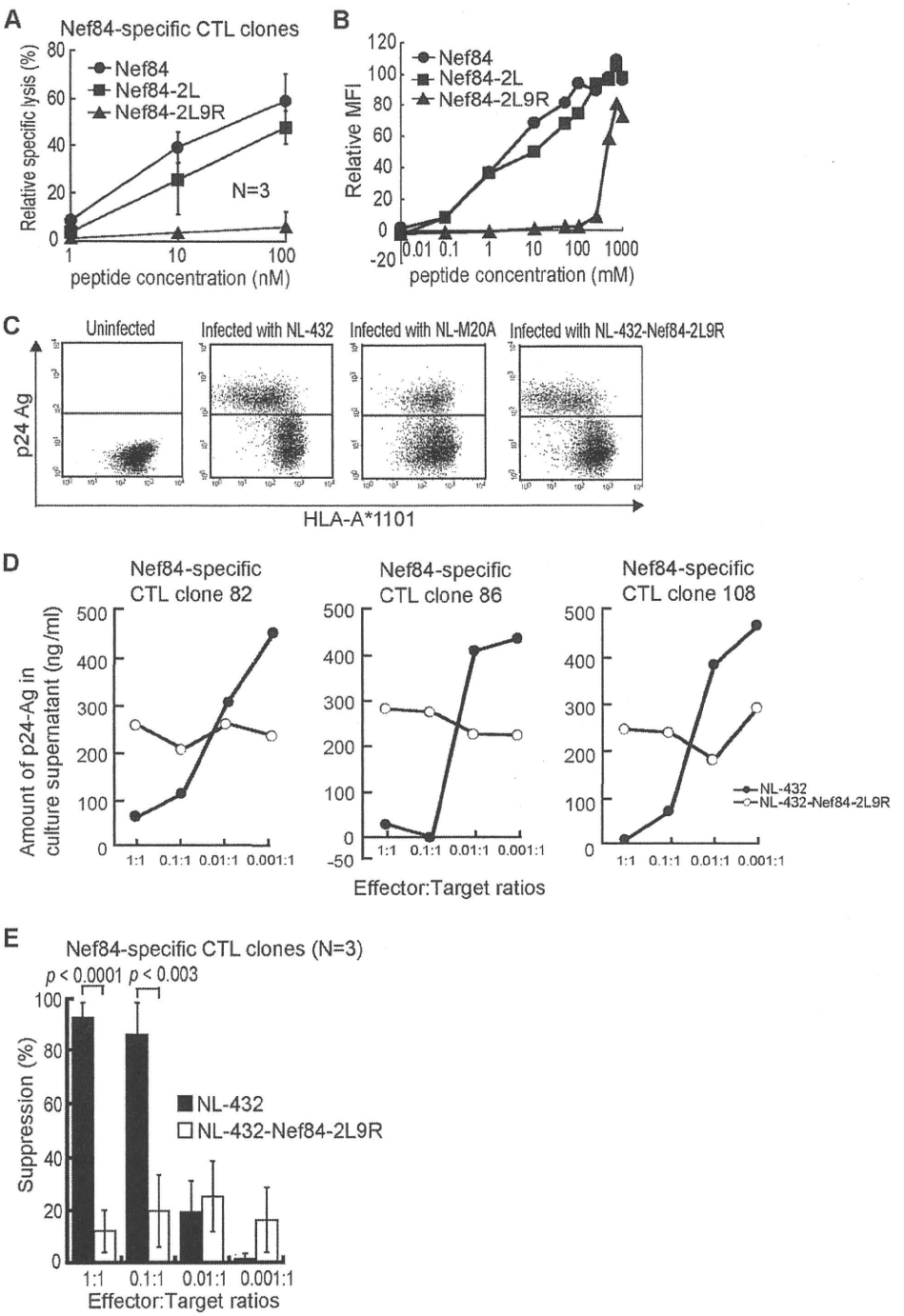

FIG. 5. Ability of Nef84-specific CTLs to suppress replication of HIV-1-Nef84-9R mutant virus. (A) Cytolytic activities of Nef84-specific CTL clones in killing C1R-A*1101 cells pulsed with Nef84-9R peptide. C1R-A*1101 cells were prepulsed with various concentrations of Nef84, Nef84-2L, or Nef84-2L9R peptide. Cytolytic activities of Nef84-specific CTL clones were measured at an effector-to-target ratio of 2:1. (B) Ability of Nef84-2L9R peptide to bind HLA-A*1101. The affinity was measured by a stabilization assay using RMA-S-A*1101 cells. (C) Surface expression of HLA class I molecules on $CD4^+$ T cells infected with NL-432-Nef84-2L9R. (D) Ability of each Nef84-specific CTL clone to suppress NL-432-Nef84-2L9R replication in $CD4^+$ T cells. (E) Analysis of ability of all 3 Nef84-specific CTL clones to suppress replication of NL-432 or NL-432-Nef84-2L9R.

Different functional abilities between *ex vivo* Nef73-specific and Nef84-specific CTLs. We speculated that Nef84-specific CTLs have a stronger functional ability *in vivo* than Nef73-specific ones. Therefore, we investigated whether Nef84-specific CTLs from *ex vivo* PBMC would respond to the specific epitope more effectively than Nef73-specific ones. To compare functional abilities between these 2 CTLs, we selected 5 individuals who had both Nef73-specific and Nef84-

