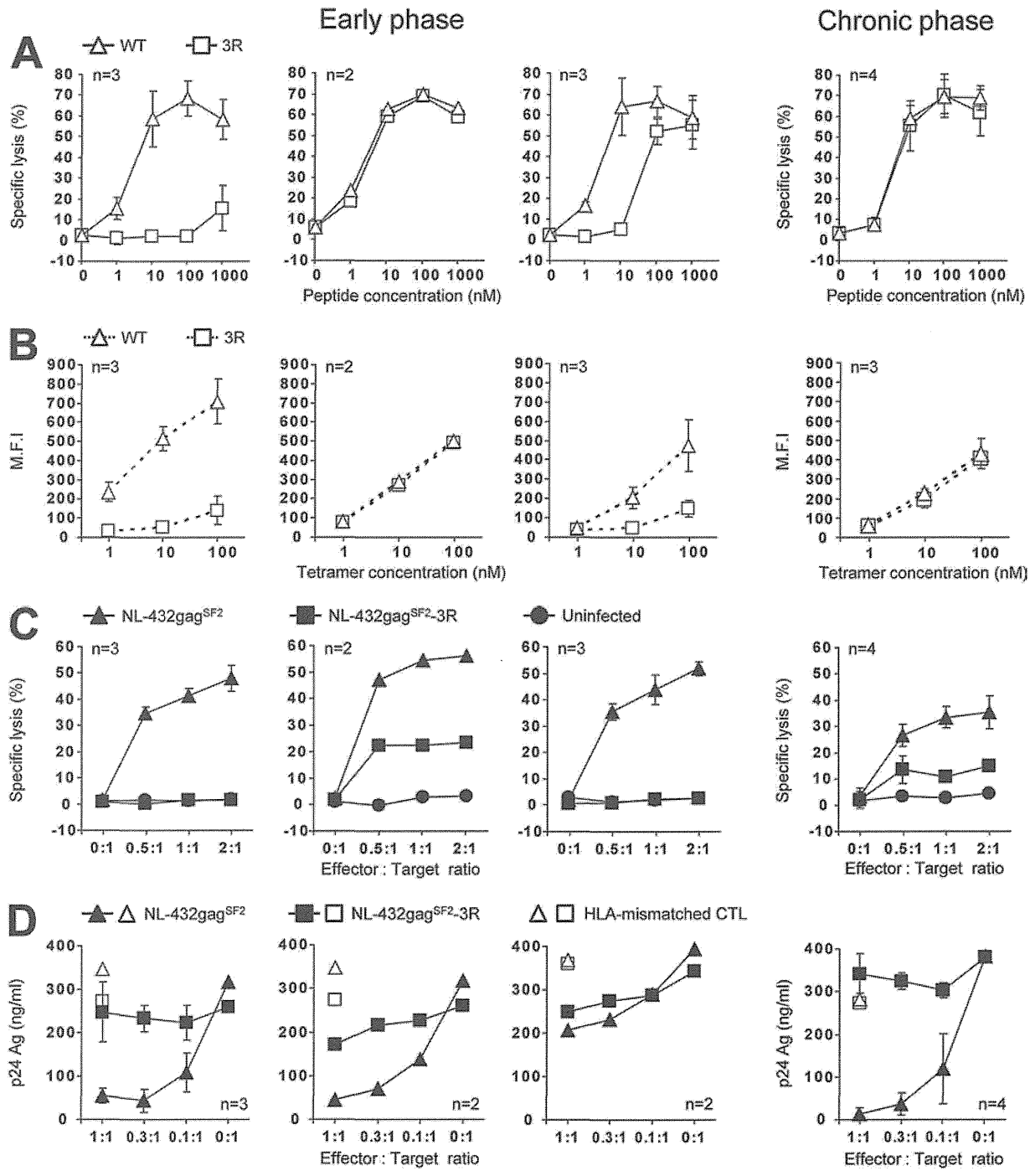

FIG 3 Antiviral activities of Gag28-specific CTL clones generated from PBMCs of patient KI-161, infected with WT virus. Gag28-specific CTL clones were generated from early-phase and chronic-phase PBMCs isolated from KI-161 after stimulating them with the WT and 3R peptides, respectively. Three types of Gag28-36-specific CTL clones, i.e., WT specific (left), cross-reactive (middle), and WT dominant (right), were generated from the early-phase PBMCs. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected (49.0% of total cells were p24 Ag$^+$) and 3R-virus-infected (50.0% of total cells were p24 Ag$^+$) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The ability was tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

been accumulating during the past 30 years in the Japanese population.

It is well known that some escape mutations affect replication capacity and that HIV-1 containing such mutations reverts to WT in individuals not carrying HLA class I restriction alleles (23, 32). We previously showed that the 3R mutation does not affect replication capacity when 2 T cell lines are used in an assay measuring it (46). Since a different effect of mutations on replication capacity

TABLE 3 Responses of CD8$^+$ T cells from individuals infected with 3R virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN-γ-producing cells specific for each peptide among CD8$^+$ T cells[a]		
	Early phase	Chronic phase			Without	WT	3R
KI-091	12/13/2000 (3R)	8/4/2005 (3R)	12/13/2000	WT	0.2	**74.6**	**71.2**
				3R	0.3	**55.4**	**71.9**
			9/29/2004	WT	0.2	**77.7**	**65.5**
				3R	0.2	**61.1**	**69.3**
KI-134	10/25/2001 (3R)	6/30/2004 (3R)	10/25/2001	WT	0.4	0.6	0.8
				3R	1.0	1.1	**5.7**
			1/21/2004	WT	0.8	1.0	0.7
				3R	0.7	0.6	**2.0**
KI-136	10/29/2001 (3R)	7/10/2003 (3R)	10/29/2001	WT	0.1	0.4	0.2
				3R	0.1	0.2	0.2
			5/15/2003	WT	0.4	0.8	0.4
				3R	0.1	0.2	**24.8**
KI-151	2/15/2002 (3R)	6/16/2005 (3R)	11/21/2001	WT	0.3	0.7	0.8
				3R	0.7	0.6	**10.8**
			7/28/2004	WT	0.4	0.7	1.3
				3R	0.1	0.1	**44.5**
KI-163	8/30/2002 (3R)	9/27/2004 (3R)	8/30/2002	WT	0.2	0.3	0.2
				3R	0.2	0.4	0.2
			8/29/2005	WT	0.3	0.5	0.2
				3R	0.4	0.6	**6.9**

[a] Without, without peptide. Boldface, positive IFN-γ-producing response.

between cell lines and CD4$^+$ T cells from a healthy individual is known (23), we measured the replication capacity of the 3R virus by using CD4$^+$ T cells from a healthy individual. The results confirm that this mutation did not affect the replication capacity (Fig. 6), suggesting that the 3R mutant could not revert in HLA-A*24:02$^-$ individuals.

DISCUSSION

It is known that CTLs recognizing escape mutants are elicited after the escape mutant had been selected by WT epitope-specific CTLs (2, 4, 12, 15, 33, 39) or in new escape mutant virus-infected hosts having the same restricted HLA allele (15). However, since the CTLs recognizing escape mutants have been not well analyzed, the role of these CTLs in the control of HIV-1 infections remains unclear. In the present study, we investigated 2 groups, HLA-A*24:02$^+$ individuals infected with WT virus and those infected with 3R escape mutant virus. We found that both WT-specific and cross-reactive CD8$^+$ T cells were elicited in individuals infected with WT virus. Interestingly, cross-reactive T cells had been elicited before the emergence of the 3R escape mutant virus, though a similar finding was made in previous studies that analyzed other epitope-specific CTLs (18, 25, 26, 34). The present study shows that WT-specific CD8$^+$ T cells were predominantly elicited in an early phase of the infection and that the number of cross-reactive CD8$^+$ T cells increased in the chronic phase. The CTL clones from early and chronic phases in KI-161 showed similar abilities to kill WT virus-infected or 3R virus-infected cells and activities to suppress both viruses, suggesting that cross-reactive CD8$^+$ T cells elicited at the early phase were expanded via antigen presentation by 3R virus-infected cells at the chronic phase.

WT-specific and cross-reactive CTL clones from KI-092 and KI-161 at an early phase of the infection effectively killed WT-virus-infected cells and suppressed the replication of the WT virus, whereas they exhibited no and weak ability, respectively, to suppress that of the 3R virus. Cross-reactive CTL clones had the same ability to suppress the replication of WT virus as did the WT-specific CTL clones. These results strongly suggest that both CTLs selected the 3R virus in these individuals infected with the WT virus. The 3R virus was not selected within at least 1 year after Gag28-specific CTLs had been detected in the individuals infected with the WT virus. This finding indicates that the 3R mutation was more slowly selected by these CTLs than escape mutants selected at an acute phase of the infection (16, 19, 34, 44, 45). On the other hand, a previous study suggested that acute accumulation of mutations in this epitope occurs after an HIV-1 infection (6). However, the data shown in that study concerned mutations contained at position 1 of the epitope. In addition, those data may have included cases in which the individuals had been infected with the 3R mutant virus, because it may be assumed that 3R virus had accumulated in the cohorts analyzed. Cross-reactive CTL clones established from PBMCs at both early and chronic phases of KI-161 killed 3R virus-infected cells, though the killing activity against the 3R virus-infected cells was weaker than that against the WT virus-infected cells. These CTL clones weakly suppressed the replication of the 3R virus (Fig. 3C). This weak ability to suppress it might have delayed the emergence of the 3R mutation in these patients.

WT-specific CTLs were not induced by stimulation of early- or chronic-phase PBMCs from the 5 individuals in which the 3R mutation had been detected at the early phase with WT peptides. This finding supports the possibility that these individuals had been infected with the 3R virus. Only KI-091 had cross-reactive T cells at early and chronic phases of the infection. All CTL clones established from this patient had cross-reactivity, implying that the patient had been infected with WT virus and that 3R had been selected at an early phase. However, WT-specific CTL clones were not established from this patient. In addition, the cross-reactive

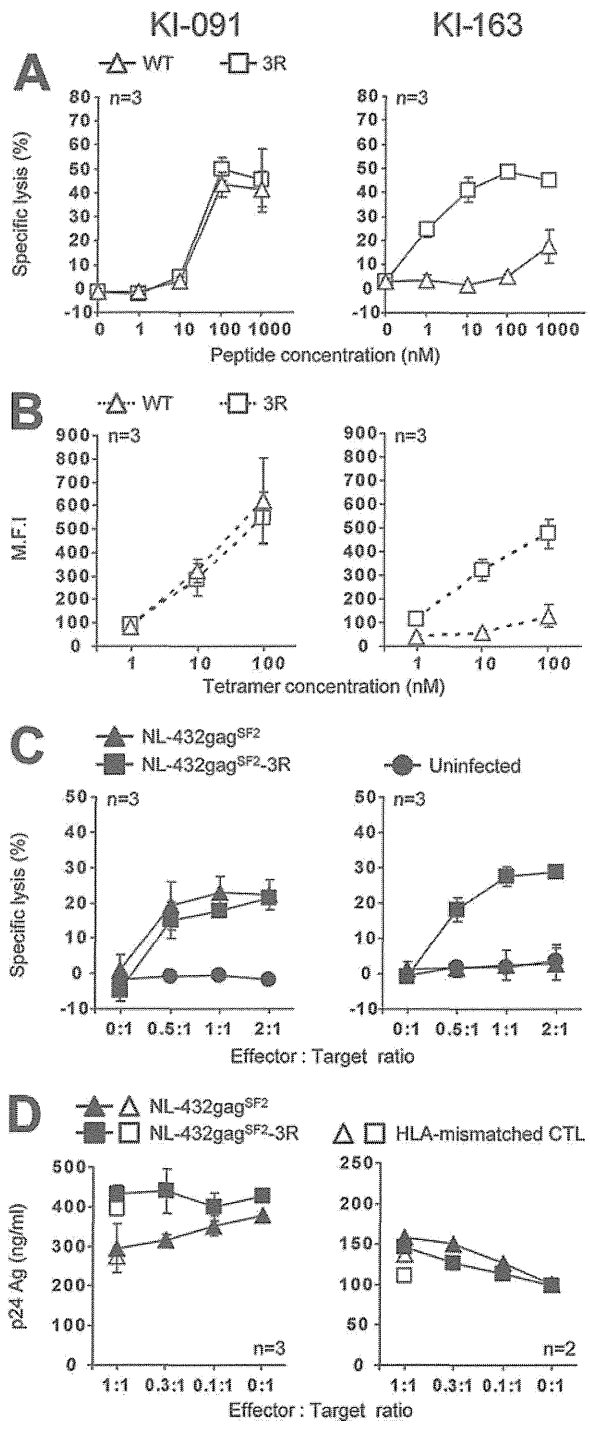

FIG 4 Antiviral activities of cross-reactive and 3R-specific CTL clones generated from patients KI-091 and KI-163 infected with 3R virus. Gag28-specific CTL clones were generated from chronic-phase PBMCs isolated from patients KI-091 and KI-163 after their stimulation with 3R peptide. The following activities of these CTL clones were analyzed. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected and 3R virus-infected cells were used as target cells. The frequency of p24 Ag$^+$ cells among the HIV-1-infected cells was as follows: WT-virus-infected cells, 49.1% and 43.1% for CTL clones from KI-091 and KI-163, respectively, and 3R-virus-infected cells, 48.6% and 45.6% for CTL clones from KI-091 and KI-163, respectively. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The abilities were tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

