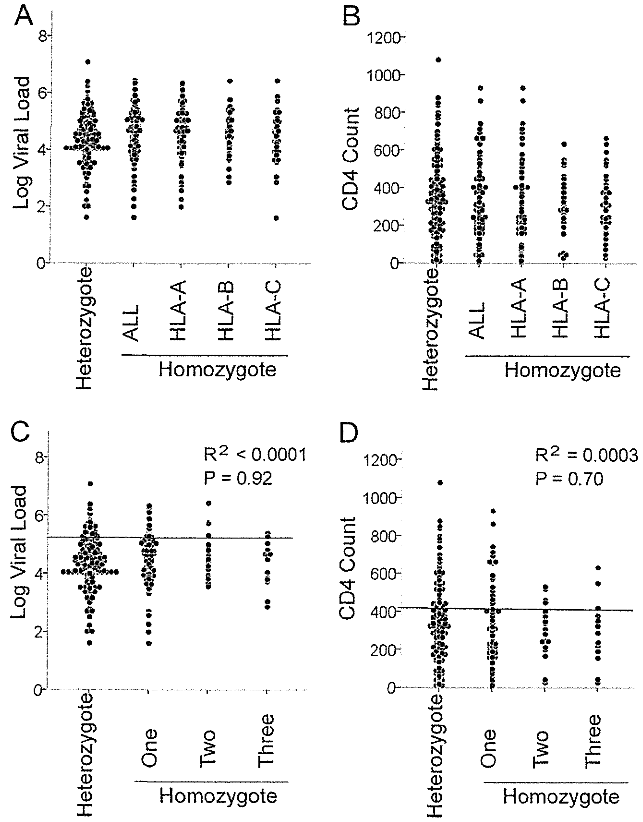

FIG 3 Effect of HLA class I homozygosity on VL and CD4. The comparison between heterozygotes at all three class I loci ($n = 349$) and homozygotes ($n = 155$) for at least one HLA allele is shown (A and B). (A and B) Homozygotes are grouped according to homozygosity for the HLA-A ($n = 106$), -B ($n = 42$), 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	H**P**VHAGPI	**A**	Gag 216-224	Gag HA9*
	C	--------	-		
	B	N**P**PIPVGEI	**Y**	Gag 253-262	Gag NY10
	C	-------D-	-		
RT	B	T**V**LDVGDA	**Y**	Pol 262-270	RT TY9
	C	--------	-		
	B	V**P**LDKDFRK	**Y**	Pol 273-282	RT VY10
	C	----E---	-		
	B	N**P**DIVIYQ	**Y**	Pol 330-338	RT NY9
	C	--E-----	-		
	B	E**P**IVGAETF	**Y**	Pol 587-596	RT EY10*
	C	---A-----	-		
Int	B	I**P**AETGQETA**Y**		Pol 804-814	Int IY11*
	C	-----------			
Rev	B	K**T**VRLIKFL	**Y**	Rev 14-23	Rev KY10, Rev QY10*
	C	Q**A**--I--I-	-		
gp120	B	V**P**VWKEATTT**L**		Env 42-52	Env VL11
	C	-----K--	-		
	B	D**P**NPQEVV	**L**	Env 78-86	Env DL9
	C	------M-	-		
gp41	B	T**A**VPWNAS	**W**	Env 606-614	Env TW9
	C	------S-	-		
Nef	B	V**P**LRPMT	**Y**	Nef 73-81	Nef VY8, Nef VF8
	C	-------	**F**		
	B	Y**P**LTFGWC	**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-

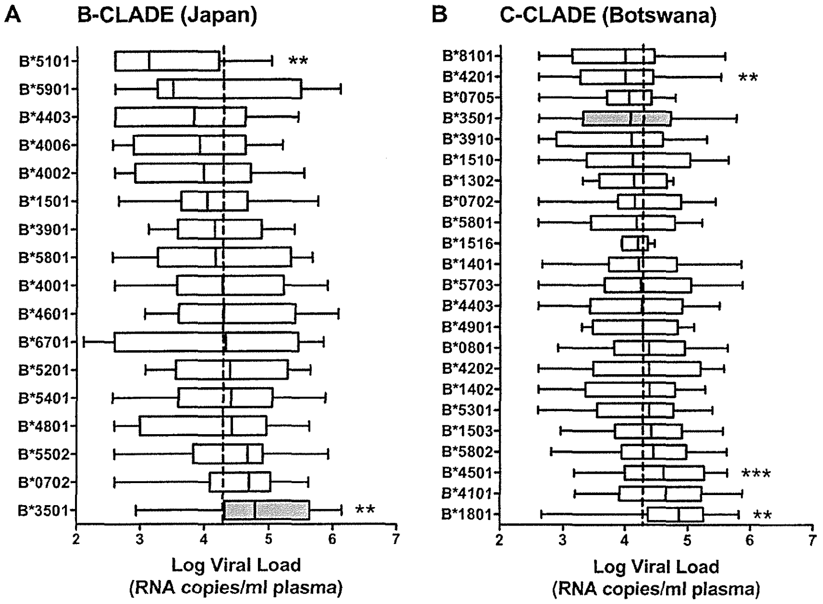

FIG 1 Ranking of HLA-B alleles with respect to median viral load (VL) in chronic HIV-1 infection in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. HLA-B*3501 is highlighted in gray. Dashed lines indicate median VL for 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, $P = 0.06$;

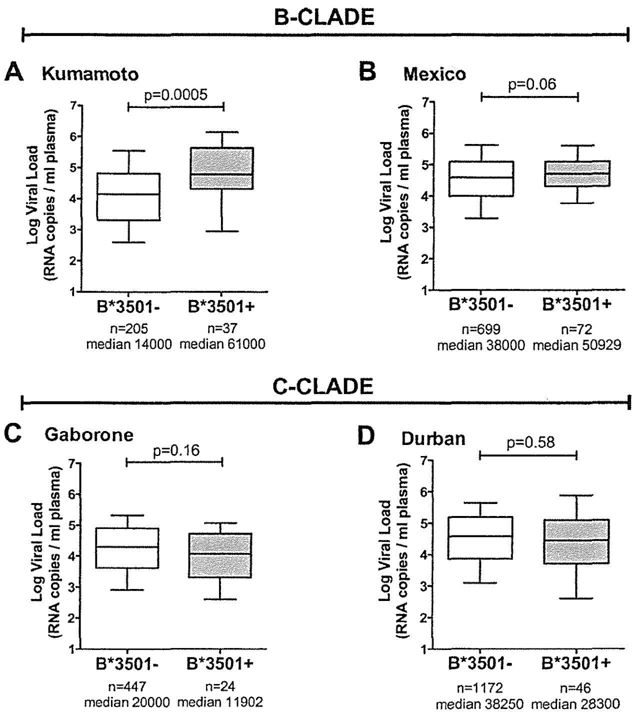

FIG 2 Median viral load in subjects with and without HLA-B*3501 in B- and C-clade-infected study cohorts. Boxes show median and 25th and 75th centiles; whiskers show 10 to 90% confidence intervals. (A) Kumamoto, Japan (B clade); (B) Mexico City, Mexico (B clade); (C) Gaborone, Botswana (C clade); (D)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

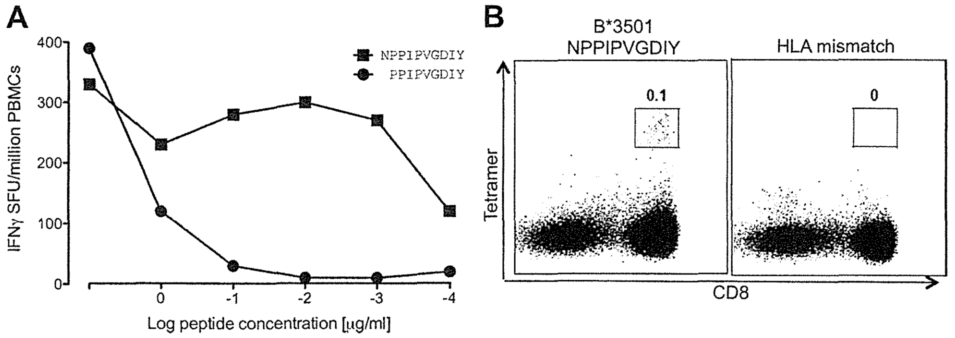

FIG 3 Optimization of the NY10 Gag epitope (NPPIPVGDIY). (A) IFN-γ ELISpot responses to titrated amounts of the 9-mer PPIPVGDIY versus the 10-mer NPPIPVGDIY peptides made by an HLA-B*3501-positive adult subject with chronic B-clade HIV-1 infection (Thames Valley subject H033, HLA-A*3601, -A*7401, -B*3501, -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.

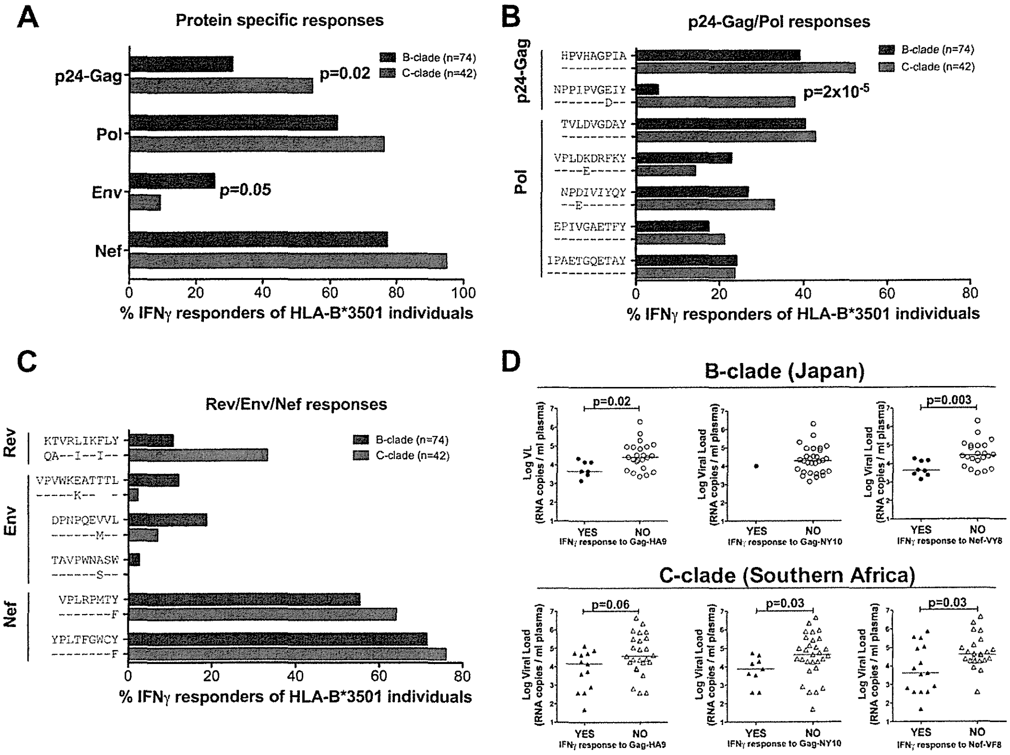

FIG 4 Percentage of HLA-B*3501-positive subjects making IFN-γ ELISpot responses to 13 HLA-B*3501-restricted epitopes in B- and C-clade infection and the impact on viral load to HA9 Gag, NY10 Gag and VY/VF8 Nef responses controlled by HLA-B*3501 matched nonresponding individuals. (A) Responses expressed 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.