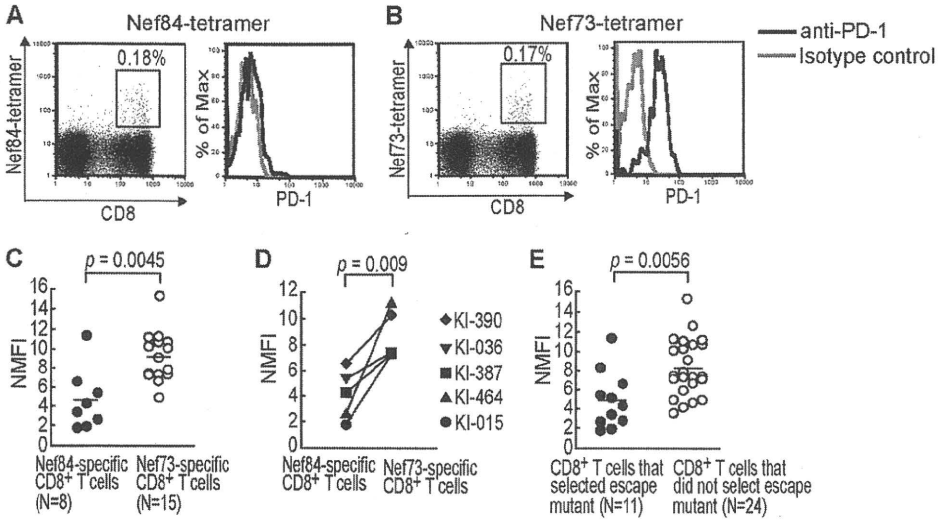

FIG. 6. PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells. (A and B) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells among PBMCs from an HIV-1-infected individual (KI-015). PBMCs from KI-015 were stained with anti-CD3, anti-CD8, anti-PD-1 MAb, and the tetramer. The frequency of $tetramer^+$ $CD8^+$ T cells in the lymphocyte population was plotted (left). The histogram shows PD-1 expression on the specific $CD8^+$ T cells (right). (C) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells in PBMCs of HIV-1-infected individuals. PD-1 expression on the cells from each individual was normalized by the mean fluorescence intensity of the isotype control (NMFI). (D) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells from the same individuals (KI-015, -036, -387, -390, and -464). (E) PD-1 expression on $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* and to select escape mutants. The left part of the plot shows 8 HLA-A*1101-restricted Nef84-specific and 3 HLA-A*2402-restricted Nef-138-specific $CD8^+$ T cells that select escape mutants, and the right part shows 15 HLA-A*1101-restricted Nef73-specific and 9 HLA-A*26-restricted Gag169-specific $CD8^+$ T cells that do not select them.