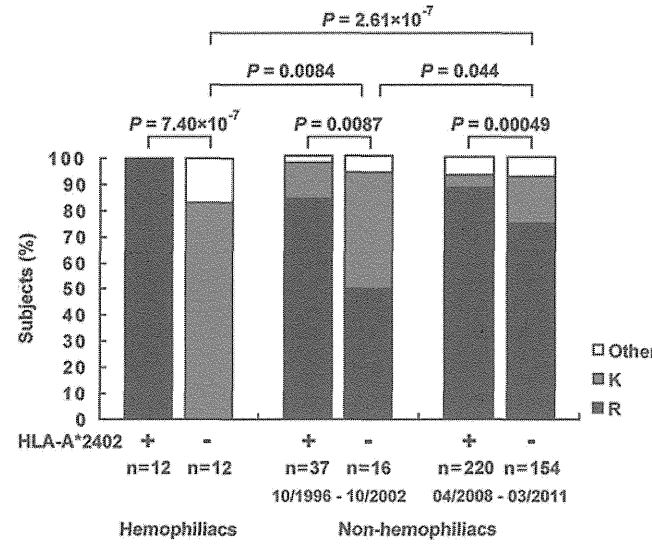

FIG 5 Frequencies of the 3R mutation in a Japanese hemophiliac cohort and nonhemophiliac cohorts recruited from 1996 to 2002 and from 2008 to 2011. The frequencies of mutations at position 3 of the Gag28 epitope in chronically HIV-1-infected HLA-A*24:02$^+$ or HLA-A*24:02$^-$ hemophiliac individuals and nonhemophiliac individuals recruited from 1996 to 2002 or from 2008 to 2011 are shown. The consensus sequence of this epitope in HIV-1 subtype B is KYKLKHIVW. The frequency of the 3R mutation between HLA-A*24:02$^+$ and HLA-A*24:02$^-$ subjects in each cohort or that in HLA-A*24:02$^+$ or HLA-A*24:02$^-$ subjects among the 3 cohorts was statistically analyzed by using Fisher's exact test.

CTL clones established from KI-091 did not have the ability to suppress the replication of the WT virus, although the CTL clones from individuals who had been infected with the WT virus had strong ability to suppress it. These findings suggest that this patient had been infected with the 3R virus rather than with the WT virus. However, it remains unknown why 3R-specific CTLs were elicited in the other 4 individuals but not in this patient. Thus, the abilities of CTLs to respond to WT peptide and to suppress the replication of WT virus together supported the idea that the individuals who had 3R virus in the early phase had been infected with 3R virus, although the possibility that they had been infected with WT virus cannot be completely excluded.

The 3R mutant epitope peptide would have been processed and presented to 3R-specific CTLs in 3R virus-infected cells, since 3R-specific and cross-reactive CTL clones effectively killed 3R virus-infected cells. However, these CTL clones failed to suppress the replication of the 3R virus. 721.221-CD4-A2402 cell lines were used as target cells for the killing assay, whereas CD4$^+$ T cells from healthy individuals were used for the replication suppression assay. The former cells express HLA-A*24:02 to a much higher degree than the latter cells. This difference between the 2 cell lines may account for the discrepancy of the results between the 2 assays. 3R-specific CTL clones failed to suppress the replication of the 3R virus, whereas cross-reactive CTLs from the individuals

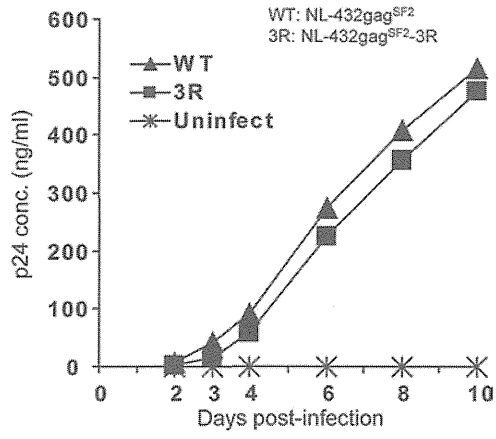

FIG 6 Replication kinetics of WT and 3R viruses in CD4$^+$ T cells. CD4$^+$ T cells (2×10^5) isolated from PBMCs from a healthy donor were infected with WT or 3R virus in triplicate at a blue-cell-forming unit of 500 (in MAGIC-5 cells) in a total volume of 0.2 ml and then incubated at 37°C for 2 h. The infected cells were washed twice with R10 and then cultured in 1 ml of complete medium plus rIL-2 at 37°C. A 0.1-ml volume of the culture supernatants was collected at days 2 to 10 postinfection. The concentration of p24 Ag was measured by using ELISA.

infected with WT virus effectively suppressed the replication of the WT virus but failed to suppress that of the 3R virus. These findings suggest that 3R virus-infected CD4$^+$ T cells could not effectively present the 3R mutant epitope. This finding also suggests that 3R virus-infected CD4$^+$ T cells were not the main source of antigen-presenting cells in 3R virus-infected individuals. A previous study showed that HIV-1-infected macrophages effectively present HIV-1 epitopes more than HIV-1-infected CD4$^+$ T cells (14), implying that 3R virus-infected macrophages are the main antigen-presenting cells and contribute to the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals. A further study should clarify the role of macrophages in the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals.

Cross-reactive CTLs were found in individuals infected with the WT virus or with the 3R virus. The CTL clones established from individuals infected with the WT virus had a strong ability to kill WT-virus-infected cells and to suppress the replication of the WT virus, whereas those established from an individual infected with the 3R virus showed moderate ability to kill WT-virus-infected cells and no ability to suppress the replication of WT virus. These findings indicate that cross-reactive CTLs from an individual infected with the 3R virus may have had less ability to recognize the WT epitope than those from an individual infected with the WT virus. Indeed, the former CTL clones exhibited lower sensitivity to reaction with WT peptide-pulsed cells than the latter CTLs, indicating that cross-reactive CTLs elicited in individuals infected with the WT virus had higher-affinity TCRs for WT peptide than those in an individual infected with the 3R virus. In addition, the latter CTL clones weakly killed 3R virus-infected cells, whereas the former clones showed the same killing activity against 3R virus-infected cells as against WT-virus-infected cells. Thus, cross-reactive CTLs in individuals infected with 3R virus have different characteristics than those in individuals infected with the WT virus. This finding suggests that cross-reactive CTLs elicited in individuals infected with the WT virus had TCRs with higher affinity for WT and 3R peptides than those in individuals infected with the 3R virus.

Japanese hemophiliacs were infected with HIV-1 via blood products from the United States around 1983, and HLA-A*24:02 is a rare allele in North America. Therefore, it may be speculated that HIV-1 in the blood product had not yet accumulated escape mutations. Indeed, the 3R mutation was not found in the 12 HLA-A*24:02$^-$ hemophiliacs tested, though other amino acid variants at position 3 were detected in 2 of these hemophiliacs. This mutation was found in 50.0% of HLA-A*24:02$^-$ individuals in the 1996 to 2002 cohort and in 74.7% of those in the 2008 to 2011 cohort, indicating that the mutation had accumulated in the Japanese population. The frequency of this mutation in HLA-A*24:02$^-$ individuals thus increased about 1.5-fold during the approximately 10-year period between these 2 nonhemophiliac cohorts. Thus, the mutation greatly accumulated over the last 10 years. Since HLA-A*24:02 is found in approximately 70% of Japanese, the high prevalence of the allele is the cause of the high accumulation of the 3R mutation in the Japanese population. In addition, this high accumulation resulted not only from a strong selection of the 3R mutation by WT-specific and cross-reactive CTLs elicited in the donors infected with WT virus, but also from a lack of reversion of the mutation in the HLA-A*24:02$^-$ individuals.

Our previous study concerning HLA-A*24:02-restricted Nef138-specific CTLs demonstrated that only WT epitope-dominant CTLs, which suppress the replication of WT virus but fail to suppress that of mutant virus, are elicited at an early phase in HLA-A*24:02$^+$ individuals infected with the WT virus and that mutant-epitope-dominant CTLs but not cross-reactive CTLs are elicited after the emergence of the mutant virus in them (15). In addition, only mutant-epitope-dominant CTLs are elicited in those individuals infected with the mutant virus. The mutant-epitope-dominant CTLs suppress the replication of WT virus but weakly suppress that of mutant virus (15). Thus, Nef138-specific CTLs elicited in individuals infected with WT or mutant viruses had different characteristics in terms of the recognition of WT and mutant epitopes than the Gag28-specific CTLs analyzed in the present study. The difference between Nef138-specific and Gag28-specific CTLs might be explained by a different CTL repertoire elicited at an early phase. These 2 studies suggest the elicitation of various HIV-1-specific CTLs in regard to recognition of escape mutations.

In the present study, we demonstrated that WT-specific and cross-reactive CTLs were elicited at an early phase in individuals infected with the WT virus and that cross-reactive CTLs were dominant in Gag28-specific CTLs after the emergence of the 3R virus. On the other hand, 3R-specific and cross-reactive CTLs were elicited in individuals infected with the 3R virus, though the former CTLs were predominantly elicited in these individuals. The CTLs elicited in the individuals infected with the WT virus, which had a strong ability to suppress the replication of WT virus, played a central role in the accumulation of the 3R mutation. In contrast, the CTLs elicited in those infected with 3R virus, which failed to suppress the replication of WT and 3R viruses, did not contribute to the control of the 3R virus infection. In addition, the high prevalence of HLA-A*24:02 and lack of effect of the 3R mutation on viral fitness may have strongly contributed to the high accumulation of the mutation in HIV-1-infeceted Japanese individuals.

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Minor contribution of HLA class I-associated selective pressure to the variability of HIV-1 accessory protein Vpu

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ABSTRACT

Host HLA class I (HLA-I) allele-associated immune responses are major forces driving the evolution of HIV-1 proteins such as Gag and Nef. The viral protein U (Vpu) is an HIV-1 accessory protein responsible for CD4 degradation and enhancement of virion release by antagonizing tetherin/CD317. Although Vpu represents one of the most variable proteins in the HIV-1 proteome, it is still not clear to what extent HLA-I influence its evolution. To examine this issue, we enrolled 240 HLA-I-typed, treatment naïve, chronically HIV-infected subjects in Japan, and analyzed plasma HIV RNA nucleotide sequences of the vpu region. Using a phylogenetically-informed method incorporating corrections for HIV codon covariation and linkage disequilibrium among HLA alleles, we investigated HLA-associated amino acid mutations in the Vpu protein as well as in the translational products encoded by alternative reading frames. Despite substantial amino acid variability in Vpu, we identified only 4 HLA-associations in all possible translational products encoded in this region, suggesting that HLA-associated immune responses had minor effects on Vpu variability in this cohort. Rather, despite its size (81 amino acids), Vpu showed 103 codon–codon covariation associations, suggesting that Vpu conformation and function are preserved through many possible combinations of primary and secondary polymorphisms. Taken together, our study suggests that Vpu has been comparably less influenced by HLA-I-associated immune-driven evolution at the population level compared to other highly variable HIV-1 accessory proteins.

1. Introduction

Immune-mediated adaptation occurs during an HIV-1 infection. The HLA class I (HLA-I)-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response is one of the major forces driving HIV evolution, resulting in the selection of CTL escape mutants [1,2]. Despite the extensive genetic diversity of both HIV-1 and HLA-I alleles, escape pathways are reproducible and broadly predictable based on host HLA-I alleles [3–6]. Moreover, analysis of linked HLA-I and HIV datasets from large cohorts of HIV-infected subjects has facilitated our ability to map the landscape of immune escape mutations across HIV-1, identify immunogenic regions, and identify novel CTL epitopes [3,7].

Viral protein U (Vpu) is an accessory protein that is unique to HIV-1 and a subset of related simian immunodeficiency viruses. The HIV-1 Vpu protein has two major functions: degradation of newly synthesized CD4 molecules in the endoplasmic reticulum and enhancement of the release of progeny virions from infected cells by antagonizing tetherin/CD317, a host restriction factor that directly binds and retains viral particles on the surface of infected cells (reviewed in [8,9]). As such, Vpu is thought to play a role in virus spread and pathogenesis *in vivo*. Interestingly, Vpu is the most variable protein among all HIV proteins as evidenced by a cross-sectional comparison of HIV-1 sequences isolated from HIV-infected individuals [10], raising the possibility that Vpu undergoes adaptation in response to host immune responses. However, Vpu has been shown to be a minor target for CTLs as revealed by IFN-γ Elispot assays with overlapping peptides based on the subtype B consensus sequence [11]. Considering the highly variable nature of Vpu, it is possible to miss responses if the autologous virus sequence is markedly different from the peptide sequence when using this Elispot assay system [12].