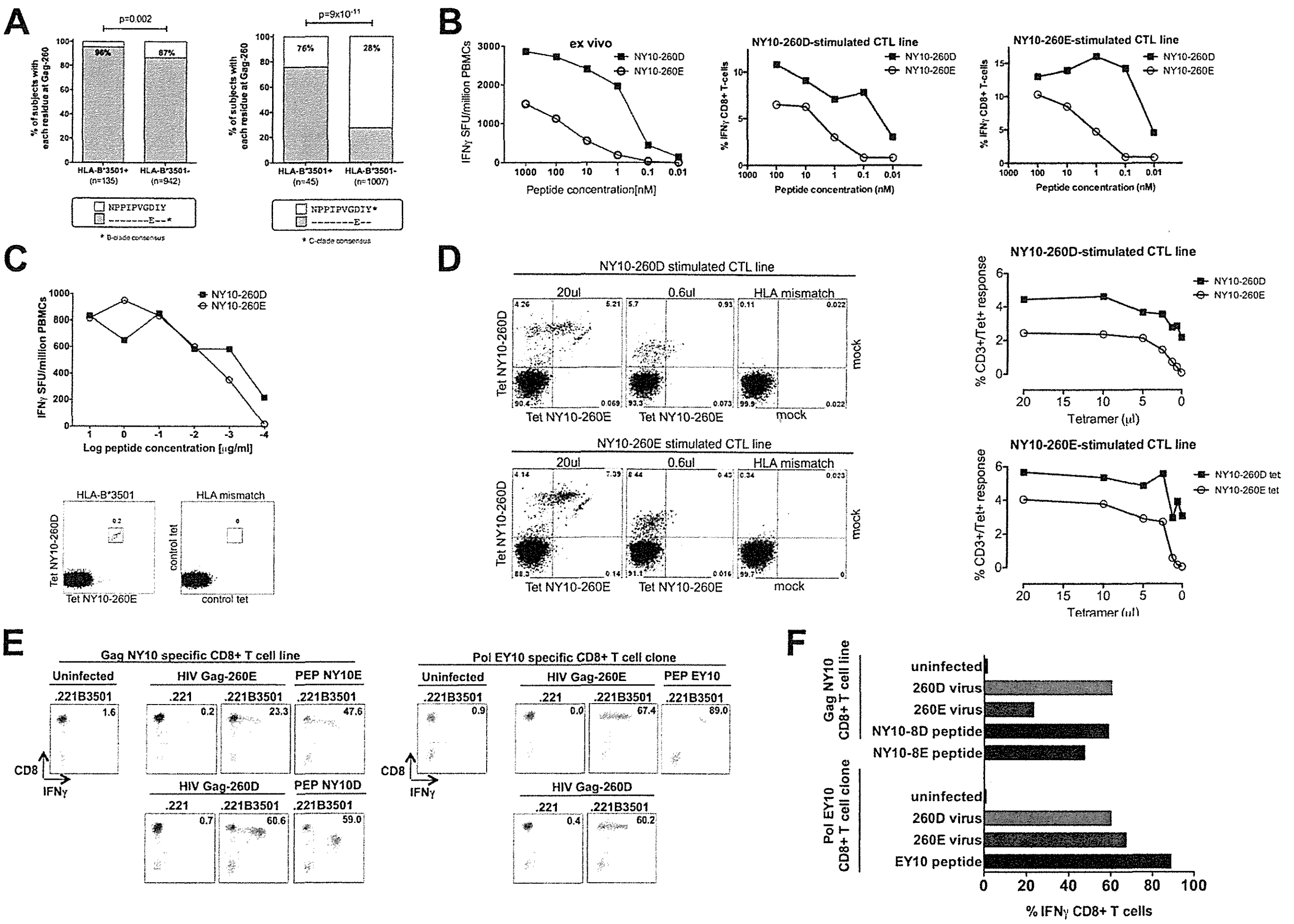

FIG 5 Selection of Gag-D260E substitution in C-clade infection and effect of this polymorphism on CD8$^+$ T-cell recognition and lack of NY10-260E specific CD8$^+$ T cells. (A) Selection of Gag-D260E polymorphism in subjects with HLA-B*3501 from an extended B-clade data set as previously published (24) (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-

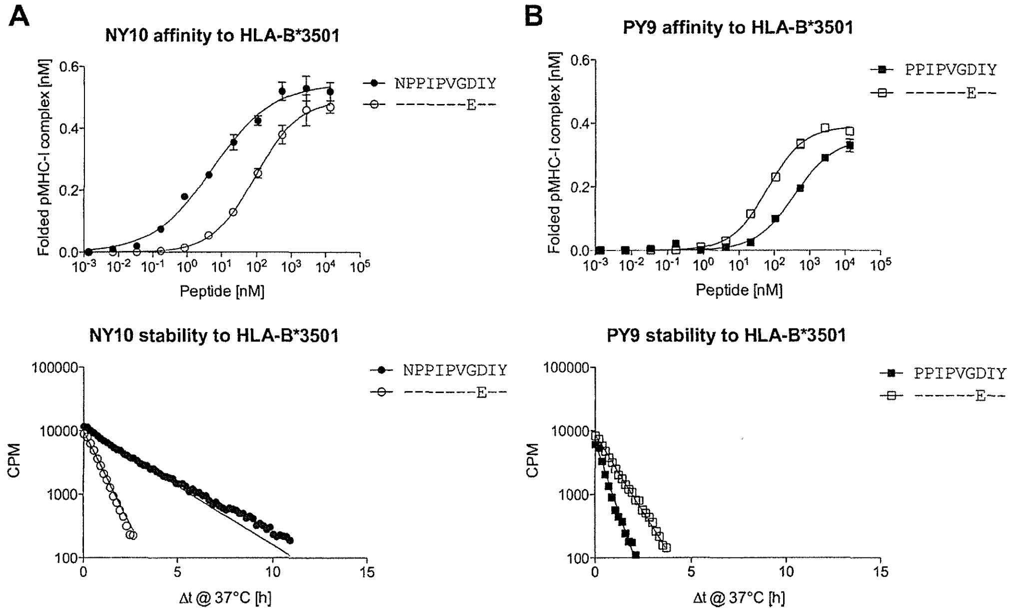

FIG 6 Binding of NY10 Gag and PY9-Gag to the HLA-B*3501 molecule. Strength of binding affinity (K_d, nM) of HLA-B*3501 was determined using the luminescent oxygen channeling immunoassay, as previously described (29) (top panels), and stability half-life ($t_{1/2}$) of binding (h) was determined using scintillation proximity assay, as previously described (30) (bottom panels), for NY10 (A) and PY9 (B). Results from one representative of four independent experiments are shown.

ant can make an NY10-260D-specific response, from which the viral escape mutant D260E is selected.

To test this hypothesis, i.e., that NY10 responses in B-clade-infected subjects are either cross-reactive between the two NY10-260D and NY10-260E variants or specific to the NY10-260D form but are never specific for the NY10-260E variant, we generated HLA-B*3501-NY10-260D and HLA-B*3501-NY10-260E tetramers with which to stain NY10-specific $CD8^+$ T cells. Staining of PBMCs and antigen-specific cell lines with these two HLA-B*3501-NY10 tetramers was consistent with the hypothesis (Fig. 5C and D). *In vitro* expansion of NY10-specific $CD8^+$ T cells in the rare B-clade-infected persons showing a response to this epitope showed, irrespective of which variant had been used to stimulate PBMCs, preferential recognition of the NY10-260D (C-clade version) of the epitope (Fig. 5B). Where there is apparent cross-reaction of NY10-260D-specific $CD8^+$ T cells to the NY10-260E variant (Fig. 5C), following *in vitro* expansion of these cells using either the NY10-260D or the NY10-260E peptide, preferential recognition of the NY10-260D epitope consistently emerges. Dual NY10-260D and NY10-260E tetramer staining confirms that only cross-reactive or NY10-260D-specific $CD8^+$ T cells exist, with no detection of NY10-260E-specific $CD8^+$ T cells (Fig. 5D). To test whether the 260E escape version has a reduced recognition compared to the 260D version using intracellular processed epitopes, rather than peptide-pulsed cells, we infected HLA-B*3501-positive or HLA class I-negative cells with HIV containing either the 260D or the 260E virus and determined the level of NY10 epitope recognition by assaying the activation of an NY10-specific $CD8^+$ T-cell line after coculture with cells infected for 6 days (Fig. 5E and F). We detected almost 3-fold-higher activation after infection with the 260D virus compared to the 260E virus ($CD8^+$/IFN-γ^+, 60.6% versus 23.3%) but equal activation of the control Pol EY10-specific $CD8^+$ T-cell clone. Thus, infection with the 260E virus results in a markedly reduced recognition of the nonimmunogenic NY10-260E compared to the immunogenic NY10-260D epitope processed from the 260D virus.