specific CTLs. IFN-γ production from these T cells among *ex vivo* PBMC was measured after they had been stimulated with Nef84 peptide or Nef73 peptide (Fig. 7A). The results showed that the frequency of IFN-γ-producing cells was higher for Nef84-specific $CD8^+$ T cells than for Nef73-specific ones from each individual. That is, it is significantly higher for the former T cells than for the latter ones (Fig. 7B; see also Fig. S3 in the supplemental material). These results support the idea that Nef73-specific T cells can partially function *in vivo*.

DISCUSSION

Previous studies showed an inverse correlation between the plasma viral load (pVL) and the frequency of some HIV-1-specific CTLs in HIV-1-infected individuals, indicating that these CTLs control HIV-1 *in vivo* (5, 28, 33). However, this correlation was not found in the case of many other HIV-1-specific CTLs (16, 25, 26), suggesting the possibility that the quality of HIV-1-specific CTLs is a critical factor for the control of HIV-1 *in vivo*. However, it is not easy to assess the quality of HIV-1-specifc CTLs. An assay to directly measure the ability of the CTLs to suppress HIV-1 replication *in vitro* is a very useful method to evaluate the ability of the CTLs to control HIV-1. A previous study using this assay demonstrated that the ability of HLA-B*5101-restricted HIV-1-specific CTLs to suppress HIV-1 replication is dependent on the epitope recognized by these CTLs (43). In addition, a recent study showed that HLA-A*2402-restricted Nef138-specific CTLs have a strong ability to suppress HIV-1 replication, whereas HLA-A*2402-restricted Gag133-8-, Pol797-8-, or Gag263-10-specific CTLs showed a weak ability or no ability to suppress HIV-1 replication (18).

TABLE 1. Sequences of Nef73 and Nef84 epitopes in HIV-1 from the 5 subjects whose Nef73- and Nef84-specific CD8 T cells were analyzed for PD-1 expression

Patient ID or sequence description	Sequence[a]																	
	Nef73										Nef84							
Wild type	Q	V	P	L	R	P	M	T	Y	K	A	V	(L)	D	L	S	H	F	L	K
KI-015[b]	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
KI-036[c]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	-
KI-387[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	-
KI-390[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	R
KI-464[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	K/R[d]

[a] Sequences were analyzed by the direct sequencing method. "-" indicates agreement with wild-type sequence.
[b] The same sample was analyzed for sequencing and PD-1 expression.
[c] This patient was analyzed for the sequence of HIV-1 on 6 October 2005 and for PD-1 expression on the T cells on 14 July 1999.
[d] The mixture of sequences carrying K or R at position 9 was detected.

The Nef138-specific CTLs select the 2F escape mutation within 1 to 2 years after the start of an HIV-1 infection (18). The frequency of the Nef138-specific CTLs is inversely correlated with pVL in individuals infected with wild-type virus before the virus with the 2F mutant (the 2F virus) is selected. In contrast, it did not correlate with pVL in them after the 2F virus appeared or in individuals originally infected with the 2F virus (18). These observations strongly suggest that Nef138-specific CTLs have a strong ability to suppress the replication of wild-type HIV-1 *in vivo*, such that they can select the 2F escape virus. Thus, a strong ability of HIV-1-specific CTLs to suppress HIV-1 replication is necessary to select CTL escape mutants *in vivo*.