In the present study, we sought to identify HLA-associated polymorphisms in Vpu and alternate reading frames and examine to

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what extent they are involved in Vpu amino acid variability at the population level. We utilize a published phylogenetic dependency network model [13], a comprehensive evolutionary model that considers all important confounding effects such as HIV phylogeny, HIV codon covariation, and linkage disequilibrium of HLA alleles.

2. Materials and methods

2.1. Patient samples

A total of 240 chronically HIV-1-infected, treatment-naïve subjects (CD4, median 237; IQR, 160-397; viral load, median 33,200; IQR, 222,000-55,400) followed at the AIDS Clinical Center, International Medical Center of Japan were enrolled in this study. All participants provided written informed consent. HLA-I typing was performed as previously described [14]. The most frequently observed *HLA-A, B,* and *C* alleles in this cohort were *HLA-A*24:02*, *HLA-B*52:01*, and *HLA-C*01:02*, respectively, consistent with HLA class I allelic frequencies of the Japanese people [14].

2.2. Sequence analysis of vpu

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 15 min) of patients' plasma, after which the viral RNA was extracted using standard methods. Following reverse transcription, DNA fragments encoding Vpu proteins were amplified by nested PCR, and gel purified as previously described [15,16]. The primers used were as follows: the primers for the first round of amplification were VVVa-F (5′-TTAAAAGAAAAGGGGG GATTGGGGG-3′) and VVVb-R (5′-ATTCCATGTGTACATT GTACTGT-3′); and those for the second round, VVVc-F (5′-AGATAATAGTGAC ATAAAAGTAGTGCCAAGAAG-3′) and VVVd-R (5′-CCATAATAGACT GTGACCCACAA-3′). The *vpu* sequence was then directly analyzed with an automated sequencer (Applied Biosystems 3500xL) and aligned to the *vpu* sequence of the HIV-1 subtype B reference strain HXB2 (Accession No. K03455). More than 90% of the subjects were infected with subtype B, as determined by phylogenetic analysis of concatenated sequences of *vif*, *vpr*, and *vpu* reading frames.

2.3. Analysis of amino acid sequence variability

A Shannon entropy score for each position in the Vpu protein was calculated and used to analyze amino-acid sequence variability, as described previously [10]. Entropy is a measure of the amino acid variability at a given position that takes into account both the number of possible amino acid residues allowed and their frequency.

2.4. Analysis of association between Vpu sequence polymorphisms and host HLA class I alleles

To identify HIV-HLA polymorphism associations, we employed a phylogenetically dependency network model [13], which comprehensively includes all confounding effects of the analysis, such as HIV founder effects, HIV codon co-variation, and linkage disequilibrium of HLA-I alleles. Multiple comparisons are addressed using q-values (refer the detailed methods given in refs. [4,5,13]); in the present study, a cutoff of $q < 0.2$ was used to denote statistically significant associations. HLA-associated polymorphisms were classified into two categories. "Nonadapted" amino acids are enriched in the absence of the restricting HLA of interest. Usually, "nonadapted" forms represent the consensus amino acid at that position, and they can be thought of as the "wild-type" or "susceptible" form particular to that allele. Conversely, "adapted" amino acids are those enriched in the presence of the HLA allele; these can be thought of as the escape variant particular to that allele.

3. Results and discussion

3.1. Genetic variability of the vpu gene

We successfully amplified DNAs encompassing the *vpu* region from 216 of 240 samples (90%). Firstly, we analyzed the amino acid variability at each codon of Vpu by determining its Shannon entropy score. Two amino acid residues, Trp23 and Arg49, showed highly conserved (>98%) among individual sequences. Instead, most codons displayed substantial variability, with the average of the entropy score reaching 0.58 (Fig. 1A), confirming the findings by Yusim et al., which showed that Vpu is a highly variable protein [10]. Also, the amino acid variability of each codon in the present study correlated strongly with that of published subtype B sequence data from the Los Alamos database (Fig. 1B), suggesting that our observed pattern of amino acid variation in Vpu was generally representative of the variation observed in HIV-1 subtype B. In fact, the consensus amino acid sequences of subtype B and the present dataset were identical except for 3 amino-acid residues: positions 3, 5, and 24 (Fig. 1C). These amino acid residues were highly variable (Fig. 1A) and not directly associated with known Vpu functions (Fig. 1C).

3.2. HLA-associated polymorphisms in Vpu

As HLA-I-mediated selective contributes to HIV-1 sequence variability, especially the accessory protein Nef [4], we sought to examine whether HLA-I-mediated selective pressure substantially influenced the evolution of Vpu, another accessory protein. We applied a phylogenetic dependency network model [13], which adjusts for the confounding effects of HIV phylogeny, HIV codon covariation and linkage disequilibrium of HLA-I alleles.

In our dataset of 216 individuals, we identified only three HIV-HLA associations in Vpu: a nonadapted association between C*03 and Glu-5, a nonadapted association between A*33:03 and Arg-37, and an adapted association between A*33:03 and Lys-37. The presence of both nonadapted and adapted A*33:03-associated polymorphisms at Vpu codon 37 is consistent with an Arginine-to-Lysine escape mutation occurring at the C-terminus of the immunodominant HLA-A*33:03-restricted epitope in Vpu, ^{29}EYRKILRQR^{37} [11]. However, there was no HLA-restricted T cell epitopes around Vpu position 5 have been reported. Although we might have missed some polymorphisms due to the limited sample size in this study, these data suggest that HLA-I-mediated selective pressure toward Vpu does not substantially drive Vpu variability at the population level in this cohort.

3.3. HLA-associated polymorphisms in alternating reading frames

CTLs can recognize epitopes encoded by alternate reading frames including the antisense-strand sequences of HIV-1 *gag*, *pol*, and *nef* [17,18]. Therefore, we also investigated HIV-HLA polymorphism associations in peptide sequences encoded by alternative reading frames of the *vpu* gene. We observed no statistically significant HLA-associated polymorphisms in alternate or antisense reading frames, except for a single HLA-B*40:01 associated "adapted" lysine polymorphism at codon 2 of the overlapping Envelope reading frame which is initiated in the middle of the *vpu* gene (ORF + 2; Table 1, Fig. 2). Although this association was between Lys-2 of Env and *HLA-B*40:01*, no CTL epitopes have been reported in the context of HLA-B*40:01 in this region. Using bioinformatic prediction programs Epipred [19] and BIMAS [20] we

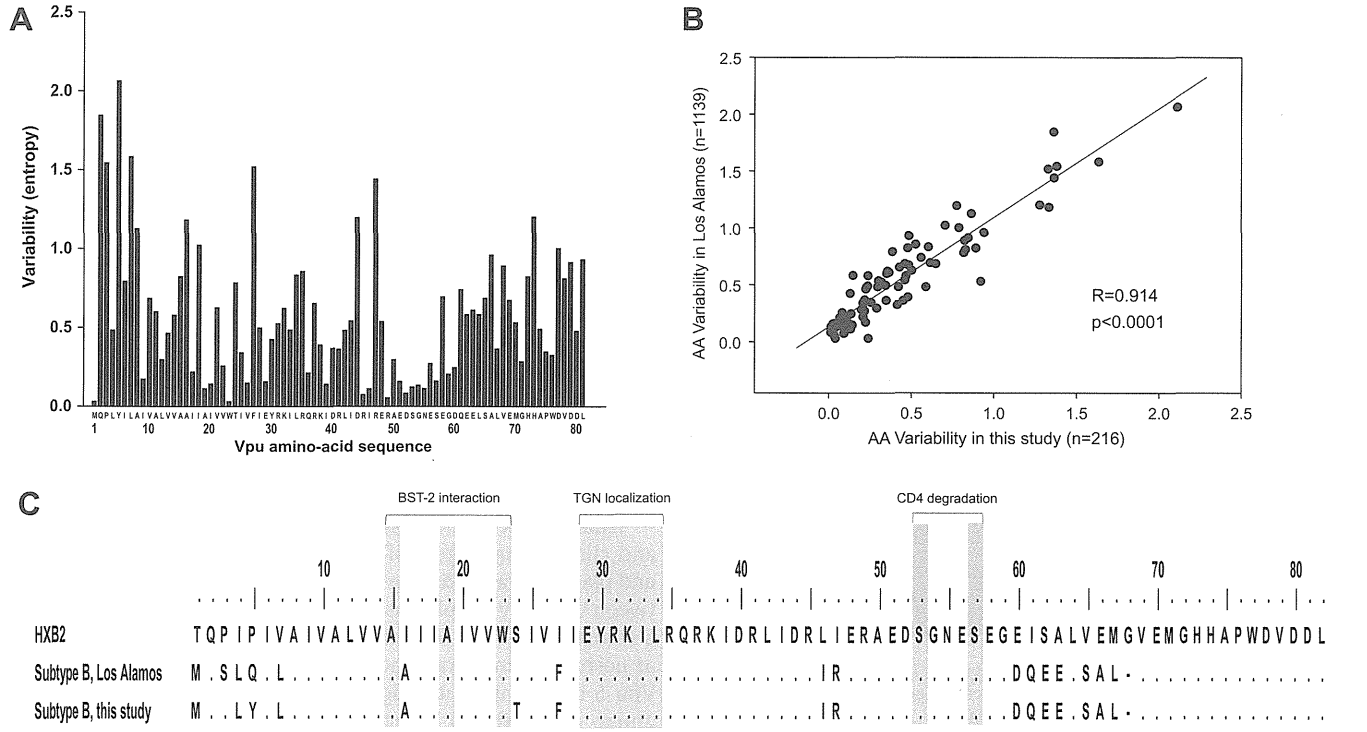

Fig. 1. Variability of the amino acid residues of HIV-1 Vpu. The amino acid sequence of Vpu was analyzed based on the cross-sectional studies on 216 HIV-infected subjects. The amino acid variability at each position of Vpu was analyzed by determining its Shannon entropy score (panel A). The Vpu sequence (subtype B, $n = 1139$) was retrieved from the Los Alamos HIV sequence database, analyzed for its amino acid variability, and compared with subtype B obtained from this study using Spearman Rank Order Correlation (panel B). The consensus sequences of Vpu obtained from Los Alamos database and this study were aligned with reference strain HXB2 and regions responsible for some key Vpu functions highlighted (panel C).

Table 1
Summary of HIV-HLA associations in the Vpu-encoded region.

RF	Protein	Pos HXB2	aa	HLA	Association	*p*-Value	*q*-Value	Known epitope	
								Sequence	Reference
+1	Vpu	5	E	C*03	Nonadapted	2.13×10^{-5}	1.52×10^{-1}	none	–
		37	R	A*22:03	Nonadapted	3.40×10^{-6}	5.50×10^{-2}	^{29}EYRKILRQR^{37}	[11]
		37	K	A*33:03	Adapted	2.80×10^{-5}	1.52×10^{-1}	^{29}EYRKILRQR^{37}	[11]
+2	Env	2	K	B*40:01	Adapted	1.63×10^{-5}	1.67×10^{-1}	none	–

RF, reading frame; Pos HXB2, amino acid position when aligned to HXB2 sequence.

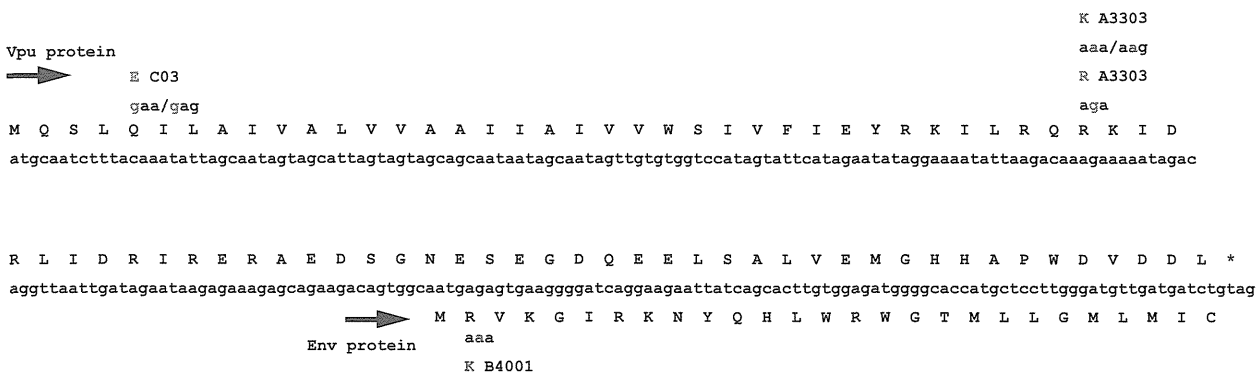

Fig. 2. The Vpu and a part of Env proteins and their associations with host HLA class I alleles. The nucleotide sequence and its deduced amino acid sequence of Vpu and of an overlapping part of Env with reference to the subtype B consensus sequence of Los Alamos database is shown. The amino acid residues associated with the indicated HLA class I alleles ($p < 0.05$, $q < 0.2$) are shown with adapted (red) and nonadapted (blue) residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attempted to predict B*40:01-restricted CTL epitopes, but found none (data not shown). This failure is most likely due to the presence of several basic amino acids, such as Arg and Lys, in this region of Env (Fig. 2), as it has been shown that HLA-B*40:01

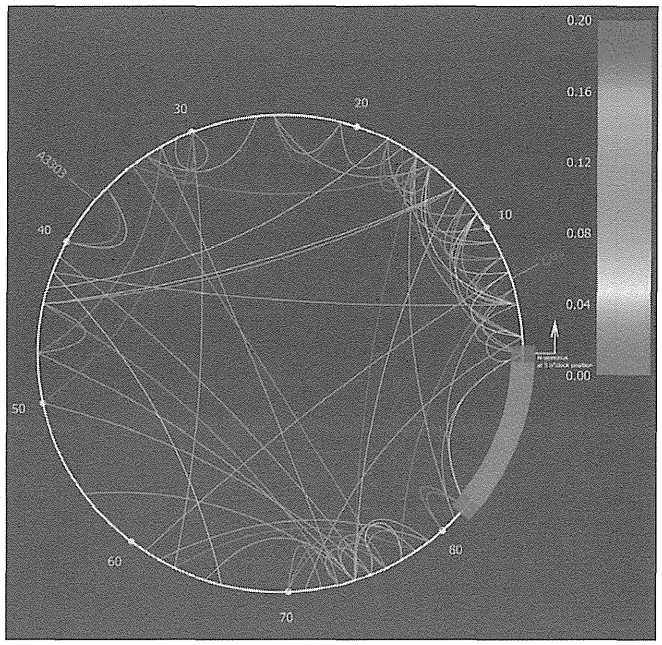

Fig. 3. Amino acid codon–codon covariation in Vpu. The circular map, generated by the PhyloDv software [13], shows Vpu codon–codon covariation associations as inner arcs connecting the association sites, with the HLA associations as tags pointing to their corresponding sites. Q values of individual codon pairs are represented as a heat map shown at the right.

preferentially binds peptides with acidic residues at their anchors [21]. This issue needs to be clarified in further studies using immunologic assays. Taken together, our results suggest that HLA-I-mediated selective pressure do not contribute to a large extent to population-level sequence variation in HIV-1 Vpu.