NY10-260E nonimmunogenicity results from lack of HLA-B*3501–peptide binding affinity and stability. Given that the peptide-binding motif for HLA-B*3501 does not show any preference for particular residues at position 8 (P8) in the epitope, our initial hypothesis was that nonimmunogenicity of the NY10-260E variant might be related to the low TcR repertoire available for HLA-B*3501-restricted T-cell responses, as proposed by Kosmrlj et al. (41). However, to determine whether that NY10-260E nonimmunogenicity might be more readily explained as a result of weak HLA-B*3501 binding affinity and/or stability, we first performed these MHC binding studies. We found that the immunogenic, NY10-260D (C-clade) version of the peptide had a >10-fold-greater binding affinity to the HLA-B*3501 molecule than the NY10-260E (B-clade) variant and was more than three times more stable in complex with the HLA-B*3501 molecule than the NY10-260E version (half-life, 1.6 h versus 0.5 h) (Fig. 6A). Previous studies suggest that, with rare exceptions, a peptide-MHC stability half-life of >1 h is required for peptides to be immunogenic (31). The low peptide-MHC binding stability of the NY10-260E variant (half life, 0.5 h) would therefore explain the lack of NY10-specific responses observed for the B-clade cohorts studied here. This is also consistent with reduced recognition of the NY10-260E versus the NY10-260D version of the epitope shown in Fig. 5.

It is noteworthy that had PY9 as opposed to NY10 been the optimal epitope in this case, it would not have been able to explain lack of immunogenicity of the B-clade variant in this way. Both B- and C-clade versions of PY9 had low peptide-binding affinities to HLA-B*3501, in particular the Gag-260D (C-clade) version (K_d = 76 and 407 nM for PY9-260E and PY9-260D, respectively), and very low peptide-B*3501 binding stabilities, again lower for the

Gag-260D C-clade version of PY9 (half-life of 0.62 h and 0.34 h for PY9-260E and PY9-260D, respectively).

Together these data suggest that the observed differential HLA-B*3501 association with HIV disease progression in B- and C-clade infection may hinge on a single Gag epitope, NY10, and that the lack of immunogenicity of this epitope in B-clade infection rests on the presence of Glu at Gag-260 in the consensus B-clade sequence, in contrast to Asp at Gag-260 in the consensus C-clade sequence.

DISCUSSION

The data presented here demonstrate that subjects with HLA-B*3501 control HIV-1 more effectively in C-clade than in B-clade infection. This difference was associated with greater targeting of p24 Gag epitopes and less frequent targeting of Env epitopes overall. However, the single epitope significantly targeted differentially was the Gag NY10 epitope, targeted by 38% of HLA-B*3501-positive subjects with chronic C-clade infection and only 5% of HLA-B*3501-positive subjects with chronic B-clade infection. The reason for this difference is the replacement of Asp by Glu at Gag-260, position 8 within the NY10 epitope: in C-clade infection, ~75% of sequences carry Asp at Gag-260, whereas in B-clade infection, ~90% of sequences carry Glu at Gag-260. NY10-260E is nonimmunogenic and insufficiently recognized from infected cells (<25% CD8$^+$ T-cell activation) because this variant fails to bind sufficiently stably to HLA-B*3501. In contrast, the NY10-260D version is recognized more efficiently (>60% CD8$^+$ T-cell activation) and binds relatively stably to HLA-B*3501 (off-rate half-life of 1.6 h, compared to 0.5 h for NY10-260E). The binding affinity of HLA-B*3501 for NY10-260D was also substantially higher than that for NY10-260E (K_d of 10 nM versus 113 nM, respectively), consistent with the difference in antigen processing of this epitope. These findings provide a plausible explanation for why NY10-260E is an escape variant in B- and C-clade infection and why only the NY10-260D variant is immunogenic.

Several hypotheses have previously been proposed to explain the rapid disease progression of HLA-B*3501-positive subjects infected with B-clade HIV, including a paucity of HLA-B*3501-restricted Gag-specific CD8$^+$ T-cell epitopes (39), failure to optimize antiviral NK activity (51, 52) and narrowness of the TcR repertoire available to counter epitope sequence variability (41). The data presented here support the "Gag hypothesis" (39), in that even the addition of a single extra Gag response appears to significantly alter the impact of HLA-B*3501 in HIV infection. This is consistent with previous findings that increasing Gag-specific CD8$^+$ T-cell breadth is correlated with increasing viral suppression (39) and that the p24 Gag protein is infrequently targeted by HLA-B*3501-restricted CD8$^+$ T-cell responses in B-clade infection (67). These data also support previous studies that have suggested that even one effective CTL response can mediate long-term immune control of immunodeficiency virus infection, such as the KK10 (Gag positions 263 to 272) response in HIV-infected subjects with HLA-B*27 (27) or the SW9 (Gag positions 241 to 249) response in simian immunodeficiency virus (SIV)-infected Burmese macaques expressing the MHC 90-120-Ia haplotype (35, 70).

These data show that inadequate HLA-B*3501 binding of the peptide, as opposed to TcR paucity, as has been proposed as a mechanism for HLA-B*3501-associated rapid progression (41), may provide the explanation for the lack of a response to NY10-260E in B-clade infection. The two hypotheses are not mutually exclusive, and it remains possible that HLA-B*3501 is associated with some degree of protection against C-clade progression in spite of TcR paucity. However, the distinction is of direct relevance to vaccine design, since we show here that HLA-associated disease outcome is dependent on the epitopes being targeted, irrespective of any deficiencies attributed to the respective HLA molecule. Furthermore, it is striking that HLA-B*0702 and HLA-B*3501, the two alleles proposed to predispose to rapid HIV progression as a result of TcR paucity (41), both have a more successful impact on the viral set point in C-clade infection (Fig. 1), as do many other alleles within the HLA-B7 supertype whose peptide-binding motifs are very similar, namely, HLA-B*8101, B*4201, B*0705, and B*3910 (45).

These studies also draw attention to caveats associated with epitope prediction approaches using peptide-binding motifs or even those using the most sophisticated software that takes account of the possible contribution to MHC binding of every amino acid of every peptide known to bind to a particular MHC class I molecule. Although PPIPVGDIY (PY9) has appeared in the "A" list of HIV-specific CD8$^+$ T-cell epitopes since 1995 (48) and epitope prediction programs predict that PY9 would bind better than NY10 to HLA-B*3501 (17), nonetheless PY9 is not the epitope. It is significant that 0/377 peptides eluted from HLA class I molecules and sequenced have Pro at P1 (44). Bearing in mind the specificity of ERAP-1, which cleaves neither at X-P nor at P-X bonds (32), it seems that epitopes carrying Pro at P1, if they exist at all, are rare. The importance of defining the precise optimal epitope correctly is underlined by this study, in the demonstration that the 10-mer NY10 could only be immunogenic with Asp at P8 (Fig. 5). In contrast, although PY9-260E bound with stronger avidity than PY9-260D to HLA-B*3501, neither version of the 9-mer PY9 appeared to bind HLA-B*3501 with adequate stability to be immunogenic. It may be helpful in the future to confirm the identification of novel epitopes using peptide-MHC I tetramers, as now can be done readily (40, 43).

The critical contribution to MHC binding of the residue at P8 in an HLA-B*3501-restricted epitope was unexpected, given the peptide-binding motif of HLA-B*3501, which describes proline at P2 and Tyr at PC as the primary anchor residues, with various residues less strongly preferred at P2, P3, P4, and PC (18, 33). Explanation of this awaits the solution of the crystal structure of the HLA-B*3501–NY10-260D complex. However, an HLA-B*3501-EBV epitope structure has been solved (71), and modeling the HLA-B*3501-NY10 structure based on these data suggests that Asp at P8 in the NY10 epitope indeed points into the groove (Fig. 7). The model suggests that replacement of Asp by Glu at P8 would lead to steric hindrance between the longer side chain of Glu and the side chain of Ala-150 in the MHC α2 helix. The resulting altered conformation of the peptide would explain the observed reduction in stability of NY10-260E (Fig. 6B). This is consistent with the reduced but detectable processing of the NY10-260E peptide (data not shown) and is directly explained by the reduced affinity to the MHC molecule and thereby suggests that the limiting step in processing of the NY10-260E peptide occurs when the fully trimmed epitope is loaded onto the HLA-B*3501 molecule by the peptide-loading complex. This reduction in processing of the NY10-260E epitope may be critical to distinguish immunogenicity, especially at low infection levels of pri-

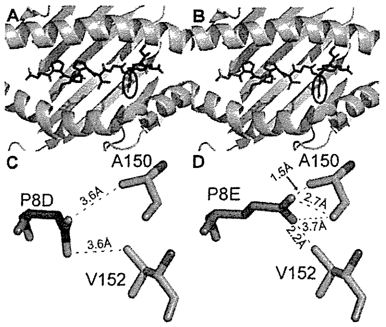

FIG 7 Modeled B3501-NY10 structure using the B3501-EPLPQGQLTAY complex (71). (A) HLA-B*3501 (shown in gray cartoon)-NPPIPVGDIY (shown in blue sticks), looking down at the MHC-binding groove. Position P8D in the peptide is circled. (B) HLA-B*3501 (shown in gray cartoon)-NPP IPVGEIY (shown in red sticks), looking down at the MHC-binding groove. P8E is circled. (C) Modeled interaction with NY10 residue P8D (blue stick) and MHC residues A150 and V152 (green sticks). (D) Modeled interaction with NY10 residue P8E (red stick) and MHC residues A150 and V152 (green sticks). The longer side chain of E in the escape mutant NY10 compared to D in the wild-type NY10 could generate steric hindrance with MHC residue A150. This could destabilize, and change the conformation of, the NY10 escape mutant peptide.

mary CD4$^+$ T cells *in vivo*, in contrast to the higher multiplicity of infection used *in vitro* in this assay.

The high frequency (~75%) of the D260E selection in C-clade infection suggests a highly functional Gag NY10-specific CD8$^+$ T-cell response *in vivo*. However, when we undertook sequencing of the Gag NY10 region from position 253 to 262 of 17 HLA-B*3501-positive recipients with known Gag NY10 sequences of their linked donor viruses, we did not find any selection of D260E escape mutation at very early viral load set points (CD4 count nadir) in 10 HLA-B*3501 individuals infected with the 260D virus (0/10) (data not shown). This suggests that the D260E selection occurs after the CD4 nadir during chronic infection and that the Gag NY10 response therefore may operate during chronic infection rather than during acute infection. This is consistent with a previous study showing that the HLA-B*3501-D260E mutation is selected outside acute infection (54). Moreover, we did not observe any change in the viral load set point for individuals carrying 260D versus 260E within linked recipients (37,720 versus 39,740 RNA copies/ml plasma; $P = 0.6$) early after infection or during chronic infection (17,550 versus 26,600 RNA copies/ml plasma; $P = 0.58$) (data not shown). However, the small numbers in combination with the potential compensatory mutations identified, which may restore viral fitness, may mask differences in viral load set point.