In the present study, we showed that 2 HLA-A*1101-restricted Nef-specific CTLs had a strong ability to suppress

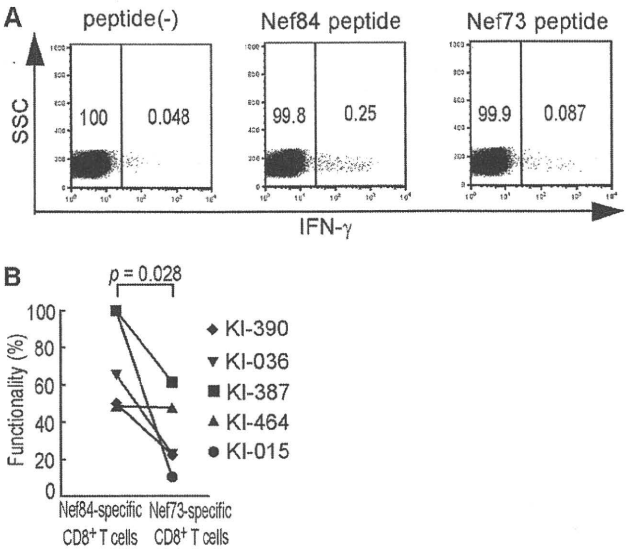

FIG. 7. Functional analysis of *ex vivo* Nef84- and Nef73-specific $CD8^+$ T cells. (A) IFN-γ production of Nef84- and Nef73-specific $CD8^+$ T cells among PBMCs from an HIV-1-infected individual, KI-036. PBMCs from KI-036 were stimulated with Nef73 peptide or Nef84 peptide and stained with anti-CD8, followed by intracellular staining for IFN-γ. The frequency of IFN-γ^+ $CD8^+$ T cells among total $CD8^+$ T cells was plotted. (B) Frequency of Nef84- and Nef73-specific $CD8^+$ T cells producing IFN-γ. The percent functionality was calculated as follows: (frequency of IFN-γ^+ $CD8^+$ T cells among total $CD8^+$ T cells/that of tetramer$^+$ $CD8^+$ T cells among total $CD8^+$ T cells) × 100.

HIV-1 replication. Nef84-specific CTLs selected the escape mutant 9R, whereas Nef73-specific ones did not select any escape mutant. There are several hypotheses to explain the difference in the abilities of these CTLs to select escape mutants. One is that the frequency of mutations is much lower in a part of the Nef73 epitope and its flanking region than in that of the Nef84 epitope and its flanking region. This idea is not likely to be true, however, because the analysis of sequences of HIV-1 isolates reported in the Los Alamos HIV-1 Sequence Database showed that the frequency of mutations in the Nef73 epitope is almost the same as that in the Nef84 one (data not shown). Another possibility is that Nef73-specific CTLs can have a strong ability to suppress HIV-1 replication *in vitro* but not *in vivo*. We analyzed the ability of HIV-1-specific CTLs to suppress HIV-1 replication by using the specific CTL clones. Since CTL clones are established from a small part of the memory or memory effector T-cell population that can effectively proliferate, they may not reflect the CTLs *in vivo*.