3.4. Codon–codon covariation of Vpu

Given that Vpu is functionally important in viral replication *in vivo*, the highly variable nature of Vpu amino acid sequences could be explained by complex networks of codon–codon covariation and/or secondary/compensatory mutation pathways. We therefore examined the codon–codon covariation of Vpu by using the phylogenetic dependency network model. Although Vpu consists of only 81 amino acids, we identified 103 covarying codon pairs in Vpu, displayed in Fig. 3. The covariation network in Vpu showed an uneven distribution, with a large number of codon–codon covariation networks at the N-terminal membrane-spanning region, a region responsible for BST-2 interaction [22]. Interestingly, the 3 HIV-HLA associations (Table 1, Fig. 2) were not significantly linked to any other amino acid residues. These data suggest that the conformation and function of Vpu may be preserved through many possible combinations of primary and secondary polymorphisms and that the HLA-I-associated immune-mediated selective pressure may have only a minor effect on such Vpu polymorphisms.

3.5. Association between Vpu polymorphisms and clinical parameters

Finally, we explored associations between Vpu polymorphisms and clinical parameters of HIV-infected patients (i.e., CD4 counts and plasma viral load). We observed no significant associations between Vpu polymorphisms and CD4 counts. However we identified a statistically significant association between amino acid residues at position 5 and viral load (Fig. 4). The patients harboring Val at Vpu-5 had significantly higher viral loads compared to those with amino acid residues other than Val at this position. Thus, amino acid residues at position 5 of Vpu showed several interesting features, i.e., the highest variability of all Vpu amino acids (Fig. 1A), nonadapted association of Glu-5 with *HLA-Cw*03*, and association of Val-5 with the increased viral load. Considering that the amino acid residue at this position is located in close proximity to the membrane-spanning region and that this region is functionally important for BST-2 binding, it would be interesting to examine functional effects of amino acid polymorphisms at position 5, whether they are mediated by host immune responses or otherwise.

In summary, we report here comparably fewer HLA-associated mutations in Vpu in this cohort although host HLA class I allele-associated immune responses are major forces driving the evolution of HIV-1 accessory proteins, such as Nef. Taken together, we conclude that the influence of immune selection on evolution of Vpu

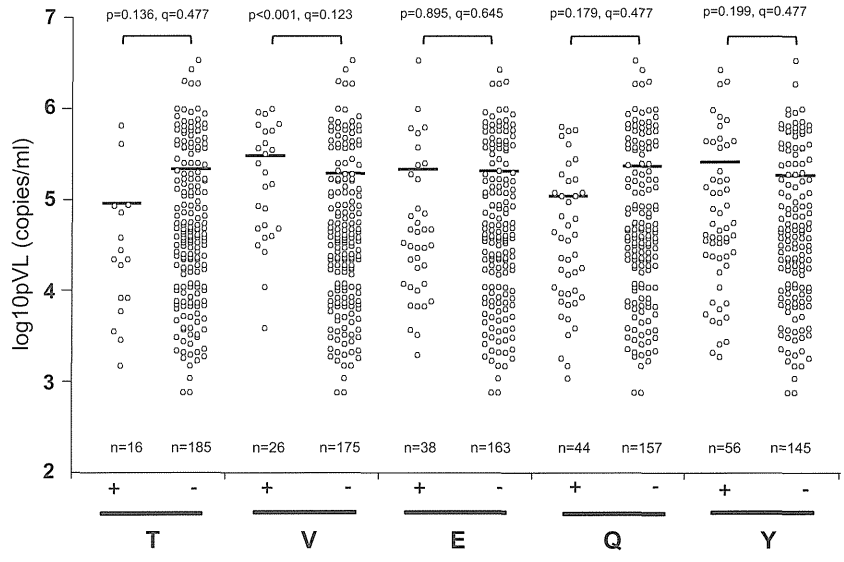

Fig. 4. Association between plasma viral load and amino acid polymorphism at position 5 of Vpu. HIV plasma viral loads, stratified by amino acid expression at Vpu codon 5, are shown. Vpu codon 5 exhibited 11 different amino acids positioning in our dataset; only those observed in >10 patients are shown here. Horizontal bars indicate medians. Statistical analysis was performed using the Mann-Whitney U-test.

at the population level may be reduced compared to other highly variable HIV-1 proteins, providing us with additional insight into differential evolutional pathways among viral accessory proteins.

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HLA Class I-Mediated Control of HIV-1 in the Japanese Population, in Which the Protective HLA-B*57 and HLA-B*27 Alleles Are Absent

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We investigated the effect of HLA class I alleles on clinical parameters for HIV-1 disease progression in the Japanese population, where two strongly protective alleles, HLA-B*57 and HLA-B*27, are virtually nonexistent. HLA-B alleles showed a dominant role, primarily through HLA-B*67:01 and the HLA-B*52:01-C*12:02 haplotype. Neither a rare-allele nor a heterozygote advantage was found, suggesting that the effect of HLA alleles in the Japanese population is either different from those observed in Africans and Caucasians or undetectable due to limited power.

The presence of particular HLA class I alleles is associated with the rate of progression to AIDS and/or with clinical markers of disease progression, such as viral load (VL) and CD4 T cell count (2, 3, 7, 11, 13). HLA-B*57 and HLA-B*27 are well-known to associate with successful control of HIV-1 or slow progression to disease in Caucasians and Africans (1, 4, 5, 9–11) but not in Asians, since the frequencies of these alleles are very low (<1%) in this population. There is growing evidence that HIV-1-specific CTL responses restricted by HLA-B allotypes play a key role in determining disease outcomes relative to HLA-A and -C (7, 9), but it remains possible that the association between HLA-B and HIV-1 control is biased by the extremely strong effects of only a few HLA-B alleles, particularly the protective HLA-B*57 and -B*27 alleles and the detrimental HLA-B*58:02 and -B*35:02/B*35:03 alleles (6–8). The exceptional protection afforded by HLA-B*57 and -B*27 may mask weaker effects of other HLA alleles, in part due to the potential for inappropriate correction for the effects of these two alleles. Since both of these alleles are virtually nonexistent (i.e., present in 0% and 0.5% of the population, respectively) in Japan (12), studies of Japanese cohorts provide an unbiased means of determining the effects of other HLA alleles on HIV control. Therefore, we analyzed the association of HLA alleles with markers of disease progression, VL and CD4 count, in Japanese individuals chronically infected with HIV-1.

We recruited 504 chronically HIV-1-infected, antiretroviral therapy (ART)-naïve Japanese individuals (464 men and 40 women) who visited our hospital during 2000-2010 and who did not meet any criteria for clinical AIDS. The median (interquartile ranges) VL and CD4 count are 35,000 (9,175/100,000) and 288 (183/402), respectively. HLA alleles of these individuals were determined at the 4-digit level. Associations between HLA alleles and VL or CD4 count obtained at the first visit were determined among these individuals. We considered 48 HLA class I alleles occurring at a frequency of greater than 1% (11 HLA-A, 22 HLA-B, and 15 HLA-C), which excluded both B*57 (0 identified) and B*27:05 (1 identified) (see Table S1 in the supplemental material). The differential contribution of HLA-A, -B, and -C alleles on VL in the cohort as a whole was determined using the Kruskal-Wallis statistic H, which is a nonparametric measure of variation between data groups (i.e., among alleles at a given locus). The range of effects (protective to susceptible) across alleles at each given class I locus on VL was largest for HLA-B ($H = 81$, $P = 0.0005$) but was also significant for both HLA-A ($H = 37$, $P = 0.006$) and HLA-C ($H = 52$, $P = 0.0001$) (Table 1). HLA-B also showed the greatest range of effects on CD4 counts, and for this outcome, it was the only locus that showed significance ($H = 71$, $P = 0.004$ for HLA-B; $H = 18$, $P = 0.43$ for HLA-A; $H = 29$, $P = 0.084$ for HLA-C). These results indicate that the HLA-B locus has the greatest effect on HIV-1 control in Japanese and confirmed previous findings in Caucasians and Africans.

We found that 36% (4 of 11) of HLA-A alleles, 41% (9 of 22) of HLA-B alleles, and 27% (4 of 15) of HLA-C alleles were associated significantly with VL before correction for multiple tests (Fig. 1). Associations with CD4 count were observed for a more limited number of HLA class I alleles than those with VL: 18% (2 of 11) for HLA-A, 18% (4 of 22) for HLA-B, and 13% (2 of 15) for HLA-C (Fig. 1). Three alleles, HLA-B*67:01, -B*52:01, and -C*12:02, were significantly associated with both low VL and high CD4 count and two alleles, HLA-A*02:07 and -B*35:01, associated with both higher VL and lower CD4 count. After local false discovery rate estimation, the numbers of HLA alleles associated with VL and CD4 count were 14 and 5, respectively (see Table S2 in the supplemental material). HLA-B*52:01 and -C*12:02 form a known haplotype, which reaches a frequency of approximately 20% of the Japanese population (12), and given the protection associated with the individual alleles, it is possible that CTL or NK cell responses restricted by this haplotype play an important role in control of HIV-1 in Japanese. HLA-B*35:01 associated with high VL and low CD4 counts, which is consistent with data from Cauca-

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TABLE 1 Kruskal-Wallis test for associations of viral load and absolute CD4 count with alleles at three HLA class I loci in Japanese individuals chronically infected with HIV-1[a]

	VL		CD4 cell count	
Allele (n)	P	H	P	H
HLA-A (504)	**0.006**	37	0.43	18
HLA-B (504)	**0.0005**	**81**	**0.004**	**71**
HLA-C (503)	**0.0001**	**52**	0.084	29

[a] Values in bold are statistically significant.

sians, in which this allele was more commonly observed among HIV noncontrollers (5). On the other hand, HLA-B*35:02 and -B*35:03 associate with rapid disease progression in Caucasians, whereas HLA-B*35:01 does not (6), highlighting distinctions across allelic associations depending on the clinical outcome being considered. HLA-A*02:07 (frequency = 0.073), an allele that is

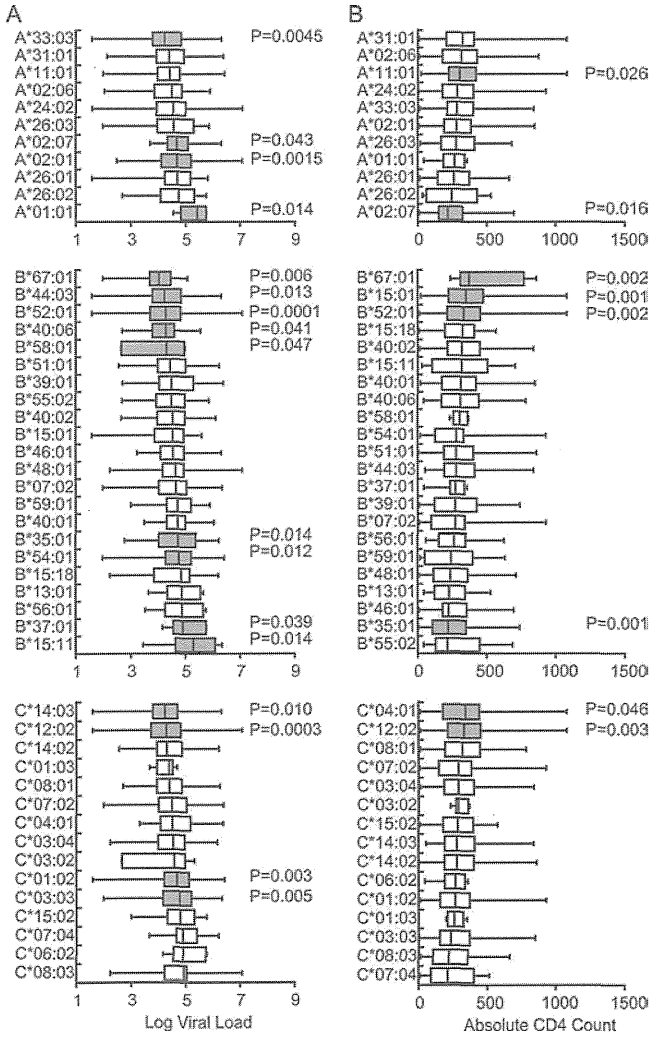

FIG 1 Association of HLA allele expression with viral load and absolute CD4 count in Japanese individuals chronically infected with HIV-1. All HLA alleles occurring at a phenotypic frequency greater than 1% were examined for their associations with viral load (A) and absolute CD4 count (B) in a cohort of 504 chronically HIV-1-infected Japanese individuals recruited from 2000 to 2010. Associations with a P value of <0.05 are highlighted in gray.