Although the residue at Gag-260 appears to play an important part in immunogenicity of the HLA-B*35:01-NY10 epitope, it is also important to note that, as with many amino acid substitutions in p24 Gag, this single-amino-acid substitution at Gag-260 is often observed in association with a number of other variations elsewhere in p24 Gag. In a covariation analysis (14), we identified 9 statistically significant associations ($q < 0.05$) between Gag-260D and variation at other positions (see Table S3 in the supplemental material), which may indicate that the D260E escape in C-clade virus may require compensatory mutations to minimize the impact on viral replicative capacity.

One further observation with respect to epitope definition highlighted by this study is the value of using a panel of overlapping peptides to comprehensively map responses made by HIV-infected subjects, as opposed to using epitope prediction. The other p24 Gag epitope defined here in HLA-B*3501-positive subjects, HPVHAGPIA (HA9), may have gone unnoticed previously because HLA-B*3501 typically shows a binding preference for Tyr or a larger hydrophobic residue than Ala at the C terminus. Between 40 and 60% of subjects studied here with HLA-B*3501 made a response to HA9, and, like for the NY10 Gag response, responders had significantly lower viral loads than nonresponders. Thus, a critical epitope within p24 Gag would have remained undetected had we used an approach based on predicted epitopes only.

Of note, we unexpectedly showed that a response to one of the Nef epitopes, NY8, was also associated with a lowered viremia in both B- and C-clade infection. CD8$^+$ T-cell responses to Nef have not typically been associated with disease control (39), but the data presented here suggest that specific responses within Nef may also mediate viremic suppression. In a previous study, it was observed that a substantial number of the Nef escape mutations revert following transmission to an HLA-mismatched host (54), suggesting a cost to viral fitness; the escape polymorphism itself may therefore contribute to disease control via an effect on viral replicative capacity. This finding is also consistent with data describing effective control of SIV in Mamu-B*08- and Mamu-B*17-positive rhesus macaques, which tend to target dominant epitopes not in Gag but in proteins such as Nef and Vif (49, 57). Thus, although a broad Gag-specific CD8$^+$ T-cell response may be more likely to be effective against HIV, it remains possible that CD8$^+$ T-cell responses targeting epitopes in non-Gag proteins may also be effective in containment of immunodeficiency virus infection.

It is important also to consider the limitations of this study. In particular, attention should be drawn to the fact that optimal HLA-B*3501-restricted epitopes 8 to 11 amino acids in length were tested for recognition in the B-clade-infected Japanese study subjects, whereas the C-clade-infected subjects were tested for recognition of the 18-mer overlapping peptides containing those optimal epitopes. Although responses to the 18-mer and to the optimal epitope have been strongly correlated (16) ($r = 0.85$; $P < 0.0001$ [H. N. Kloverpris et al., unpublished data]), the magnitude of response to the 18-mer tends to be somewhat lower than that to the optimal epitope, particularly if the location of the optimal epitope is in the central part of the 18-mer peptide (16, 55). However, this likely underestimation of the responses in the C-clade-infected study subjects, where response frequencies were determined using the 18-mer overlapping peptides, would likely have reduced the estimates of the frequency of Gag NY10 responses and of Gag HA9 responses, detected in 38% and 52% of subjects, respectively. Therefore, the difference in targeting of p24 Gag epitopes that exists between B- and C-clade-infected subjects is likely, if anything, to be even greater than shown in Fig. 4.

In summary, the impact of HLA alleles such as HLA-B*3501 on HIV disease outcome differs according to clade of infection. These data suggest that the critical difference in C-clade infection is the ability of HLA-B*3501-positive subjects to make two p24 Gag-specific responses restricted by this allele, NY10 and HA9, compared to only one (HA9) in B-clade-infected subjects. This result provides the clearest data yet that HLA-associated disease outcome is dependent on the epitopes being targeted, irrespective of the nature of the restricting HLA molecule (55), and this provides hope that a vaccine that can induce effective $CD8^+$ T-cell responses can successfully bring about immune control even in people who carry HLA alleles traditionally regarded as associated with rapid disease progression.

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We declare that no competing interests exist.

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Distinct HIV-1 Escape Patterns Selected by Cytotoxic T Cells with Identical Epitope Specificity

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Pol283-8-specific, HLA-B*51:01-restricted, cytotoxic T cells (CTLs) play a critical role in the long-term control of HIV-1 infection. However, these CTLs select for the reverse transcriptase (RT) I135X escape mutation, which may be accumulating in circulating HIV-1 sequences. We investigated the selection of the I135X mutation by CTLs specific for the same epitope but restricted by HLA-B*52:01. We found that Pol283-8-specific, HLA-B*52:01-restricted CTLs were elicited predominantly in chronically HIV-1-infected individuals. These CTLs had a strong ability to suppress the replication of wild-type HIV-1, though this ability was weaker than that of HLA-B*51:01-restricted CTLs. The crystal structure of the HLA-B*52:01-Pol283-8 peptide complex provided clear evidence that HLA-B*52:01 presents the peptide similarly to HLA-B*51:01, ensuring the cross-presentation of this epitope by both alleles. Population level analyses revealed a strong association of HLA-B*51:01 with the I135T mutant and a relatively weaker association of HLA-B*52:01 with several I135X mutants in both Japanese and predominantly Caucasian cohorts. An *in vitro* viral suppression assay revealed that the HLA-B*52:01-restricted CTLs failed to suppress the replication of the I135X mutant viruses, indicating the selection of these mutants by the CTLs. These results suggest that the different pattern of I135X mutant selection may have resulted from the difference between these two CTLs in the ability to suppress HIV-1 replication.

HIV-1-specific cytotoxic T cells (CTLs) play an important role in the control of HIV-1 replication (1–8); however, they also select immune escape mutations (9, 10). Population level adaptation of HIV to human leukocyte antigen (HLA) has been demonstrated (11–15), suggesting that HIV-1 can successfully adapt to immune responses previously effective against it.

It is well known that particular mutations can be selected by CTLs specific for a single HIV-1 epitope. On the other hand, studies on HLA-associated HIV-1 polymorphisms have revealed examples of particular mutations associated with multiple HLA class I alleles (16–21), suggesting that the same mutation can be selected by CTLs carrying different specificities in some cases. However, the selection of the same mutation by CTLs specific for different HIV-1 epitopes has rarely been reported. The change from Ala to Pro at residue 146 of Gag (A146P) is a well-analyzed case. A146P is an escape selected by not only HLA-B*57-restricted, ISW9-specific CTLs (22) but also by HLA-B*15:10-restricted and HLA-B*48:01-restricted CTLs (15, 23, 24), although the latter CTLs selected it by different mechanisms. The replacement of Thr with Asn at residue 242 (T242N) of Gag is another case. This mutant is selected by HLA-B*58:01-restricted and HLA-B*57-restricted CTLs specific for the TW10 epitope in HIV-1 clade B- and C-infected individuals (25–27).

The presence of Pol283-8(TAFTIPSI: TI8)-specific, HLA-B*51:01-restricted CTLs is associated with low viral loads in HIV-1-infected Japanese hemophiliacs, supporting an important role in the long-term control of HIV-1 infection (28). We previously showed that the frequency of a mutation at position 135 (I135X) of reverse transcriptase (RT) is strongly correlated with the prevalence of HLA-B*51 among nine cohorts worldwide and that this mutation is selected by Pol283-8(TAFTIPSI: TI8)-specific, HLA-B*51:01-restricted CTLs (15). Of these cohorts, a Japanese one showed the highest frequency of the I135X mutation in HLA-B*51:01 negatives (66% in a Japanese cohort and 11 to 29% in other cohorts). This finding may be explained by the fact that the Japanese cohort has the highest prevalence of HLA-B*51:01 among these cohorts. Another possibility is that this mutation is selected by HIV-1-specific CTLs restricted by other HLA alleles, which are highly frequent among Japanese individuals but infrequent in or absent from other populations. To clarify the latter possibility, we first analyzed the association of the I135X mutation with other HLA class I alleles in a Japanese cohort and found this mutation also to be associated with HLA-B*52:01. We next sought to identify an HLA-B*52:01-restricted CTL epitope including RT135 and found that both HLA-B*51:01 and -B*52:01 can present the same epitope, Pol283-8. Using population level analyses of Japanese and Caucasian cohorts, we identified HLA-B*51:01- and HLA-B*52:01-specific polymorphisms at RT codon 135 (position 8 of this epitope) and characterized differential pathways of escape between these two alleles. In addition, we assessed the *in vitro* ability of HLA-B*52:01- and HLA-B*51:01-restricted CTLs to se-

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lect I135X mutants and elucidated the crystal structure of the HLA-B*52:01-Pol283-8 peptide complex.

MATERIALS AND METHODS

Patients. Two hundred fifty-seven chronically HIV-1-infected, antiretroviral-naïve Japanese individuals were recruited for the present study, which was approved by the ethics committees of Kumamoto University and the National Center for Global Health and Medicine, Japan. Written informed consent was obtained from all subjects according to the Declaration of Helsinki.

In addition, HLA-associated immune selection pressure at RT codon 135 was investigated in the International HIV Adaptation Collaborative (IHAC) cohort, comprising >1,200 chronically HIV-infected, antiretroviral-naïve individuals from Canada, the United States, and Western Australia (19). The majority of the IHAC participants were Caucasian, and the HIV subtype distribution was >95% subtype B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, and its mutant form pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef) were previously reported (29). Pol283-8 mutant viruses (Pol283-8L, -8T, -8V, and 8R) were previously generated on the basis of pNL-432 (15, 28).

Generation of CTL clones. Pol283-8-specific, HLA-B*52:01-restricted CTL clones were generated from HIV-1-specific, bulk-cultured T cells by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark). Each well contained 200 μl of the cloning mixture (about 1 × 10^6 irradiated allogeneic peripheral blood mononuclear cells (PBMCs) from healthy donors and 1 × 10^5 irradiated C1R-B*52:01 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant interleukin-2).