Recent studies showed that PD-1 expression on HIV-1-specific T cells is associated with dysfunction of the T cells and disease progression (15, 35, 44, 47). PD-1 is a regulator of virus-specific T-cell survival (4, 8, 24, 31, 38). Therefore, we speculated that Nef73-specific $CD8^+$ T cells express a higher level of PD-1 on their cell surface, such that they lose their ability to suppress HIV-1 replication *in vivo*. Indeed, the expression of PD-1 on Nef73-specific $CD8^+$ T cells was significantly higher than that on Nef84-specific ones. This difference was found in the case of both Nef73-specific and Nef84-specific $CD8^+$ T cells present in the same individuals. In addition, the *ex vivo* analysis of both Nef138-specific and Gag169-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* confirmed that PD-1 was expressed significantly at a lower level on the former T cells, which can select escape mutants, than on those unable to select escape mutants. Thus, since PD-1 expression on the latter cells was much higher than that on the former ones, it is likely that the former could not proliferate and promptly died *in vivo* so that they failed to select escape mutants. A recent study showed that PD-1 expression on HIV-1-specific $CD8^+$ T cells decreased after the variation appeared in the target epitope sequences (39), suggesting that reduced signaling via T-cell receptors (TCR) decreased PD-1 expression. However, the present study showed that lower expression of PD-1 was also found in 4 individuals who had HIV-1 carrying the wild-type Nef84 epitope. Therefore, the T cells in these individuals may not indicate that reduced signaling via TCR decreased the PD-1 expression, because they have wild-type HIV-1. Recent studies suggested that PD-1 expression is a marker of homeostatic stimulation or T-cell differentiation (9, 21, 27, 29, 37). The analysis of the CD27 CD28 CD45RA phenotype of Nef73-specific and Nef84-specific T cells in the 5 individuals excluded the possibility that the difference in expression of PD-1 between these T cells was due to that in differentiation status between these T cells. On the other hand, the present study could not exclude another interpretation, i.e., that the difference between these T cells in ability to suppress HIV-1 replication *in vivo* is due to some mechanism other than that involving PD-1 expression. We showed that *ex vivo* Nef84-specific $CD8^+$ T cells had a stronger ability to recognize the epitope than Nef73-specific ones, suggesting that Nef84-specific $CD8^+$ T cells had a stronger ability to suppress wild-type HIV-1 *in vivo*. Further study of these T cells is necessary to clarify what determines a weak function of Nef73-specific T cells and a strong function of Nef84-specific T cells *in vivo*.

We showed in the present study that 1 of 2 HIV-1-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* selected escape mutants. In addition, we recently found that 1 of 2 Pol epitope-specific HLA-B*5101-restricted $CD8^+$ T cells and 1 Nef epitope-specific HLA-A*2402-restricted $CD8^+$ T cell having a strong ability to suppress HIV-1 replication *in vitro* could select escape mutants (6; our unpublished observation). Thus, half of HIV-1-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro*, which were previously and presently analyzed, can select escape mutants *in vivo*, whereas the other half of these $CD8^+$ T cells lose this ability. High expression of PD-1 on the $CD8^+$ T cells may be one explanation for this difference. The mechanism responsible for the presence of 2 types of $CD8^+$ T cells in HIV-1-infected individuals remains unknown.

In the present study, we showed that out of the HIV-1-specific CTLs having the ability to suppress HIV-1 replication *in vitro*, only those having a strong ability to recognize an HIV-1 epitope can select escape mutants. Thus, it is not true that CTL escape mutations are simply selected by CTLs having a strong ability to suppress HIV-1 replication *in vitro*. It is still unknown why a given HIV-1-specific CTL can have a strong ability to recognize the epitope *in vivo* and others cannot, even though both have a strong ability to suppress HIV-1 *in vitro*. Further analysis of the function of HIV-1-specific CTLs *in vivo* will be necessary for clarification of the immunopathogenesis

of AIDS and the development of immunotherapy and an effective AIDS vaccine.

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Long-Term Control of HIV-1 in Hemophiliacs Carrying Slow-Progressing Allele HLA-B*5101▽†

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HLA-B*51 alleles are reported to be associated with slow disease progression to AIDS, but the mechanism underlying this association is still unclear. In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome for Japanese hemophiliacs who had been infected with HIV-1 before 1985 and had been recruited in 1998 for this study. HLA-B*5101$^+$ hemophiliacs exhibited significantly slow progression. The analysis of HLA-B*5101-restricted HIV-1-specific cytotoxic T-lymphocyte (CTL) responses to 4 HLA-B*-restricted epitopes in 10 antiretroviral-therapy (ART)-free HLA-B*5101$^+$ hemophiliacs showed that the frequency of Pol283-8-specific CD8$^+$ T cells was inversely correlated with the viral load, whereas the frequencies of CD8$^+$ T cells specific for 3 other epitopes were positively correlated with the viral load. The HLA-B*5101$^+$ hemophiliacs whose HIV-1 replication had been controlled for approximately 25 years had HIV-1 possessing the wild-type Pol283-8 sequence or the Pol283-8V mutant, which does not critically affect T-cell recognition, whereas other HLA-B*5101$^+$ hemophiliacs had HIV-1 with escape mutations in this epitope. The results suggest that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs is associated with a Pol283-8-specific CD8$^+$ T-cell response and that lack of control of HIV-1 is associated with the appearance of Pol283-8-specific escape mutants.