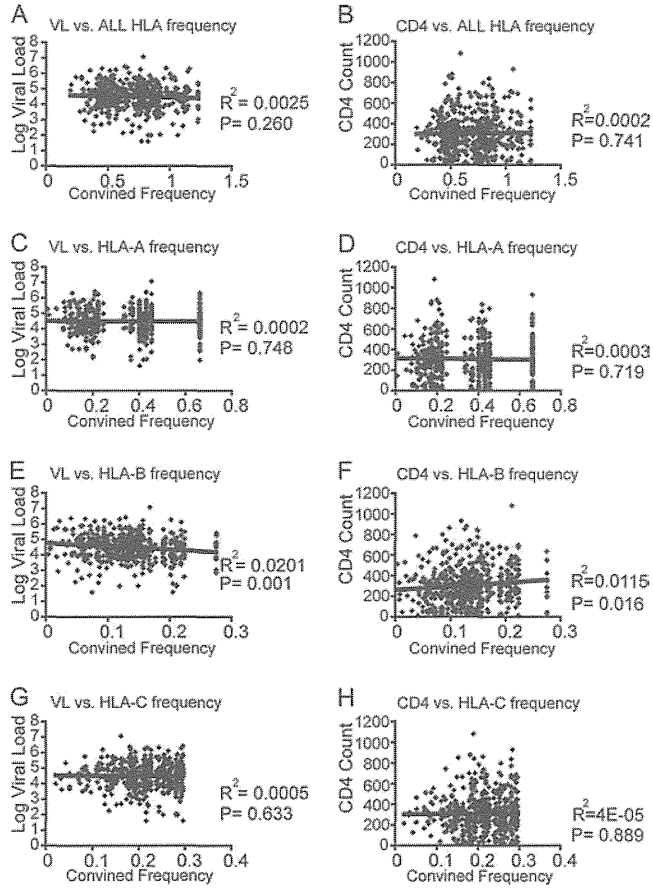

FIG 2 HLA frequency-dependent effects on viral load and CD4 counts. The correlations between VL or CD4 count with combined frequencies for all subjects (n = 504) are shown. HLA frequencies used to calculate the combined frequencies for each individual were derived from the overall allele frequencies in the entire cohort of 504 subjects. Linear regression lines are included in the plots. The distribution of HLA class I frequencies among 504 chronically HIV-1-infected Japanese individuals used in this analysis is shown in Table S4 in the supplemental material.

missing in Caucasians and Africans, also associated with susceptibility.

An advantage of rare HLA alleles in HIV-1 disease progression has been reported previously (13). Therefore, we investigated the effect of HLA frequency on VL or CD4 count in our cohort by comparing these clinical measurements first to the sum of the frequencies of the two alleles at each HLA locus individually (HLA-A, -B, and -C separately) and second to the sum of the frequencies of all HLA class I alleles (HLA-A, -B, and -C combined) for each subject. No significant correlation was observed between the overall HLA allele frequency and VL or CD4 count (Fig. 2A and B), nor was there a correlation between HLA-A allele frequencies or HLA-C allele frequencies with these clinical measurements (Fig. 2C, D, G, and H). In contrast to previous observations, the frequencies of HLA-B alleles were positively and negatively associated with CD4 count and VL, respectively (Fig. 2E and F). We also analyzed the effect of HLA supertype frequency on VL and found no effect (see Fig. S1 in the supplemental material). These results indicate no advantage of rare alleles on VL and CD4 count in the Japanese cohort. Further, we detected no significant

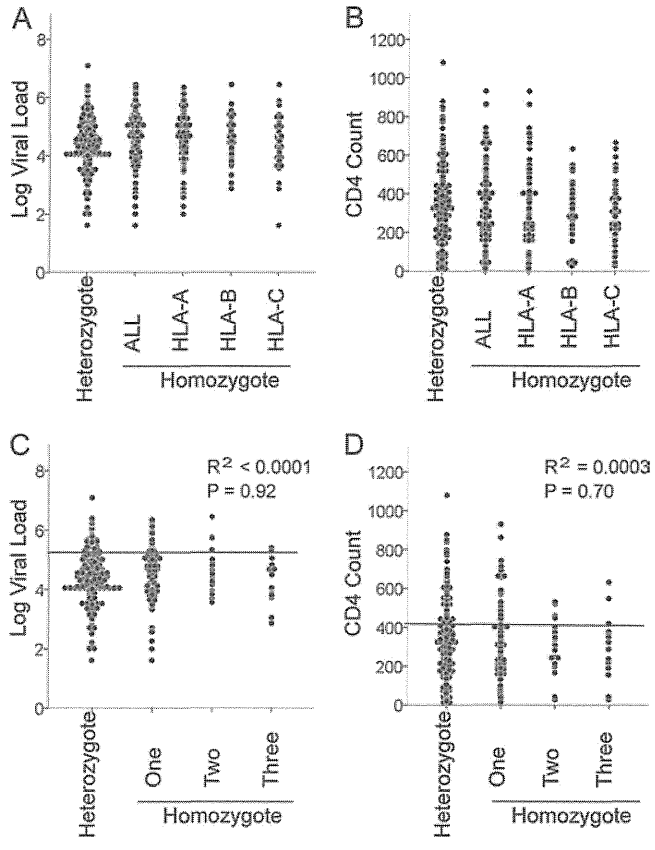

FIG 3 Effect of HLA class I homozygosity on VL and CD4. The comparison between heterozygotes at all three class I loci (n = 349) and homozygotes (n = 155) for at least one HLA allele is shown (A and B). (A and B) Homozygotes are grouped according to homozygosity for the HLA-A (n = 106), -B (n = 42), or -C (n = 64) locus. No significant difference was observed between these groups. Median VLs and CD4 counts, respectively, are 32,500 and 289 (fully heterozygous), 44,000 and 280 (HLA-A homozygous), 35,500 and 290 (HLA-B homozygous), 29,500 and 305 (HLA-C homozygous), and 42,000 and 288 (homozygous for at least one HLA locus). (C and D) Homozygotes were grouped according to homozygosity for one (n = 115), two (n = 23), or three (n = 17) HLA class I loci. Median VLs and CD4 counts, respectively, are 32,500 and 289 (fully heterozygous), 47,000 and 284 (homozygous at a single locus), 28,000 and 335 (homozygous at two loci), and 31,000 and 288 (homozygous at all three loci). No significant difference was observed between these groups. The lines in panels C and D are linear regression lines.

differences in either VL or CD4 count between heterozygotes and homozygotes at any individual HLA locus (Fig. 3A and B) or homozygosity at one, two, or all three class I loci (Fig. 3C and D). Thus, a heterozygote advantage of HLA class I was not observed in this cohort.

We also analyzed 147 ART-naïve Japanese individuals with clinical AIDS. There were no strong associations of HLA alleles with either VL or CD4 count (see Table S3 in the supplemental material). We excluded these individuals in the present study for the following reasons: (i) There were radical differences in VL and CD4 count between AIDS and non-AIDS groups, and (ii) since AIDS represents an effective breakdown of the immune response, a putative association observed in the non-AIDS group would not be comparable to that in the AIDS group even if HLA class I alleles had an effect on VL and CD4 count.

In summary, the HLA-B locus appears to have the strongest allelic effects on VL and CD4 counts in this Japanese cohort, with the HLA-B*52:01-C*12:02 haplotype showing the greatest significance. These findings in a Japanese cohort highlight the differences of the effects of HLA class I alleles on HIV-1 control between Japanese and Africans/Caucasians.

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Differential Clade-Specific HLA-B*3501 Association with HIV-1 Disease Outcome Is Linked to Immunogenicity of a Single Gag Epitope

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The strongest genetic influence on immune control in HIV-1 infection is the HLA class I genotype. Rapid disease progression in B-clade infection has been linked to HLA-B*35 expression, in particular to the less common HLA-B*3502 and HLA-B*3503 subtypes but also to the most prevalent subtype, HLA-B*3501. In these studies we first demonstrated that whereas HLA-B*3501 is associated with a high viral set point in two further B-clade-infected cohorts, in Japan and Mexico, this association does not hold in two large C-clade-infected African cohorts. We tested the hypothesis that clade-specific differences in HLA associations with disease outcomes may be related to distinct targeting of critical $CD8^+$ T-cell epitopes. We observed that only one epitope was significantly targeted differentially, namely, the Gag-specific epitope NPPIPVGDIY (NY10, Gag positions 253 to 262) ($P = 2 \times 10^{-5}$). In common with two other HLA-B*3501-restricted epitopes, in Gag and Nef, that were not targeted differentially, a response toward NY10 was associated with a significantly lower viral set point. Nonimmunogenicity of NY10 in B-clade-infected subjects derives from the Gag-D260E polymorphism present in ~90% of B-clade sequences, which critically reduces recognition of the Gag NY10 epitope. These data suggest that in spite of any inherent HLA-linked T-cell receptor repertoire differences that may exist, maximizing the breadth of the Gag-specific $CD8^+$ T-cell response, by the addition of even a single epitope, may be of overriding importance in achieving immune control of HIV infection. This distinction is of direct relevance to development of vaccines designed to optimize the anti-HIV $CD8^+$ T-cell response in all individuals, irrespective of HLA type.

Several genome-wide association studies now indicate that the host HLA class I genotype is the major genetic determinant of HIV-1 disease progression (19, 20, 61). Previously it had been established that differences in HLA allele expression have a substantial impact on HIV disease outcome, in both B-clade (10, 19, 20, 59) and C-clade (38, 44, 54, 63) infection. Variation at the HLA-B locus has the greatest impact on viral set point (20, 38). This may result from the increased diversity of HLA-B compared to non-HLA-B alleles (28), affecting the repertoire, protein specificity, and peptide-binding characteristics of epitopes presented by HLA-B alleles (38, 39, 41). In addition, HLA-Bw4 alleles can act as KIR ligands and modulate an NK response, with certain HLA-KIR combinations resulting in selection pressure on HIV and/or significantly influencing viral set point (2, 3, 52, 68).

The mechanisms by which certain HLA alleles are consistently linked with particular HIV disease outcomes remain unresolved. Several possible mechanisms have been proposed. First, HLA-associated immune control has been linked to the specificity of the $CD8^+$ T-cell response (39, 54). In this way, HLA alleles such as HLA-B*57 or HLA-B*27, associated with immune control (4, 46, 59), restrict dominant Gag-specific responses, escape from which results in a substantial reduction in viral replicative capacity (13, 15, 46, 53, 65). In contrast, HLA alleles such as HLA-B*35, associated with rapid disease progression (12), restrict dominant epitopes in Nef, Env, and other non-Gag proteins (7, 39, 58, 67, 69, 72, 73).

A second mechanism proposed for the association of particular HLA types with characteristic HIV disease outcomes is through an impact on antiviral NK activity, since certain HLA alleles have the

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potential to act as KIR ligands. The HLA alleles associated with lowest viral set point tend to be HLA-Bw4 alleles (22). HLA-Bw4 alleles expressing Ile at HLA residue 80 significantly reduce the viral set point in combination with either KIR3DS1 or KIR3DL1 (51, 52). However, the impact of HLA-KIR combinations only partially explains the effect of protective alleles such as HLA-B*27 and HLA-B*57 or of disease susceptibility alleles such as HLA-B*35 (5, 52).

A third mechanism, more recently proposed (41), suggests that disease susceptibility (61) HLA alleles such as HLA-B*0702 and HLA-B*3501 have peptide-binding motifs such that large numbers of self peptides can bind, and hence a relatively large proportion of the T-cell receptor (TcR) repertoire would be lost through negative selection of autoreactive T cells in the thymus. In contrast, protective alleles such as HLA-B*2705 and HLA-B*5701 have more restrictive peptide-binding motifs, with a requirement for Arg at P2 in the case of HLA-B*2705 and a strong preference for Trp at the C-terminal position in the case of HLA-B*5701 (50). This would result in fewer autoreactive T cells being deleted in the thymus via negative selection and therefore a relatively large TcR repertoire remaining to accommodate the challenge of epitope variation inevitably presented by viruses such as HIV.