Intracellular cytokine staining (ICS) assay. PBMCs from HIV-1-seropositive HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals were cultured with each peptide (1 μM). Two weeks later, the cultured cells were stimulated with C1R-B*52:01 cells or those prepulsed with Pol283-8 peptide (1 μM) for 60 min, and then they were washed twice with RPMI 1640 containing 10% fetal calf serum (RPMI 1640-10% FCS). Subsequently, brefeldin A (10 μg/ml) was added. After these cells had been incubated for 6 h, they were stained with an anti-CD8 monoclonal antibody (MAb; Dako Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde, and then permeabilized with permeabilization buffer. Thereafter, the cells were stained with an anti-gamma interferon (IFN-γ) MAb (BD Bioscience). The percentage of CD8$^+$ cells positive for intracellular IFN-γ was analyzed by using a FACS-Cant II (BD Biosciences, San Jose, CA). All flow cytometric data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Identification of 11-mer peptide recognized by HLA-B*52:01-restricted CD8$^+$ T cells. We identified an 11-mer peptide recognized by HLA-B*52:01-restricted CD8$^+$ T cells as follows. We stimulated PBMCs from a chronically HIV-1-infected HLA-B*52:01$^+$ donor (KI-069) with a peptide cocktail including overlapping 17-mer peptides covering RT135 and cultured the cells for 14 days. The cells in bulk culture were assessed by performing an ICS assay for C1R-HLA-B*52:01 cells prepulsed with each of these 17-mer peptides. The bulk-cultured cells recognized the target cells prepulsed with two of the 17-mer peptides assessed, Pol17-47 (KDFRKYTAFTIPSINNE) and Pol17-48 (TAFTIPSI NNETPGIRT). Further analysis with 11-mer overlapping peptides covering the Pol17-48 sequence showed that these bulk-cultured cells recognized the target cells prepulsed with Pol11-142 (TAFTIPSINNE) but not those prepulsed with Pol11-143 (FTIPSINNETP).

Assay of cytotoxicity of CTL clones to target cells prepulsed with the epitope peptide or infected with a vaccinia virus–HIV-1 recombinant. The cytotoxicity of Pol283-8-specific, HLA-B*52:01-restricted CTL clones to C1R cells expressing HLA-B*52:01 (C1R-B*52:01), which were previously generated (30), and prepulsed with peptide or infected with a vaccinia virus-HIV-1Gag/Pol recombinant was determined by the standard ^{51}Cr release assay described previously (31). In brief, the infected cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min and then washed three times with RPMI 1640 medium containing 10% newborn calf serum. Labeled target cells (2 × 10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark) with the effector cells at an effector-to-target (E/T) cell ratio of 2:1. The cells were then incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the number of counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the number of cpm in the supernatant in the wells containing both target and effector cells.

Enzyme-linked immunospot (ELISPOT) assay. Cryopreserved PBMCs of chronically HIV-1-infected HLA-B*52:01$^+$ individuals were plated in 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been precoated with 5 μg/ml anti-IFN-γ MAb 1-DIK (Mabtech, Stockholm, Sweden). The appropriate amount of each peptide (100 or 10 nM) was added in a volume of 50 μl, and then PBMCs were added at 1 × 10^5 cells/well in a volume of 100 μl. The plates were incubated for 40 h at 37°C in 5% CO_2 and then washed with phosphate-buffered saline (PBS) before the addition of biotinylated anti-IFN-γ MAb (Mabtech) at 1 μg/ml. After the plates had been incubated at room temperature for 100 min and then washed with PBS, they were incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 40 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA). The spots were counted by an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). PBMCs without peptide stimulation were used as a negative control. Positive responses were defined as those greater than the mean of the negative-control wells plus 2 standard deviations (SD) (the number of spots in wells without peptides).

HIV-1 replication suppression assay. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (32). CD4$^+$ T cells isolated from PBMCs derived from an HIV-1-seronegative individual with HLA-B*52:01, HLA-B*51:01, or both were cultured. After the cells had been incubated with the desired HIV-1 clones for 4 h at 37°C, they were washed three times with RPMI 1640-10% FCS medium. The HIV-1-infected CD4$^+$ T cells were then cocultured with Pol283-8-specific CTL clones. From day 3 to day 7 postinfection, culture supernatants were collected and the concentration of p24 antigen (Ag) in them was measured by use of an enzyme-linked immunosorbent assay kit (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

HLA stabilization assay with RMA-S cells expressing HLA-B*52:01 or HLA-B*51:01. The peptide-binding activity of HLA-B*52:01 or HLA-B*51:01 was assessed by performing an HLA stabilization assay with RMA-S cells expressing HLA-B*52:01 (RMA-S-B*52:01) or HLA-B*51:01 (RMA-S-B*51:01) as described previously (33). Briefly, RMA-S-B*51:01 and RMA-S-B*52:01 cells were cultured at 26°C for 16 to 24 h. The cells (2 × 10^5) in 50 μl of RPMI 1640 supplemented with 5% FCS (RPMI–5% FCS) were incubated at 26°C for 3 h with 50 μl of a solution of peptides at 10^{-3} to 10^{-7} M and then at 37°C for 3 h. After having been washed with RPMI–5% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of TP25.99 MAb. After two washings with RPMI–5% FCS, they were incubated for 30 min on ice with an appropriate dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig antibodies. Finally, the cells were washed three times with RPMI–5% FCS and the fluorescence intensity of the cells was measured by the FACS-Cant II. Relative mean fluorescence intensity (MFI) was calculated by subtracting the MFI of cells not peptide pulsed from that of the peptide-pulsed ones.

Sequencing of plasma RNA. Viral RNA was extracted from the plasma of chronically HIV-1-infected Japanese individuals by using a QIAamp

Mini Elute Virus spin kit (Qiagen). cDNA was synthesized from the RNA with Superscript II and random primer (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR with RT-specific primers 5′-CCAAAAGTTAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGGAGG-3′ or 5′-CCTTGCCCCTGCTTCTGTAT-3′ for the first-round PCR and 5′-AGTTAGGAATACCACACCCC-3′ and 5′-GTAAATCCCCACCTCAACAG-3′ or 5′-AATCCCCACCTCAACAGAAG-3′ for the second-round PCR and integrase-specific primers 5′-ATCTAGCTTTGCAGGATTCGGG-3′ and 5′-CCTTAACCGTAGTACTGGTG-3′ or 5′-CCTGATCTCTTACCTGTCC-3′ for the first-round PCR and 5′-AAAGGTCTACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATGGCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second-round PCR. PCR products were sequenced directly or cloned with a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator v1.1. cycle sequencing kit (Applied Biosystems) and analyzed by an ABI PRISM 310 Genetic Analyzer.

Statistical analysis with phylogenetically corrected odds ratios. Strength of selection was measured by using a phylogenetically corrected odds ratio as previously described (19). Briefly, the odds of observing a given amino acid (e.g., 135V) was modeled as $P/(1 - P) = (a \times X) + (b \times T)$, where P is the probability of observing 135V in a randomly selected individual, X is a binary (0/1) variable representing whether or not an individual expresses the HLA allele in question (e.g., B*52:01), and T equals 1 if the transmitted/founder virus for that individual carried 135V and −1 otherwise. Because the transmitted/founder virus is unknown, we averaged over all possibilities by using weights informed by a phylogeny that was constructed from the RT sequences of all of the individuals in the study. The parameters a and b were determined by using iterative maximum-likelihood methods. The maximum-likelihood estimate of a is an estimate of the natural logarithm of the odds ratio of observing 135V in individuals expressing X versus individuals not expressing X, conditioned on the individuals' (unobserved) transmitted/founder virus. P values are estimated by using a likelihood ratio test that compares the above model to a null model in which a equals 0.

To compare the odds of selection between two cohorts, we modified the phylogenetically corrected logistic regression model to include a cohort term, $Z = X \times Y$, where X is the HLA allele, and Y is a 0/1 variable that indicates cohort membership, yielding $P/(1 - P) = (a \times X) + (b \times T) + (c \times Z)$, as previously described (19, 34). A P value testing if the odds of escape are different in the two cohorts was estimated by using a likelihood ratio test that compared this model to a null model where c equals 0.

Generation of HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramer) were synthesized as described previously (31, 35). The Pol283-8 peptide was used for the refolding of HLA-B*51:01 or HLA-B*52:01 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for generation of the tetramers.

Tetramer binding assay. HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones were stained at 37°C for 30 min with PE-conjugated HLA-B*51:01-tetramer and HLA-B*52:01-tetramer, respectively, at concentrations of 5 to 1,000 nM. After two washes with RPMI 1640 medium supplemented with 10% FCS (RPMI 1640–10% FCS), the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min, followed by 7-amino-actinomycin D at room temperature for 10 min. After two more washes with RPMI 1640–10% FCS, the cells were analyzed by the FACS-Cant II flow cytometer. The tetramer concentration that yielded the half-maximal MFI (the EC_{50}) was calculated by probit analysis.

Crystallization, data collection, and structure determination. Soluble HLA-B*52:01 (with beta-2 microglobulin and peptide TAFTIPSI) was prepared as described above. Prior to crystallization trials, HLA-B*52:01 was concentrated to a final concentration of 20 mg ml^{-1} in 20 mM Tris-HCl (pH 8.0) buffer containing 250 mM NaCl. This was done with a Millipore centrifugal filter device (Amicon Ultra-4, 10-kDa cutoff; Millipore). Screening for crystallization was performed with commercially available polyethylene glycol (PEG)-based screening kits, PEGs and PEGs II suites (Qiagen). Thin needle crystals were observed from PEGs II suite 23 (0.2 M sodium acetate, 0.1 M HEPES [pH 7.5], and 20% PEG 3000). Several conditions were further screened by the hanging-drop method with 24-well VDX plates (Hampton Research) by mixing 1.5 μl protein solution and 1.5 μl reservoir to be equilibrated against reservoir solution (0.5 ml) at 293 K. Best crystals were grown from macro seeding with the initial crystals obtained with 0.2 M sodium acetate, 0.1 M Bis Tris propane [pH 7.5], and 20% PEG 3350.