Human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells play a critical role in the control of HIV-1 infections (26, 5), but HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection (6, 14). There are several mechanisms affording HIV-1 escape from the host immune system. They include the appearance of mutants that escape from HIV-1-specific cytotoxic T lymphocytes (CTLs) (6, 14) and neutralizing antibodies (27, 47, 48), impaired recognition of HIV-1-infected cells by HIV-1-specific CTLs due to Nef-mediated downregulation of HLA class I molecules (8, 42), and impaired function of HIV-1-specific T cells (3).

It is well known that long-term nonprogressors (LTNPs), who remain disease free and have very low or undetectable viral loads (VLs) in the absence of antiretroviral therapy (ART), exist as a very small population of HIV-1-infected individuals (7, 21, 38). A small minority of these LTNPs were infected by HIV-1 containing deletions in viral accessory molecules (10, 17, 24). HLA alleles such as HLA-B*57/5801, HLA-B*27, and HLA-B*51 are associated with slow progression to AIDS (19, 22, 37). Indeed, it is reported that many LTNPs carry these HLA alleles (31, 36). These findings imply that HIV-1-specific CTLs restricted by these alleles may play an important role in the control of HIV-1 replication in LTNPs. The mechanism of control of HIV-1 replication has been analyzed in LTNPs and slow progressors carrying HLA-B*57/5801, HLA-B*27, or HLA-B*13, and has been related to the Gag-specific CD8$^+$ T-cell epitopes presented by these alleles (9, 11, 14, 16, 34). On the other hand, the mechanism underlying the association between HLA-B*5101 and slow progression remains unclear. To date, no study of the mechanism of control of HIV-1 in HLA-B*5101$^+$ LTNPs has been reported.

Since the data indicate that HIV-1 replication can be controlled for more than 20 years in LTNP hemophiliacs, analysis of HIV-1-specific immune responses and HIV-1 in these patients is useful for investigating the immunological control of HIV-1. In Japan, HLA-B*57/58 and HLA-B*27 are very rare alleles (18). Therefore, it was speculated that only HLA-B*51 would play an important role in the control of HIV-1 replication in HIV-1-infected Japanese donors.

We showed previously that 2 Pol peptides and 1 Gag peptide were HLA-B*5101-restricted immunodominant CTL epitopes (45). Two Pol-specific CTLs are known to have strong abilities to suppress HIV-1 replication *in vitro* (43). Our recent study using 9 cohorts showed that of these T cells, Pol283-specific CTLs select mutations at position 8 (position 135 of reverse transcriptase [RT]) in the epitope (20). A Thr mutation at position 8 (8T) was found predominantly in HIV-1-infected HLA-B*5101$^+$ donors, whereas the 8R, 8L, and 8V mutations were also found in these donors. The 8T, 8L, and 8R mutants had fitness similar to

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that of the wild-type virus, whereas the 8V mutation had a higher fitness cost than the others.

In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome in Japanese hemophiliacs infected with HIV-1. In addition, we investigated the role of HLA-B*5101-restricted HIV-1-specific CTLs *in vivo* in HLA-B*5101$^+$ LTNP and slow-progressing Japanese hemophiliacs who had not been treated with antiretroviral therapy for approximately 25 years. Our results revealed a role for Pol283-8-specific HLA-B*5101-restricted HIV-1-specific CTLs in the long-lasting (approximately 25 years) control of HIV-1 replication.

MATERIALS AND METHODS

Patients. One hundred eight Japanese hemophiliacs who had been infected with HIV-1 before 1985, mostly around 1983, 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. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Patient HLA type was determined by standard sequence-based genotyping. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood.