An additional mechanism proposed to explain the status of HLA-B*3503 as linked with more rapid disease progression than HLA-B*3501 (25), from which it differs by only one amino acid, derives from the observation that HLA-B*3503 binds with significantly greater affinity than HLA-B*3501 to immunoglobulin-like transcript 4 (ILT-4), an inhibitory major histocompatibility complex (MHC) class I receptor expressed on dendritic cells (34). These data suggest the possibility that dendritic cell function may be significantly affected by a variety of HLA molecules, thereby explaining a range of differential HLA associations with HIV disease outcome.

We here describe an observation that allows us to test the first of these hypothetical mechanisms. While HLA-B*3501 is associated with less rapid progression to HIV disease than the less common subtypes of HLA-B*35 in Caucasians, B*3502 and B*3503 (25), HLA-B*3501 itself has also been associated with higher-than-average viremia in B-clade HIV-1 infection (42). For example, in a recent study of 3,622 B-clade-infected study subjects, HLA-B*3501 was strongly associated with HIV disease progression (61). However, in a cohort of C-clade-infected study subjects (n = 1,210) in Durban, South Africa, we noted that HLA-B*3501 is somewhat protective: viral set points tend to be somewhat lower in HLA-B*3501-positive subjects. Indeed, having removed the effect of HLA-B*57, HLA-B*5801, HLA-B*1801, and HLA-B*5802, the alleles having the strongest impact on viral set point and absolute CD4 count (38), HLA-B*3501 was the HLA-B allele associated with the highest absolute CD4 counts (44, 54) in this C-clade-infected cohort.

We show here, first, that this observation of clade specificity of the HLA-B*3501 effect on viral set point could be replicated in two additional B-clade-infected cohorts, namely, in Japan and in Mexico, and in an additional C-clade-infected cohort in Botswana. We then tested the hypothesis that the clade-specific difference in HLA-B*3501-associated HIV disease outcome could be related to altered specificity of the $CD8^+$ T-cell response. Based on the "Gag hypothesis" as described above, HLA-B*3501-restricted responses in C-clade infection would tend to be more Gag directed and less Nef/Env directed than in B-clade infection.

MATERIALS AND METHODS

Ethics statement. Ethics approval was given by the following: the University of KwaZulu-Natal Review Board and the Massachusetts General Hospital Review Board (Durban cohort); the Office of Human Research Administration, Harvard School of Public Health, and the Health Research Development Committee, Botswana Ministry of Health (Gaborone cohort); the Oxford Research Ethics Committee (Thames Valley and other cohorts); and the Ethics Committees of Kumamoto University and National Centre for Global Health and Medicine (Kumamoto cohort). Study subjects from all cohorts gave written informed consent for their participation.

Study cohorts. We studied a total of 3,132 adults with chronic, antiretroviral therapy (ART)-naïve HIV-1 infection, recruited from six cohorts as follows: (i) Durban, South Africa (C clade; n = 1,218), as previously described (38, 39, 46, 54); (ii) Gaborone, Botswana (C clade; n = 514) via the Mma Bana study, as previously described (66); (iii) Kumamoto, Japan (B clade; n = 242), as previously described (37); and (iv) Mexico City, Mexico (B clade; n = 771), as previously described (6) (see Table S1 in the supplemental material); (v) the Thames Valley cohort, United Kingdom (mixed clades; n = 237), as previously described (60, 62); and (vi) a B-clade-infected cohort of 150 subjects drawn from multiple ethnicities, also as previously described (24). Viral loads were determined using Roche Amplicor version 1.5 assay; $CD4^+$ T-cell counts were determined by flow cytometry.

HLA typing and classification. HLA typing from genomic DNA was undertaken by sequence-based typing as previously described (38). Locus-specific PCR products of exons 2 and 3 were amplified and sequenced. In the Kumamoto cohort, 32/37 subjects with HLA-B*35 were typed to 4 digits, and all 32 of these were HLA-B*3501 positive; because of this, and because of a previous analysis of 1,018 Japanese subjects (36) which showed that 158/159 subjects with HLA-B*35 had HLA-B*3501, the remaining 5 Japanese subjects were designated HLA-B*3501 positive. Likewise, in the southern African cohorts, 96/102 HLA-B*35-positive subjects typed to 4 digits were HLA-B*3501 positive. For 23 Durban subjects in whom HLA-B*35 typing had been undertaken only to 2-digit resolution, we used an HLA completion tool (http://atom.research.microsoft.com/HLACompletion) (47) to predict the most likely 4-digit HLA-B*35 allele. In all cases HLA-B*3501 was predicted as the 4-digit type with a high level of statistical certainty (probability of B*3501, 0.86 to 0.98; median, 0.97). For this reason, we designated all 23 Durban subjects with HLA-B*35 typed to 2-digit resolution as HLA-B*3501.

Definition of HLA-B*3501-restricted epitopes. To define a comprehensive list of HLA-B*3501-restricted epitopes, we identified previously characterized epitopes from studies of predominantly B-clade-infected subjects (Los Alamos "A list"; www.lanl.gov) (48) and also identified five novel HLA-B*3501-restricted epitopes by testing recognition of 410 overlapping 18-mer peptides in a cohort of C-clade-infected subjects (see Table S2 in the supplemental material). One of these (HA9) has, since the start of this study, now been confirmed by another group (74). From this dual approach, 13 HLA-B*3501-restricted epitopes were identified for further analysis (Table 1).

IFN-γ ELISpot assays. Gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays were undertaken using fresh or cryopreserved peripheral blood mononuclear cells (PBMCs). We screened for HIV-1-specific responses statistically associated ($q < 0.05$) with the expression of HLA-B*3501 by testing a total of 1,010 chronically infected subjects (n = 795 from Durban; n = 215 from the Thames Valley) against a panel of 410 overlapping peptides (OLPs) spanning the entire HIV proteome, as previously described (38, 39, 54). Significant associations were determined using Fisher's exact test and corrected for multiple comparisons using a q value (false-detection rate [FDR]) approach as previously described (11, 40, 54).

In order to screen subjects with HLA-B*3501 for specific responses to HLA-B*3501 epitopes, B-clade-infected subjects were tested for IFN-γ responses to optimal peptides (Japan, n = 30) or against overlapping

TABLE 1 Thirteen HLA-B*3501-restricted epitopes in HIV-1 from Gag, Pol, Rev, Env, and Nef proteins[a]

Protein	Clade	Epitope sequence[b] P2 ↓ C terminus ↓	HXB2 position	Epitope designation
p24 Gag	B	HPVHAGPI **A**	Gag 216-224	Gag HA9*
	C	-------- -		
	B	NPPIPVGEI **Y**	Gag 253-262	Gag NY10
	C	-------D- -		
RT	B	TVLDVGDA **Y**	Pol 262-270	RT TY9
	C	-------- -		
	B	VPLDKDFRK **Y**	Pol 273-282	RT VY10
	C	----E--- -		
	B	NPDIVIYQ **Y**	Pol 330-338	RT NY9
	C	--E----- -		
	B	EPIVGAETF **Y**	Pol 587-596	RT EY10*
	C	---A----- -		
Int	B	IPAETGQETA **Y**	Pol 804-814	Int IY11*
	C	----------		
Rev	B	KTVRLIKFL **Y**	Rev 14-23	Rev KY10, Rev QY10*
	C	QA--I--I- -		
gp120	B	VPVWKEATTT **L**	Env 42-52	Env VL11
	C	-----K-- -		
	B	DPNPQEVV **L**	Env 78-86	Env DL9
	C	------M- -		
gp41	B	TAVPWNAS **W**	Env 606-614	Env TW9
	C	------S- -		
Nef	B	VPLRPMT **Y**	Nef 73-81	Nef VY8, Nef VF8
	C	------- **F**		
	B	YPLTFGWC **Y**	Nef 135-143	Nef YY9, Nef YF9*
	C	-------- **F**		

[a] The 13 epitopes include 8 from the Los Alamos database "A list" (www.lanl.gov) and 5 new HLA-B*3501-restricted optimal epitopes (indicated by asterisks).
[b] The B- and C-clade consensus sequences of each epitope are listed; a dash indicates no difference between clades. Residues at position 2 and at the C terminus are in bold.

peptides in a previously described B-clade cohort (23, 24) (n = 44). C-clade-infected subjects (n = 42) were tested for responses to the C-clade version of the same epitopes using the respective 18-mer peptides containing the HLA-B*3501 epitopes.

Viruses from all study subjects in the Japan cohort were sequenced to confirm clade of infection, and only those subjects who were B-clade infected were included in the study (one subject who was A-clade infected was excluded). Likewise C-clade infection was confirmed in >99% of the southern African study subjects. The B-clade-infected subjects were tested for recognition of the version of the peptides corresponding to the B-clade consensus sequence in Japan, and the C-clade-infected subjects were tested for recognition of the version corresponding to the C-clade consensus sequence (the 2006 Durban and other Southern African consensus sequence). Using previously established criteria (38, 39), a response of 100 spot-forming cells (SFC)/10^6 PBMC was defined as significantly above the background response in control wells.

Epitope fine mapping and HLA class I tetramer assay. We confirmed HA9 (HPVHAGPIA; Gag positions 216 to 224) as an HLA-B*3501-restricted optimal epitope via assays of PBMCs in subject R051 (HLA-A*0101, -A*3002, -B*1801, -B*3501, -Cw*0401, -Cw*0501) against the optimal peptide and four truncations (±1 amino acid at the C and N termini); this experiment was performed in triplicate. Likewise, NY10 (NPPIPVGDIY; Gag positions 253 to 262) was optimized against the PY9 (PPIPVGDIY; Gag positions 254 to 262) using responder PBMCs from subject H033 (HLA-A*3601, -A*7401, -B*3501, -B*5301, -Cw*0401, -Cw*0401) in an IFN-γ ELISpot peptide titration assay.

The corresponding peptide responses were validated using HLA class I tetramers and controlled by a mismatched HLA-B*4201 tetramer. A pretitrated concentration of phycoerythrin (PE)-conjugated tetramers (43) was used to stain PBMCs, which were incubated for 30 min and stained with pretitrated extracellular antibodies CD8-Pacific Blue (BD Pharmingen) and CD3-Pacific Orange (Invitrogen). Dead cells were excluded using the Vivid LIVE/DEAD marker (Invitrogen). For NY10-Gag dual-tetramer staining, PBMCs from subject OX030 were stained *ex vivo* or *in vitro* expanded for 12 days using 10 μg/ml of NY10-260D or NY10-260E in culture medium RPMI 1640 (Gibco) supplemented with 10% human serum, 1% penicillin-streptomycin (Invitrogen), and 10% T-cell growth factor (Helvetica), costained with HLA-B*3501-NPPIPVGDIY (PE conjugated) and HLA-B*3501-NPPIPVGEIY (allophycocyanin [APC] conjugated) pretitrated tetramers (*ex vivo* PBMCs) or in 2-fold titrations (cytotoxic T lymphocytes [CTLs]), and subsequently stained with extracellular antibodies as described above.

Intracellular cytokine staining. PBMCs from subject KI-705 were stimulated with NY10-260D (NPPIPVGDIY) or NY10-260E (NPPIPGV EIY) (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human interleukin-2 [IL-2]). After 14 days in culture, the cells were assessed for IFN-γ production. Briefly, bulk cultures were cocultured with C1R cells express-

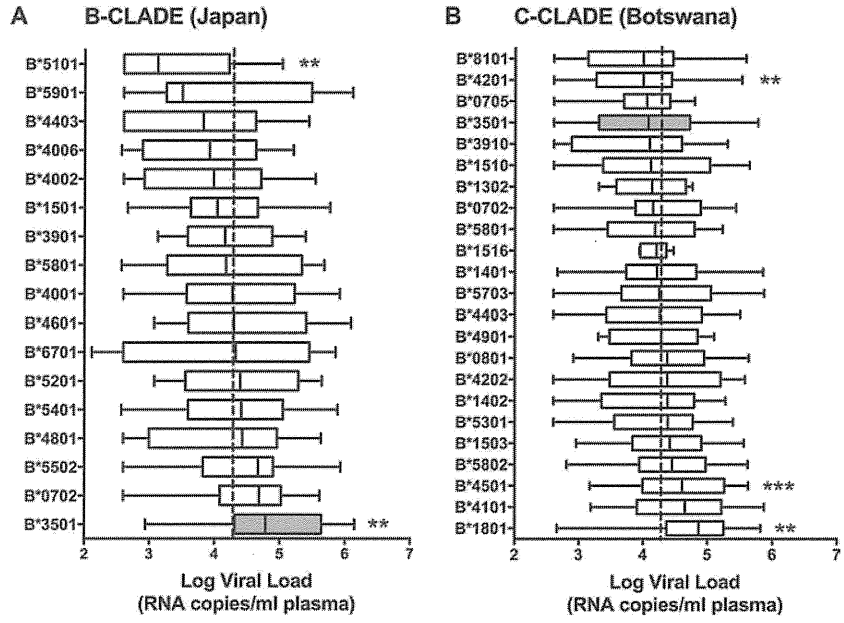

FIG 1 Ranking of HLA-B alleles with respect to median viral load (VL) in chronic HIV-1 infection in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. HLA-B*3501 is highlighted in gray. Dashed lines indicate median VL for the whole cohort. *P* values by Mann-Whitney test, comparing VL for subjects with each allele to the whole population: ***, $P < 0.0001$; **, $P < 0.001$. Alleles represented are those occurring at ≥0.5% phenotypic frequency and for which a minimum of 5 subjects had VL data available. (A) Kumamoto, Japan (median VL, 19,500 RNA copies/ml). (B) Gaborone, Botswana (median VL, 19,150 RNA copies/ml). For equivalent data for Durban, South Africa, see reference 55.

ing HLA-B*3501 pulsed with NY10-260E or NY10-260D peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the cocultures were continued for additional 4 h. Cells were stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (Dako Corporation, Glostrup, Denmark) and subsequently fixed (4% paraformaldehyde), permeabilized (0.1% saponin and 20% NCS in phosphate-buffered saline), and intracellularly stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ monoclonal antibody (MAb) (PharMingen, San Diego, CA). Samples were acquired on a FACSCalibur instrument within 24 h of staining and data analyzed using FlowJo version 8.8.6.