The data set was collected at beamline BL41XU of SPring-8 with Rayonix charge-coupled device detector MX225HE. Prior to diffraction data collection, crystals were cryoprotected by transfer to a solution containing 25% (vol/vol) glycerol and incubation in it for a few seconds, followed by flash cooling. The data sets were integrated with XDS (36) and then merged and scaled by using Scala (37). HLA-B*52:01 crystals belonged to space group $P2_12_12_1$, with unit cell parameters $a = 69.0$ Å, $b = 83.3$ Å, and $c = 170.3$ Å. Based on the values of the Matthews coefficient (V_M) (38), we estimated that there were two protomers in the asymmetric unit with a V_M value of 1.37 A^3/Da (Vsolv = 10.5%). For details of the data collection and processing statistics, see Table S1 in the supplemental material.

The structure was solved by the molecular replacement method with Molrep (39). The crystal structure of HLA-B*51:01 (PDB ID: 1E28) was used as a search model. Structure refinement was carried out by using Refmac5 (40) and phenix (41). The final model was refined to an Rfree factor of 34.7% and an R factor of 29.5% with a root mean square deviation of 0.014 Å in bond length and 1.48° in bond angle for all reflections between resolutions of 38.8 and 3.1 Å. Table S1 in the supplemental material also presents a summary of the statistics for structure refinement. The stereochemical properties of the structure were assessed by Procheck (42) and COOT (43) and showed no residues in the disallowed region of the Ramachandran plot.

Protein structure accession number. Atomic coordinates and structure factors for HLA-B*52:01 have been deposited in the Protein Data Bank under accession code 3W39.

RESULTS

Association of I135X variants with HLA-B*52:01. To clarify the possibility that CTLs restricted by other HLA alleles select the I135X mutation, we investigated the association between other HLA alleles and this mutation in 257 Japanese individuals chronically infected with HIV-1. We found an association of HLA-B*52:01 with the I135X variant, though this association was weaker than that with HLA-B*51:01 (phylogenetically corrected ln odds ratio [lnOR] of 11.76 [$P = 8.77 \times 10^{-4}$] for B*52:01 versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] for B*51:01; Table 1). We also analyzed the effects of HLA-B*52:01 and HLA-B*51:01 in chronically HIV-1-infected Japanese individuals, excluding HLA-B*51:01$^+$ and HLA-B*52:01$^+$ individuals, respectively, and found a significant association between HLA-B*52:01 and I135X variants among 200 HLA-B*51:01-negative individuals with chronic HIV-1 infection ($P = 4.7 \times 10^{-4}$; see Fig. S1A in the supplemental material) and that of HLA-B*51:01 with the variants in 202 HLA-B*52:01-negative ones ($P = 5.3 \times 10^{-8}$; see Fig. S1B in the supplemental material). These results together imply that HLA-B*52:01-restricted CTLs selected this mutation.

Identification of HLA-B*52:01-restricted, Pol283-specific CTLs. To identify the HLA-B*52:01-restricted HIV-1 epitope including RT135, we first investigated whether overlapping peptides covering RT135 could elicit CD8$^+$ T cells specific for these peptides in chronically HIV-1-infected individuals. We identified CTLs recognizing the Pol11-142 (TAFTIPSINNE) peptide in a chronically HIV-1-infected HLA-B*52:01$^+$ donor, KI-069 (see Materials and Methods). Since the C terminus of HLA-B*52:01-binding peptides is known to be a hydrophobic residue (30, 44), we speculated that TAFTIPSI (Pol283-8) was the epitope peptide.

TABLE 1 HLA-B*52:01 and HLA-B*51:01 association with variation at RT135 in Japanese and Caucasian cohorts

		PlyloLOR[a]		Within-cohort *P* value		
HLA class I allele	RT135 target variable	Japanese	IHAC	Japanese	IHAC	*P* value comparing cohorts
B*51:01	T	13.70	4.53	4.66×10^{-6}	1.70×10^{-35}	0.042
B*52:01	T	−9.77	1.25	0.464	2.04×10^{-3}	0.62
B*51:01	I	−40.00	−5.71	5.78×10^{-12}	1.58×10^{-51}	0.052
B*52:01	I	−11.76	−3.06	8.77×10^{-4}	2.95×10^{-5}	0.52
B*51:01	V	−9.76	8.52	0.884	0.41	0.85
B*52:01	V	12.21	10.15	0.076	1.82×10^{-3}	0.037
B*51:01	R	12.08	13.02	0.038	2.36×10^{-3}	0.42
B*52:01	R	0.26	8.37	0.423	0.469	0.89
B*51:01	L	−0.89	3.21	1	0.038	0.17
B*52:01	L	−0.56	3.61	1	0.231	0.29
B*51:01	K	−0.71	−40.00	1	0.53	0.99
B*52:01	K	−0.69	−40.00	1	0.779	0.99
B*51:01	M	7.76	12.00	0.894	2.10×10^{-4}	0.34
B*52:01	M	11.09	−40.00	0.034	0.517	0.12

[a] PlyloLOR, phylogenetically corrected lnOR.

Indeed, bulk-cultured T cells that had been cultured for 2 weeks after stimulation with Pol17-48 recognized C1R-B*52:01 cells prepulsed with Pol283-8 peptide at a much lower concentration than those incubated with the Pol11-142 peptide (Fig. 1A), strongly suggesting that Pol283-8 is an epitope recognized by HLA-B*52:01-restricted CTLs. These findings were confirmed by ELISPOT assay with PBMCs from two HLA-B*52:01$^+$ individuals chronically infected with HIV-1 (Fig. 1B). To clarify whether this peptide was processed and presented by HLA-B*52:01, we investigated the killing activity of bulk-cultured T cells against HLA-B*52:01$^+$ target cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant. They killed target cells infected with this recombinant but not those infected with wild-type vaccinia virus (Fig. 1C), indicating that the Pol283-8 peptide was presented by

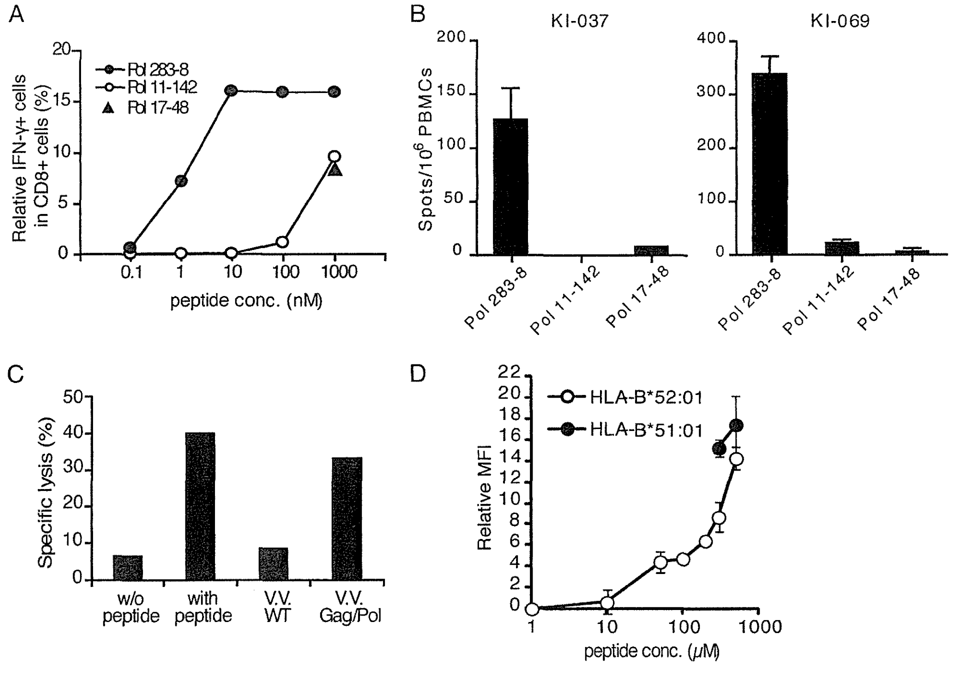

FIG 1 Identification of HLA-B*52:01-restricted Pol epitope. (A) Identification of the epitope peptide recognized by HLA-B*52:01-restricted CD8$^+$ T cells. Bulk T cells were cultured for 2 weeks after stimulation with the Pol17-48 peptide, and then the recognition of C1R-HLA-B*52:01 cells prepulsed with Pol17-48, Pol11-142, or Pol283-8 peptide was assessed by ICS assay. (B) Pol283-8 peptide recognition by T cells *ex vivo*. Recognition of the Pol17-48, Pol11-142, or Pol283-8 peptide by PBMCs from two HLA-B*52:01$^+$ individuals chronically infected with HIV-1 (KI-037 and KI-069) was analyzed by ELISPOT assay. A 100 nM concentration of each peptide was used. (C) Killing activity of Pol283-specific, HLA-B*52:01-restricted CD8$^+$ T cells against cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant. The killing activities of bulk-cultured T cells stimulated with Pol11-142 against target cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant (Gag/Pol) and against those infected with wild-type vaccinia virus (V.V. WT) are shown. (D) Binding of Pol283-8 peptide to HLA-B*52:01. Binding ability was measured by performing the HLA class I stabilization assay with RMA-S-B*52:01. RMA-S-B*51:01 cells were used as control cells for the Pol283-8 peptide.

TABLE 2 Pol283-8-specific CD8$^+$ T cells in chronically HIV-1-infected, HLA-B*52:01$^+$ individuals

Patient ID	HLA class I alleles		No. of CD4 cells/μl	No. of CD8 cells/μl	Viral load (no. of copies/ml)	Antiretroviral therapy	Relative IFN-γ^+/CD8$^+$ % in ICC assay	No. of spots/10^6 PBMCs in ELISPOT[a] assay
KI-037	A*24:02/−	B*52:01/40:02	465	973	76,000	−	64.1	150
KI-090	A*24:02/−	B*52:01/55:01	606	511	≤50	+	40.2	80
KI-106	A*24:02/33:03	B*52:01/07:01	433	890	≤50	+	1.4	<79
KI-126	A*24:02/31:01	B*52:01/40:01	465	NT[b]	36,000	−	60.4	<79
KI-130	A*24:02/−	B*52:01/07:02	351	1,275	14,000	−	0.0	<79
KI-167	A*24:02/−	B*52:01/54:01	455	909	26,000	−	0.0	<79
KI-067	A*24:02/−	B*52:01/48:01	234	1,198	89,000	−	10.9	<79
KI-071	A*24:02/31:01	B*52:01/40:06	292	1,134	48,000	−	0.7	<79
KI-076	A*02:01/24:01	B*52:01/40:01	136	252	14,000	−	61.0	80
KI-114	A*02:01/24:01	B*52:01/27:04	416	463	≤50	+	0.1	<79
KI-056	A*24:02/−	B*52:01/40:02	290	844	8,200	−	-0.1	<79
KI-108	A*24:02/−	B*52:01/−	373	481	NT	−	1.0	<79
KI-028	A*24:02/26:01	B*52:01/48:01	1,351	811	≤50	+	0.5	<79
KI-069	A*24:02/−	B*52:01/40:06	448	1,631	4,400	−	18.1	790

[a] More than the mean number of negative-control spots + 2 SD was defined as a positive response (positive response, >79 spots).