Cells. C1R and 721.221 cells expressing HLA-B*5101 (C1R-B*5101 and 721.221-B5101, respectively) were generated previously (15, 33, 44). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (1). Pol283-8 and Pol743-9 mutant (Pol283-8L, -8T, -8V, and -8R; Pol743-1I, -5I, and -415I) viruses were generated based on pNL-432 by using the GeneTailor site-directed mutagenesis system (Invitrogen).

HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as described previously (2). Four HIV-1 specific epitopes (Pol283-8, Pol743-9, Gag327-8, and Rev71-11) (45) were used for the refolding of HLA-B*5101 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for the generation of the tetramers.

Flow cytometric analysis using tetramers. PBMCs were incubated with the tetramers at 37°C for 30 min. The cells were subsequently washed twice with RPMI–10% newborn calf serum (NCS) and were then stained with an anti-CD8 monoclonal antibody (MAb). Next, they were incubated at 4°C for 30 min and were then washed twice with RPMI–10% NCS. The cells were finally resuspended in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, and then the percentage of tetramer-positive cells among the CD8$^+$ population was determined by using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

Generation of CTL clones. Pol283-8-specific CTL clones and Pol743-9-specific 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) containing 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-B*5101 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 [rIL-2]) (43).

CTL assay for target cells infected with HIV-1. The cytotoxicity of CTL clones for 721.221-B5101 cells infected with HIV-1 (>30% p24 antigen [Ag]-positive cells) was determined by the standard ^{51}Cr release assay as described previously (42). The infected cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min, and then the infected cells were washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark) with effector cells at an effector-to-target cell (E:T) 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.

Assay for suppression of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (42). CD4$^+$ T cells isolated from PBMCs were derived from an HIV-1-seronegative individual with HLA-B*5101. After the CD4$^+$ T cells had been incubated with the desired HIV-1 clones for 4 h at 37°C, they were washed three times with R10 medium. The HIV-1-infected CD4$^+$ T cells were then cocultured with HIV-1-specific CTL clones. From day 3 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

Sequencing of proviral DNA or plasma RNA. Genomic DNA was extracted from PBMCs by using a QIAamp DNA blood minikit (Qiagen). Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp Mini Elute virus spin kit (Qiagen). cDNA was synthesized from the RNA with SuperScript II and random primers (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR using RT-specific primers 5′-CCAAAAGT TAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGGAGG-3′ or 5′-CC TTGCCCCTGCTTCTGTAT-3′ for the first round of PCR and 5′-AGTTAGG AATACCACACCCC-3′ and 5′-GTAAATCCCCACCTCAACAG-3′ or 5′-AA TCCCCACCTCAACAGAAG-3′ for the second round and integrase-specific primers 5′-ATCTAGCTTTGCAGGATTCGGG-3′ and 5′-CCTTAACCGTAG TACTGGTG-3′ or 5′-CCTGATCTCTTACCTGTCC-3′ for the first round of PCR and 5′-AAAGGTCTACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATG GCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second round. PCR products were either sequenced directly or cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems), and sequences were analyzed by use of an ABI PRISM 310 genetic analyzer.

Cell surface staining and intracellular cytokine staining (ICC assay). PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12 to 14 days. These cultured PBMCs were assessed for gamma interferon (IFN-γ)-producing activity as previously described (42). After C1R-B*5101 cells had been incubated for 60 min with epitope peptides (1 μM), they were washed twice with RPMI 1640 containing 10% FCS. These C1R cells and the cultured PBMCs were incubated at 37°C for 6 h at an effector-to-stimulator ratio of 2:1 or 4:1 after the addition of brefeldin A (10 μg/ml). Next, the cells were stained with an anti-CD8 MAb (Dako Corporation, Glostrup, Denmark), fixed with 4% paraformaldehyde at 4°C for 20 min, and then permeabilized at 4°C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and were then stained with an anti-IFN-γ MAb (BD Bioscience Pharmingen, San Diego, CA). Finally, they were resuspended in PBS containing 2% paraformaldehyde, and then the percentage of CD8$^+$ cells positive for intracellular IFN-γ was determined by using a FACSCalibur flow cytometer.