Generation of mutant virus NL43-2 E260D and epitope processing assay. The NL43-2-Gag260D mutant virus was generated by introducing the Gag260D mutation into the NL43-2 Gag260E backbone using site-directed mutagenesis (Invitrogen). After virus generation, 721.221-CD4-B*3501 and 721.221 target cells were infected with NL43-2 B-clade WT(Gag260E) or NL4-32-Gag260D mutant virus. The infection rates were determined by the level of intracellular p24-positive cells stained with FITC-conjugated anti-p24 MAb (KC57-FITC; BD Biosciences) and followed over 6 days. When the level of p24-positive target cells reached 80%, the Gag NY10-specific $CD8^+$ T-cell line and the control Pol-EY10 $CD8^+$ T-cell clone was cocultured with the target cells for 5 h in the presence of brefeldin A and subsequently stained for intracellular IFN-γ as described above. The level of IFN-γ-positive $CD8^+$ T cells after coculture was used as a measure of the level of specific epitope presentation and controlled by uninfected HLA-matched and infected HLA-negative 721.221 cells. Peptide-pulsed HLA-matched target cells were used as positive controls for optimal epitope presentation. Samples were acquired on a FACSCalibur instrument within 24 h of staining and data analyzed using FlowJo version 8.8.6

Peptide-MHC binding studies. HLA-peptide binding studies were undertaken using a luminescent oxygen channeling immunoassay (LOCI) as previously described (29). We tested binding for 12 HLA-B*3501 epitopes as shown in Table S3 in the supplemental material. Binding assays were performed in quadruplicate; the reported result is the mean of the four values obtained.

Stability of binding (binding half-life) was determined as described previously (30). Briefly, biotinylated HLA-I heavy chain, ^{125}I-labeled beta-2-microglobulin (B2m), and peptide were allowed to fold into peptide–HLA-I complexes in streptavidin-coated scintillation microplates (Flashplate Plus; Perkin-Elmer, Boston, MA) for 24 h at 18°C. Excess unlabeled B2m was added, and dissociation was initiated by placing the microplate in a scintillation reader (TopCount NXT; Perkin-Elmer, Boston, MA) operating at 37°C. The scintillation signal was monitored by continuous reading of the microplate for 24 h. Half-lives were calculated from dissociation curves using the exponential decay equation in Prism v.5.0a (GraphPad, San Diego, CA). Assays were performed in duplicate; the mean value from two experiments is reported.

Statistical analysis. Statistical analysis was undertaken using GraphPad Prism v.5.0a (GraphPad, San Diego, CA). To define the sites of new putative HLA-B*3501 epitopes, relationships between HIV-1 sequence polymorphisms and HLA class I expression and between ELISpot responses and HLA class I expression were determined using Fisher's exact test (corrected for viral lineage in the case of sequence analysis) and corrected for multiple comparisons using a *q* value (false-detection rate), as previously described (11, 54).

RESULTS

Consistent differential HLA-B*3501-association with viral set point in B- and C-clade infection. We first sought to test the consistency of our initial observation that, in contrast to its impact in B-clade infection (7, 21, 59), HLA-B*3501 is not associated with high viral set point in C-clade infection (38, 44). In B-clade-infected cohorts in Mexico and in Japan, HLA-B*3501 is associated with a high viral set point ($P = 0.06$ and $P = 0.0005$, respectively) (Fig. 1 and 2). In contrast, in a C-clade-infected Botswanan cohort, HLA-B*3501 is somewhat protective, although this did not reach statistical significance (Fig. 1 and 2).

HLA-B*3501 is also associated with higher absolute $CD4^+$ T-cell counts in subjects with C-clade infection (Durban, $P = 0.06$;

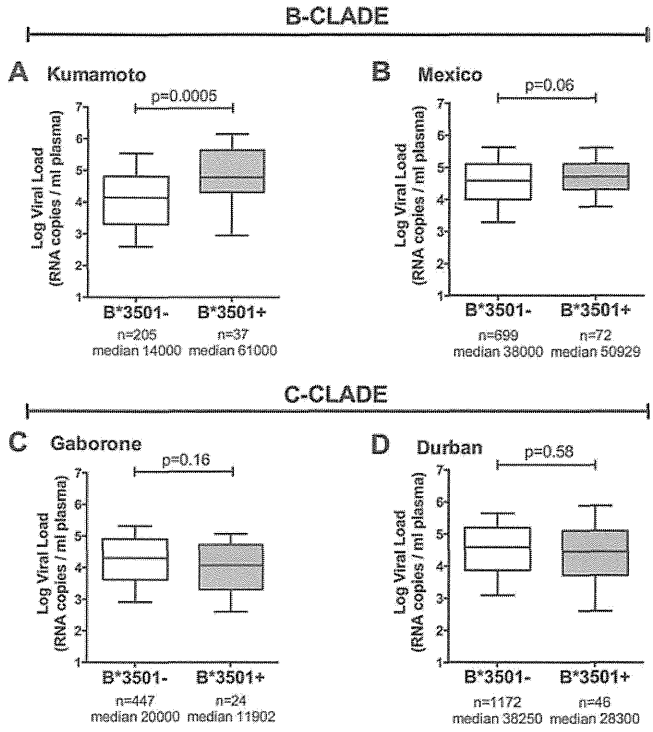

FIG 2 Median viral load in subjects with and without HLA-B*3501 in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. (A) Kumamoto, Japan (B clade); (B) Mexico City, Mexico (B clade); (C) Gaborone, Botswana (C clade); (D) Durban, South Africa (C clade). *P* values are by the Mann-Whitney test.

Gaborone, $P = 0.16$; $P = 0.01$ when data were pooled; median absolute CD4 counts, 439 versus 369 cells/mm^3 in HLA-B*3501-positive versus HLA-B*3501-negative subjects) (data not shown). In contrast, HLA-B*3501 is associated with lower absolute CD4 counts in subjects with B-clade infection (Mexico, $P = 0.01$; Japan, $P = 0.3$; $P = 0.01$ when data were pooled; median absolute CD4 counts, 249 versus 370 cells/mm^3 in HLA-B*3501-positive versus HLA-B*3501-negative subjects) (data not shown).

Thus, in two large C-clade-infected cohorts, HLA-B*3501 is associated with lower viral loads and higher CD4$^+$ T-cell counts in chronic HIV infection, whereas in B-clade-infected cohorts, such as those studied in Japan and in Mexico, HLA-B*3501 tends to be associated with a higher viral set point and lower absolute CD4 count.

HLA-B*3501-restricted CD8$^+$ T-cell responses in B- and C-clade infection. In order to investigate whether the observed difference in HLA-B*3501-associated HIV disease outcome in B- and C-clade-infected cohorts is related to clade-specific differences in the CD8$^+$ T-cell activity, we measured responses in HLA-B*3501-positive subjects infected with B- or C-clade virus to a comprehensive panel of HLA-B*3501-restricted epitopes (Table 1). This panel comprised epitopes previously defined from studies of B-clade-infected subjects with HLA-B*3501 and published in the Los Alamos Immunology database "A list" (www.hiv.lanl.gov) (48), together with 5 additional novel epitopes that were identified by analysis of CD8$^+$ T-cell responses in a cohort of 1,010 study subjects (40) to a panel of 410 overlapping 18-mer peptides (OLPs) spanning the C-clade proteome (see Table S2 in the supplemental material). An illustration of the approach that was used to identify these HLA-B*3501-restricted epitopes is shown for HPVHAGPIA (Gag positions 216 to 224) (HA9) (see Fig. S1 in the supplemental material), which was recently also described by another group (74) as a p24 Gag epitope restricted by HLA-B*3501.

For all the epitopes identified that were not listed in the Los Alamos Immunology database (www.hiv.lanl.gov) (48), in each case strong binding avidities to HLA-B*3501 (with the K_d [dissociation constant] ranging between 1 and 55 nM) were demonstrated (data not shown), and a CD8$^+$ T-cell response to each was detected in ≥2 study subjects tested (see below). In the process of validating the novel and previously published HLA-B*3501-restricted epitopes using HLA-class I tetramers (40), we noted one epitope that had been previously identified via an epitope prediction approach as PPIPVGDIY (PY9) (Gag positions 254 to 262) (64). We demonstrated that the true optimal epitope is the 10-mer NPPIPVGDIY (NY10) (Gag positions 253 to 262), which is consistently recognized at <1/1,000 of the concentration of PY9 (Fig. 3A). HLA-B*3501 tetramer staining of antigen-specific cells was readily observed using the 10-mer NY10 (Fig. 3B) but was never achieved using the 9-mer PY9. This process of distinguishing the correct epitope, NY10, from the incorrect epitope, PY9, was of crucial significance in understanding the differential impact of HLA-B*3501 in B- and C-clade HIV infection (see below).

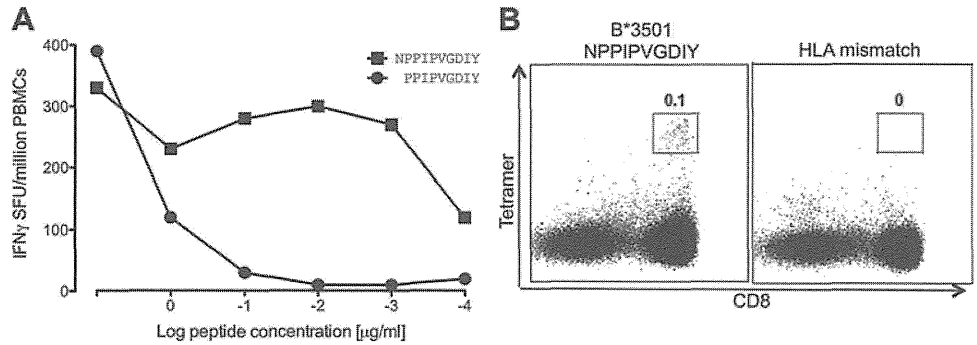

FIG 3 Optimization of the NY10 Gag epitope (NPPIPVGDIY). (A) IFN-γ ELISpot responses to titrated amounts of the 9-mer PPIPVGDIY versus the 10-mer NPPIPVGDIY peptides made by an HLA-B*3501-positive adult subject with chronic B-clade HIV-1 infection (Thames Valley subject H033, HLA-A*3601, -A*7401, -B*3501, -B*5301, -Cw*0401, -Cw*0401). (B) Unequivocal definition of the correct HLA-B*3501-restricted optimal epitope NY10 using an HLA-B*3501–NY10 tetramer to stain the NY10 responder PBMCs from the same subject (H033) as used for panel A. Results from one representative of two independent experiments are shown.