[b] NT, not tested.

HLA-B*52:01. We analyzed the binding of the Pol283-8 peptide to HLA-B*52:01 by using the HLA stabilization assay. The results demonstrated that this peptide bound to HLA-B*52:01 (Fig. 1D). These results together indicate that the Pol283-8 epitope can therefore be presented by both HLA-B*51:01 and HLA-B*52:01.

We investigated whether Pol283-8-specific CD8$^+$ T cells were elicited predominantly in chronically HIV-1-infected HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals. PBMCs from 14 of these individuals were analyzed by ICS assay with Pol283-8 peptide-stimulated culture cells, as well as by ELISPOT assay. The results of the ICS assay showed that 7 of these 14 HLA-B*52:01$^+$ HLA-B*51:01$^-$ patients had Pol283-specific CD8$^+$ T cells, whereas those of the ELISPOT assay with *ex vivo* PBMCs revealed that Pol283-specific CD8$^+$ T cells were detected in only four individuals (Table 2). These results suggest that the three individuals in whom the specific CTLs were detected by the ICS assay but not by the ELISPOT assay may have memory T cells. These results together indicate that Pol283-8 was recognized as an HLA-B*52:01-restricted immunodominant epitope in the HLA-B*52:01$^+$ individuals and support the idea that the I135X mutation was selected by HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cells.

Strong ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cells to suppress HIV-1 replication. A previous study showed that HLA-B*51:01-restricted, Pol283-8-specific T cells have a strong ability to kill HIV-1-infected target cells and to suppress HIV-1 replication (31). Therefore, we expected that the HLA-B*52:01-restricted T cells also would have this strong ability. We generated HLA-B*52:01-restricted, Pol283-8-specific T cell clones and investigated their ability to kill peptide-pulsed or HIV-1-infected target cells. Clone 1E1 effectively killed C1R-B*52:01 cells prepulsed with the Pol283-8 peptide (Fig. 2A) and NL-432-infected CD4$^+$ T cells from an HLA-B*52:01$^+$ individual (Fig. 2B). Additional T cell clones also showed strong killing activity against NL-432-infected HLA-B*52:01$^+$ CD4$^+$ T cells (data not shown). In addition, we investigated the ability of these CTL clones to suppress HIV-1 replication. CD4$^+$ T cells derived from an HLA-B*52:01$^+$ individual were infected with NL-432 or M20A mutant virus, the latter of which has an amino acid substitution at position 20 of Nef and lacks the ability to downregulate the surface expression of HLA-A and -B molecules (Fig. 2C). Representative data on the 1E1 clone and summary data on four clones are shown in Fig. 2D and E, respectively. These CTL clones strongly suppressed the replication of both the NL432 and M20A mutant viruses, indicating that the HLA-B*52:01-restricted CTLs had a strong ability to suppress HIV-1 replication, as was the case with the HLA-B*51:01-restricted ones.

Recognition of I135X mutations by Pol283-8-specific, HLA-B*52:01-restricted CTLs. Four mutations (8T, 8L, 8R, and 8V) were observed predominantly at RT135 in chronically HIV-1-infected HLA-B*52:01$^+$ individuals (Fig. 3). These mutations may have been selected by Pol283-8-specific, HLA-B*52:01-restricted CTLs in these patients. We therefore investigated the ability of HLA-B*52:01-restricted CTLs to suppress the replication of these mutant viruses *in vitro*. The CTL clones failed to suppress the replication of the 8L, 8T, or 8R mutant, though they weakly suppressed that of the 8V virus at an E/T cell ratio of 1:1 (Fig. 4A). These results support the idea that these variants were escape mutations from the HLA-B*52:01-restricted CTLs. To clarify the mechanism by which the CTL clones failed to suppress the replication of these mutant viruses, we investigated the CTL clones for recognition of C1R-B*52:01 cells prepulsed with the mutant peptides. The CTL clones effectively recognized the 8V peptide at the same level as the wild-type peptide and the 8T and 8L peptides at less than that of the wild-type one, whereas they failed to recognize the 8R peptide (Fig. 4B). An ELISPOT assay with *ex vivo* PBMCs from KI-069 showed that Pol283-8-specific CTLs effectively recognized the 8I and 8V variants but not the other three mutant peptides (Fig. 4C), suggesting that Pol283-8-specific CTLs failed to recognize the 8T, 8L, and 8R peptides *in vivo*. The lack of recognition of these mutants by CTLs may be attributable to a failure of T cell receptor (TCR) recognition, the inability of the peptide to bind to HLA-B*52:01, and/or disruption of the processing of the epitope in HIV-1-infected cells.

Different pattern of RT135 mutation selection by two HLA alleles. As described above, HLA-B*51:01 and HLA-B*52:01 were associated with I135X in a Japanese population in which the prevalence of HLA-B*51:01 and B*52:01 alleles is relatively high (21.9 and 21.1%, respectively). In a Japanese cohort, out of the five

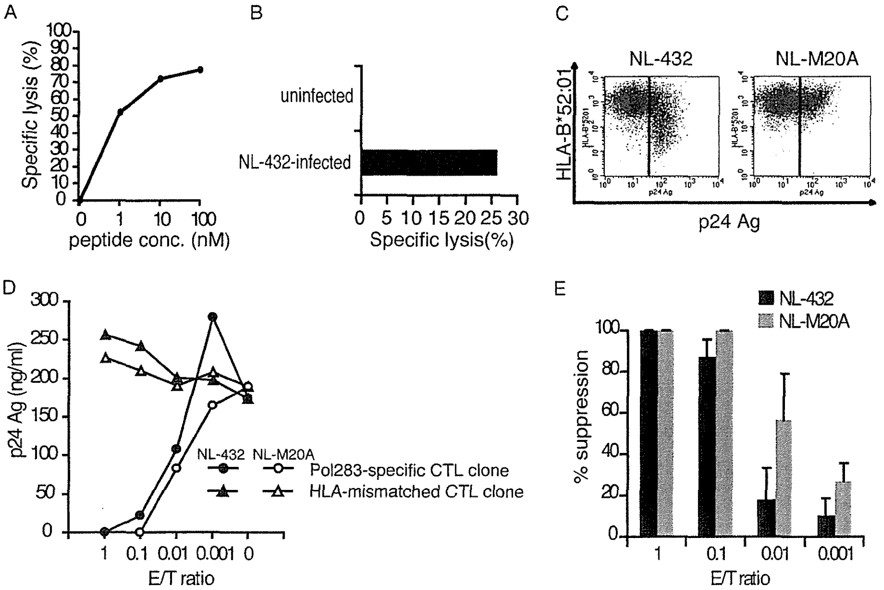

FIG 2 Abilities of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to kill HIV-1-infected CD4$^+$ T cells and to suppress HIV-1 replication. (A) Killing activity of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone against C1R-B*52:01 cells prepulsed with Pol283-8 peptides. The activity of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T clone, 1E1, to kill C1R-B*52:01 cells was measured by performing a ^{51}Cr-release assay. (B) Killing activity of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone 1E1 against CD4$^+$ T cells infected with HIV-1. The ability of the clone to kill CD4$^+$ T cells infected with NL-432 was measured by performing a ^{51}Cr-release assay. (C) Downregulation of HLA-B*52:01 in HIV-1-infected CD4$^+$ T cells. CD4$^+$ T cells derived from an HLA-B*52:01$^+$ donor (HLA-A*11:01/A*24:02, HLA-B*52:01/B*52:01, and HLA-C*12:02/C*14:02) were infected with NL-432 and then cultured for 4 days. The cultured CD4$^+$ T cells were stained with anti-p24 Ag and TÜ109 anti-Bw4 MAbs. (D) Ability of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone to suppress the replication of NL-432 and M20A mutant viruses. Suppressing ability was measured at four different E/T cell ratios (1:1, 0.1:1, 0.01:1, and 0.001:1). HIV-1-infected HLA-B*52:01$^+$ CD4$^+$ T cells were cocultured with an HLA-B*52:01-restricted, Pol283-8-specific CTL clone or an HLA-mismatched CTL clone at various E/T cell ratios. HIV-1 p24 Ag levels in the supernatant were measured on day 6 postinfection. (E) Summary of the ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones ($n = 4$) to suppress the replication of Nl-432 and M20A mutant viruses at four different E/T cell ratios.

amino acid mutations that can be generated by a one-nucleotide mutation from Ile, the T mutation was strongly associated with the presence of HLA-B*51:01 ($P = 4.66 \times 10^{-6}$), whereas HLA-B*52:01 was associated not with any single amino acid substitu-

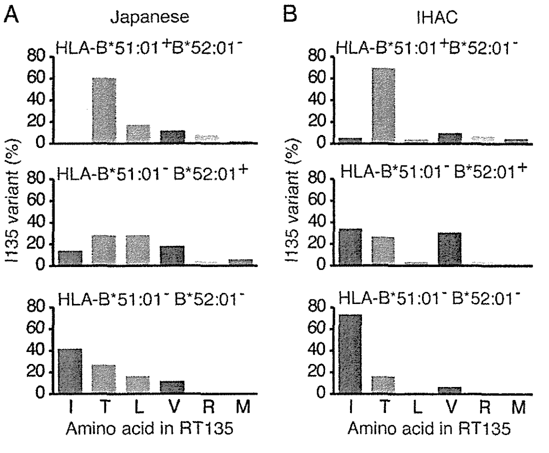

FIG 3 Amino acid variation at RT135 in Japanese individuals. (A) Frequency of the amino acid at RT135 in 51 HLA-B*51:01$^+$ HLA-B*52:01$^-$, 49 HLA-B*51:01$^-$ HLA-B*52:01$^+$, and 151 HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese subjects. (B) Frequency of the amino acid at RT135 in 131 HLA-B*51:01$^+$ HLA-B*52:01$^-$, 26 HLA-B*51:01$^-$ HLA-B*52:01$^+$, and 1195 HLA-B*51:01$^-$ HLA-B*52:01$^-$ subjects in three predominantly Caucasian cohorts from Canada, the United States, and Western Australia (IHAC).