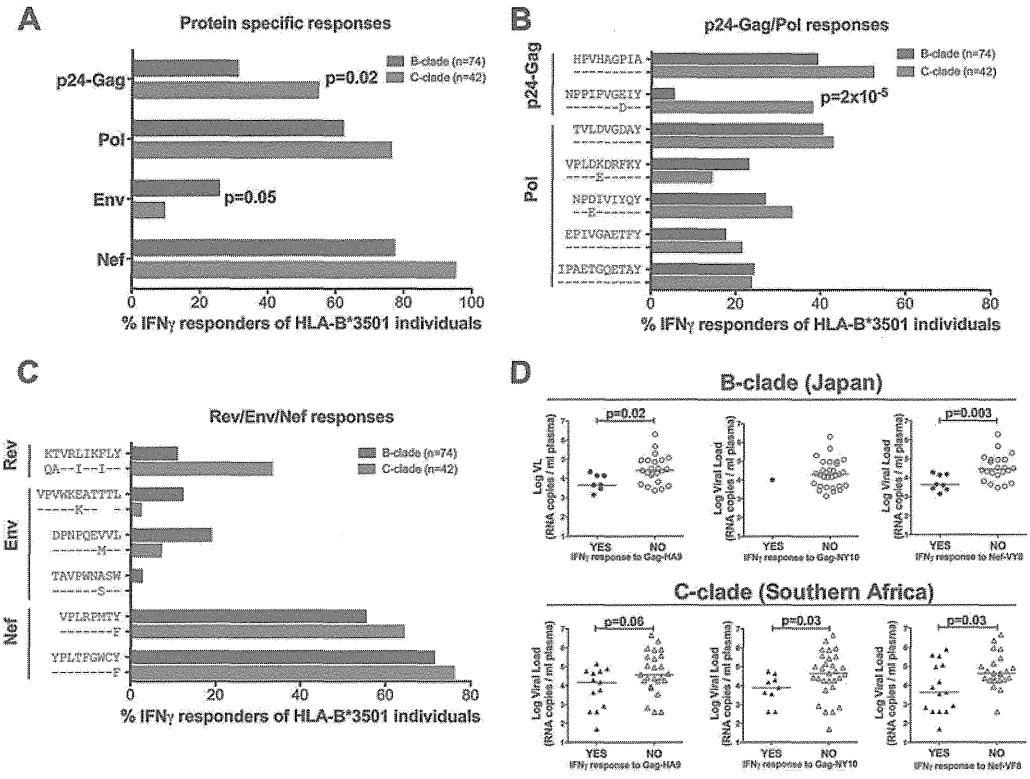

FIG 4 Percentage of HLA-B*3501-positive subjects making IFN-γ ELISpot responses to 13 HLA-B*3501-restricted epitopes in B- and C-clade infection and the impact on viral load to HA9 Gag, NY10 Gag and VY/VF8 Nef responses controlled by HLA-B*3501 matched nonresponding individuals. (A) Responses expressed as protein specific were obtained by pooling the percentage of adult HLA-B*3501-positive subjects with B-clade infection (Kumamoto, Japan) making IFN-γ ELISpot responses to individual HLA-B*3501-restricted optimal peptides ($n = 30$ subjects) pooled with another B-clade cohort (23, 44) screened against 18-mer overlapping peptides containing the optimal epitopes ($n = 44$ subjects) (blue) (total of 74 B-clade-infected subjects) and compared to adult subjects with C-clade infection (southern African subjects) tested against C-clade consensus overlapping peptides containing the corresponding optimal peptides ($n = 42$ subjects) (red). (B) Responses as in panel A but shown for individual epitopes within Gag and Pol proteins. (C) Responses as in panel A but shown for individual epitopes within Rev, Env, and Nef proteins. (D) Comparison of viral load between responders and nonresponders for B-clade-infected Japanese subjects ($n = 30$), based on responses to optimal peptides, HA9 Gag (left), NY10 Gag (middle), and VY/VF8-Nef (right) (top panels) and C-clade southern African subjects based on responses to OLPs containing the corresponding optimal peptides (bottom panels). In each case, a positive ELISpot response is defined as >100 SFC/10^6 PBMCs; P values are by Fisher's exact test (A, B, and C) (and for B and C are shown only when significant after correction for multiple comparisons) or by Mann-Whitney U test (D).

Gag NY10 is the single epitope differentially targeted by HLA-B*3501 subjects with B- and C-clade infection. Reactivity to the panel of HLA-B*3501-restricted epitopes defined was determined in HLA-B*3501-positive subjects with B-clade infection ($n = 74$) and in subjects with C-clade infection ($n = 42$) using ELISpot assays (Fig. 4). Overall, p24 Gag-specific epitopes were targeted significantly more frequently by the C-clade-infected B*3501-positive study subjects (55% versus 31%; $P = 0.02$ by Fisher's exact test), whereas Env-specific epitopes were targeted more frequently by B-clade-infected B*3501-positive study subjects (10% versus 26%; $P = 0.05$ by Fisher's exact test) (Fig. 4A). At the individual epitope level, the single statistically significant clade-specific difference was in the response to the Gag NY10 epitope (Gag positions 253 to 262; $P = 2 \times 10^{-5}$). A response to this epitope was seen in only 5% of B-clade-infected subjects, versus 38% of C-clade-infected subjects. Although the Rev epitope KY10 (Rev positions 14 to 23) was also predominantly targeted in C-clade infection, this difference in recognition in B- and C clade-infected HLA-B*3501-positive subjects did not reach statistical significance after correction for multiple comparisons.

Both p24 Gag responses and one Nef response are consistently associated with lower viral load in subjects with HLA-B*3501. Having determined which HLA-B*3501-restricted epitopes are targeted in B- and C-clade-infected subjects with HLA-B*3501, we next investigated which of these responses appear to be most effective in bringing about a low viral set point. Two responses were consistent in being associated with a lower set point in the responders compared to the nonresponders in both B- and C-clade cohorts, Gag HA9 and Nef VY8 (Fig. 4D). These two epitopes are targeted equally well in B- and C-clade infection, and therefore these responses do not help to explain why HLA-B*3501 is associated with lower viral set points in C-clade infection. In the case of Gag NY10, however, in B-clade infection there was only 1 responder among 31 B-clade subjects for whom viral loads were available. However, in the C-clade-infected cohort, a response toward Gag NY10 was also associated with a lowered viremia ($P = 0.03$ by Mann-Whitney test) (Fig. 4D), Thus, the only HLA-B*3501-restricted response associated with a lower viral set point for which there was a significant difference in epitope targeting comparing the B- and C-clade cohorts was the Gag NY10 response.

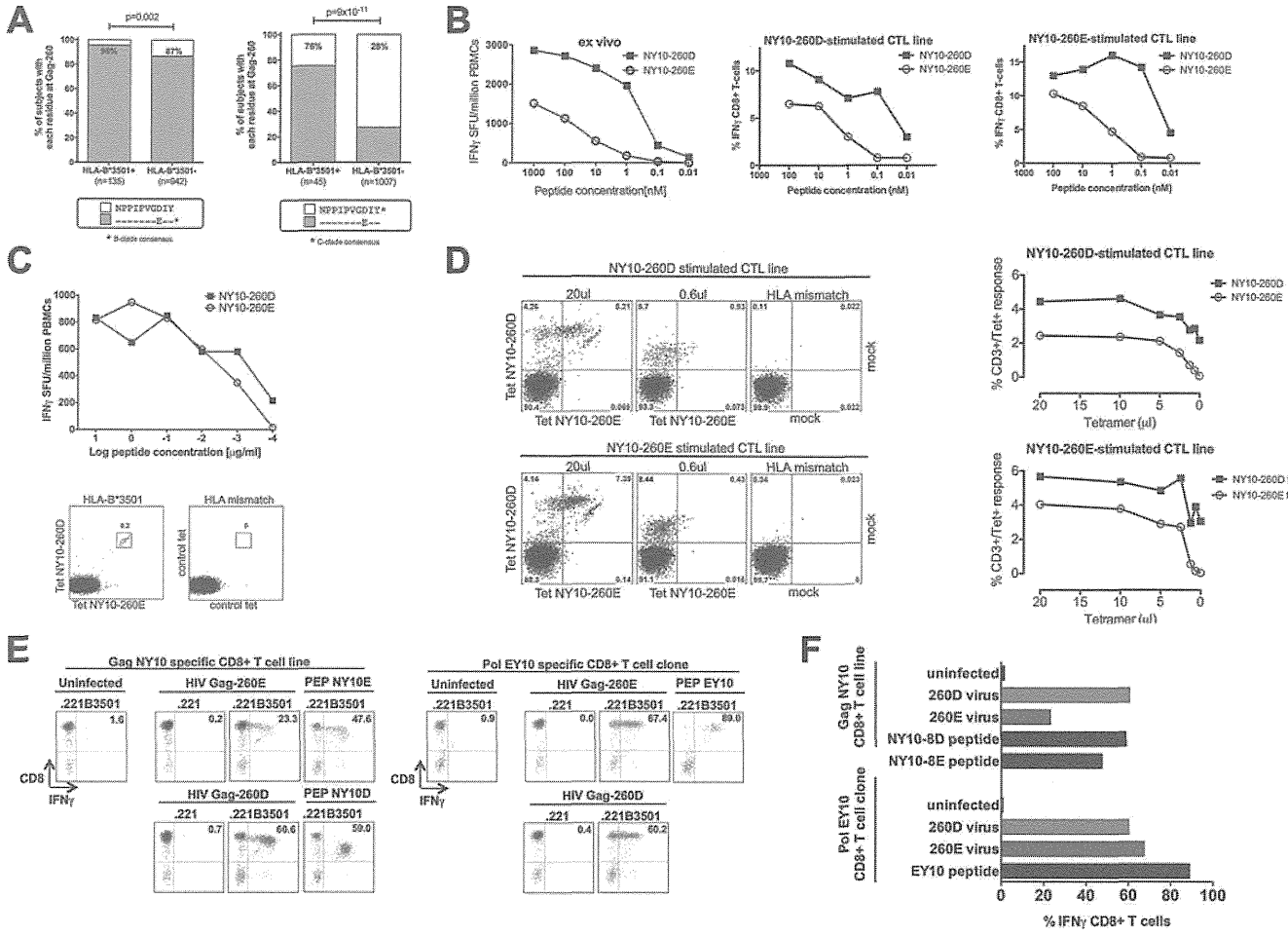

FIG 5 Selection of Gag-D260E substitution in C-clade infection and effect of this polymorphism on $CD8^+$ T-cell recognition and lack of NY10-260E specific $CD8^+$ T cells. (A) Selection of Gag-D260E polymorphism in subjects with HLA-B*3501 from an extended B-clade data set as previously published (24) (n = 1,077; total subjects with HLA-B*3501, n = 135 [12.5%]) (left) and selection of Gag-D260E polymorphism in subjects with HLA-B*3501 from an extended southern African data set (Durban, n = 695; Botswana, n = 298; Thames Valley Africans, n = 59; total subjects with HLA-B*3501, n = 45 [4.3%]) (right). (B) IFN-γ *ex vivo* ELISpot responses made by an HLA-B*3501-positive Japanese subject with chronic B-clade infection (subject KI705, HLA-A*2402, -A*2601, -B*3501, -B*5201, -Cw*0303, -Cw*1202) to optimal epitope NY10 (NPPIPVGDIY) and an escape variant containing the D260E substitution (NPPIPVGEIY) and IFN-γ intracellular cytokine staining of $CD8^+$ T cells *in vitro* expanded and tested against titrated amounts of NY10-260E and NY10-260D peptides. One experiments was performed. (C) IFN-γ *ex vivo* ELISpot responses made by an HLA-B*3501-positive subject with chronic B-clade infection (subject OX035, HLA-A*0201, -A*1101, -B*1801, -B*3501, -Cw*0401, -Cw*0501) to optimal epitope NY10-260D and an escape variant containing the D260E substitution NY10-260E and dual NY10-260E and NY10-260D HLA-B*3501 tetramer staining of *ex vivo* PBMCs controlled by HLA-B*4201 mismatch tetramer. Results from one representative of two independent experiments are shown. (D) *In vitro*-expanded PBMCs from subject OX035 using NY10-260D (top) and NY10-260E (bottom) peptides and stained with titrated amounts of dual HLA-B*3501 tetramers (260D/260E) gated on $CD8^+$ T cells (dot plots) and expressed as $CD3^+/Tet^+$ positive cells for all tetramer titrations (right) controlled by HLA-B*4201 mismatch tetramers. *P* values are by Fisher's exact test. One experiments was performed. (E and F) HLA-negative and HLA-B*3501-expressing target cells were infected with either Gag-260E or Gag-260D virus and tested for epitope recognition by specific $CD8^+$ T cells determined by IFN-γ production after coculture and shown for Gag-NY10 epitope processing (left) or the control Pol-EY10 epitope (right) by fluorescence-activated cell sorter (FACS) plots (E) and shown as horizontal bar graphs (F). Peptide-pulsed target cells (PEP) were included as a positive control for optimal epitope presentation.

Lack of immunogenicity of NY10-260E indicated by strong selection of the Gag-D260E polymorphism in B- and C-clade infection and lack of NY10-260E-specific $CD8^+$ T-cell responses. We next addressed the question of why the B-clade version of Gag NY10, which differs from the C-clade version only at position 8 in the epitope, in the replacement of Asp by Glu (Gag-D260E), appears to be nonimmunogenic, whereas the C-clade version is highly immunogenic. Although 38% of HLA-B*3501-positive subjects with chronic C-clade infection show detectable responses to NY10-260D, analysis of *gag* sequences in the cohort indicates that exactly twice that figure, 76%, of HLA-B*3501-positive subjects carry the Gag-D260E mutation, compared to 28% of the HLA-B*3501-negative study subjects (Fig. 5A) ($P = 9 \times 10^{-11}$). We confirmed that, in every case tested, the NY10-D260E variant is substantially less well recognized than the C-clade wild-type NY10-260D (Fig. 5B) and that NY10-D260E is therefore an escape mutant. Strikingly, NY10-260E is also selected in HLA-B*3501-positive subjects with B-clade infection (Fig. 5A), in spite of the fact that close to 90% of B-clade sequences carry Gag-260E (37). These data suggest that NY10-260E is nonimmunogenic and that only the small fraction of B-clade-infected HLA-B*3501-positive subjects presented with virus expressing the Gag-260D vari-