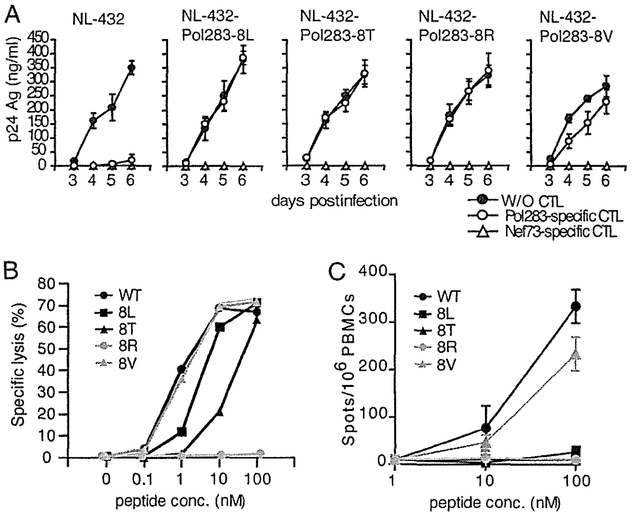

FIG 4 Ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to suppress the replication of four mutant viruses. (A) Ability of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone to suppress the replication of four (8L, 8T, 8R, and 8V) mutant viruses and NL-432. The abilities of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone and an HLA-A*1101-restricted Nef73-specific T cell clone to suppress the replication of these viruses were measured at an E/T cell ratio of 1:1 on days 3 to 6. W/O, without. (B) Recognition by an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone of C1R-B*52:01 cells prepulsed with any one of the four mutant epitope peptides or the wild-type (WT) peptide (8I). (C) Recognition of mutant epitope peptides by *ex vivo* Pol283-8-specific CTLs. The recognition of the Pol283-8 peptide (WT) or the mutant epitope peptide by PBMCs from KI-069 was analyzed by ELISPOT assay.

tion but only with the non-I mutation ($P = 8.77 \times 10^{-4}$, Table 1). The distribution of amino acid variations at RT135 in the HLA-B*51:01$^+$ HLA-B*52:01$^-$ Japanese subjects was different from that in the HLA-B*51:01$^-$ HLA-B*52:01$^+$ ones (Fig. 3). These results suggest that the HLA-B*51:01-restricted CTLs strongly selected the 135T mutation but that the HLA-B*52:01-restricted ones selected a variety of different amino acids at this position in Japanese individuals.

We also analyzed the association of I135X mutations with HLA-B*52:01 and HLA-B*51:01 in three predominantly Caucasian cohorts from Canada, the United States, and Western Australia (International HIV Adaptation Collaborative [IHAC]) (19) comprising >1,200 subjects (Table 1). HLA-B*51:01 was very strongly associated with the I135X mutation (lnOR of 5.71; $P = 1.58 \times 10^{-51}$). Although only 2.1% of the IHAC cohort subjects expressed HLA-B*52:01, this allele was also associated with I135X (lnOR of 3.06; $P = 2.95 \times 10^{-5}$). The T mutation was strongly associated with HLA-B*51:01 ($P = 1.70 \times 10^{-35}$), whereas the T and V mutations were weakly associated with HLA-B*52:01 ($0.0005 < P < 0.005$). Thus, these results showed a similar selection of RT135 mutations by HLA-B*52:01 in the predominantly Caucasian cohort, despite a substantially lower frequency of HLA-B*52:01. The magnitude of the strength of selection by HLA-B*52:01 and HLA-B*51:01 on RT135 did not differ significantly between the two cohorts (Table 1). These results indicate that HLA-B*51:01 strongly selected 135T but that HLA-B*52:01 selected a variety of substitutions at this site (designated I135X) in both the Japanese and non-Japanese cohorts.

Comparison of TCR affinity and abilities of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTLs to suppress HIV-1 replication. We investigated the TCR affinity of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones by using tetramers of the HLA-B*51:01-Pol283 peptide and the HLA-B*52:01-Pol283 peptide complex (HLA-B*51:01 and HLA-B*52:01 tetramers, respectively). The TCR affinity of these CTL clones was compared in terms of EC_{50}. The EC_{50} of the HLA-B*51:01-restricted CTL clones was significantly lower than that of the HLA-B*52:01-restricted CTL clones (Fig. 5A), suggesting that the former CTL clones had TCRs with a higher affinity for the ligand than those of the latter clones. These results imply that the HLA-B*51:01-restricted CTL clones could recognize the HIV-1-infected targets more effectively than HLA-B*52:01-restricted ones.

Since CD4$^+$ T cells derived from an HLA-B*52:01 homozygous individual were used in the experiment shown in Fig. 2D and E, the ability of the HLA-B*52:01-restricted CTL clones to suppress the replication of NL-432 may have been overestimated. To evaluate and compare the abilities of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones to suppress the replication of NL-432, we used CD4$^+$ T cells from individuals expressing HLA-B*51:01$^+$/B*52:01$^-$, HLA-B*51:01$^-$/B*52:01$^+$, or HLA-B*51:01$^+$/B*52:01$^+$ (Fig. 5B). Two HLA-B*51:01-restricted CTL clones strongly inhibited the replication of HIV-1 in cultures of NL-432-infected HLA-B*51:01$^+$/B*52:01$^-$ CD4$^+$ T cells but not in those of HLA-B*51:01$^-$/B*52:01$^+$ cells, whereas two HLA-B*52:01-restricted CTL clones strongly inhibited the replication of HIV-1 in cultures of NL-432-infected HLA-B*51:01$^-$/B*52:01$^+$ CD4$^+$ T cells but not in those of HLA-B*51:01$^+$/B*52:01$^-$ cells (Fig. 5B, left and middle). The ability of the HLA-B*51:01-restricted CTL clones to suppress the replication of HIV-1 was greater than that of the HLA-B*52:01-restricted CTL clones. This was confirmed by the experiment with HLA-B*51:01$^+$/B*52:01$^+$ CD4$^+$ T cells (Fig. 5B, right). Although both HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones strongly inhibited the replication of HIV-1 in the cultures of NL-432-infected HLA-B*51:01$^+$/B*52:01$^+$ CD4$^+$ T cells, the former clones exhibited a greater ability to suppress the replication of HIV-1 than did the latter cells. These results indicate that the HLA-B*51:01-restricted CTL clones had a stronger ability to suppress HIV-1 replication than the HLA-B*52:01-restricted clones. Taken together, both our *in vitro* and our *in vivo* (population level HLA-association) data suggest that immune pressure on RT135 by HLA-B*51:01-restricted T cells was stronger than that imposed by HLA-B*52:01-restricted cells.

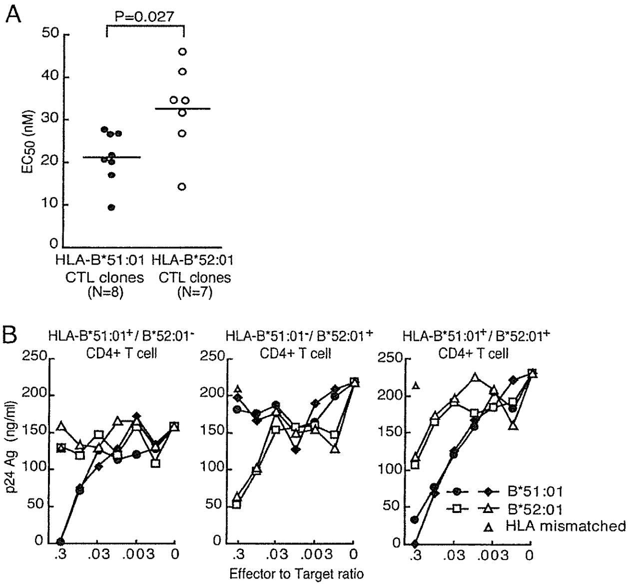

FIG 5 Differences between HLA-B*51:01-restricted and HLA-B*52:01-restricted CD8$^+$ T cell clones in TCR avidity and the ability to suppress HIV-1 replication. (A) TCR avidity of the HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones expressed as EC_{50}. The ability of the TCRs of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones to bind HLA-B*51:01 tetramers and HLA-B*52:01 tetramers, respectively, was measured in terms of the MFI of each CTL clone stained with the tetramers at concentrations of 5 to 1,000 nM. (B) The ability of two HLA-B*51:01-restricted and two HLA-B*52:01-restricted CD8$^+$ T cell clones to suppress HIV-1 was measured at six different E/T cell ratios (0.3:1, 0.1:1, 0.03:1, 0.01:1, 0.003:1, and 0.001:1). CD4$^+$ T cells from individuals expressing HLA-B*51:01$^+$/B*52:01$^-$, HLA-B*51:01$^-$/B*52:01$^+$, or HLA-B*51:01$^+$/B*52:01$^+$ were infected with NL-432 and then cocultured with a given Pol283-8-specific CTL clone or an HLA-mismatched CTL clone. HIV-1 p24 Ag levels in the supernatant were measured on day 5 postinfection.

Structural basis of the difference in recognition between HLA-B*52:01- and HLA-B*51:01-restricted CTLs. In order to investigate the structural basis of the difference in recognition between HLA-B*52:01- and HLA-B*51:01-restricted CTLs, we performed a crystallographic study of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide. The recombinant HLA-B*52:01 protein was crystallized, and by using the molecular replacement method, the three-dimensional structure of HLA-B*52:01 complexed with the Pol283-8 peptide was successfully determined. The crystal and statistical data are summarized in Table S1 in the supplemental material. The overall structure and peptide-binding mode were similar to those of HLA-B*51:01 complexed with the same Pol283-8 peptide (Fig. 6A and B), which