RESULTS

Association of HLA-B*5101 with long-term control of HIV-1 in HIV-1-infected Japanese hemophiliacs. We recruited 108 Japanese hemophiliacs who had been infected with HIV-1 before 1985. Eighteen of the patients had not been treated with any antiretroviral therapy (ART) and had CD4 counts of >350 (very-slow-progressor [VSP] group) by 1998, whereas the other 90 patients had been treated with ART and/or had a CD4 count of <350 (slow-progressor [SP] group). The frequency of HLA-B*5101 in the VSP group (9 of 18 donors [50.0%]) was higher than that in the SP group (15 of 90 donors [16.7%]), and the difference between these 2 groups was significant (P, 0.01). We analyzed the association of HLA class I alleles with disease progression during the years 1998 to 2007 in the VSP group. The 9 HLA-B*5101$^+$ VSP hemophiliacs exhibited significantly slower progression of the disease over this period than the 9 HLA-B*5101$^-$ subjects (Fig. 1), and no other HLA-B alleles or HLA-A/DR alleles showed any significant influence on the progression of the disease in this group (not shown). One HLA-B*3501$^+$ VSP hemophiliac was found in the HLA-B*5101$^+$ group, but none were found in the HLA-B*5101$^-$ group, indicating that HLA-B*3501, which is associated with rapid progression to AIDS, did not affect the results for the 2 VSP groups. Other HLA-A/B/DR alleles were not associated with the HLA-B*5101$^+$ or the HLA-B*5101$^-$ group (see Table S1 in the supplemental material). These results, taken together, show that the HLA-B*5101 allele was

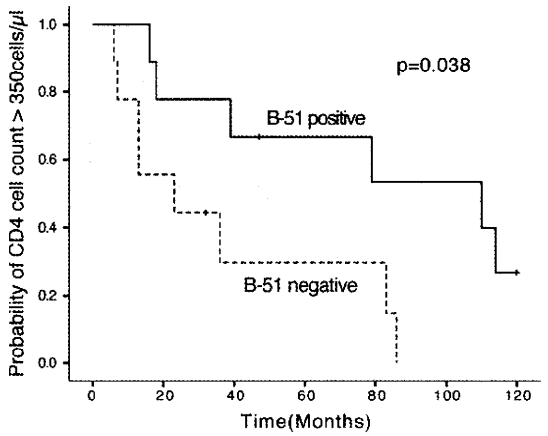

FIG. 1. Association of HLA-B*5101 with slow progression to AIDS. Kaplan-Meier survival analysis was used to estimate the time to the first CD4 cell count (24-week time-weighted average levels of CD4 cells) of <350/μl^3 for 9 HLA-B*5101-positive (solid line) and 9 HLA-B*5101-negative (dashed line) hemophiliacs who had not been treated with antiretroviral therapy (ART) and who had a CD4 count of >350/μl in 1998.

still associated with slow progression of the disease more than 20 years postinfection.

Control of HIV-1 replication by HLA-B*5101-restricted CD8$^+$ T cells. A previous study demonstrated that 2 types of HLA-B*5101-restricted CTLs, Pol283-8 (TAFTIPSI)-specific and Pol743-9 (LPPVVAKEI)-specific CTLs, suppressed HIV-1 replication *in vitro* much more strongly than did other HLA-B*5101-restricted CTLs (43), suggesting that these CTLs may play a key role in the control of HIV-1 in the HLA-B*5101$^+$ SP group. To investigate the control of HIV-1 by these CTLs, we selected 10 HLA-B*5101-positive donors (8 VSPs and 2 SPs) who had not been treated with ART by 1998 and whose PBMC samples were available for analysis of HLA-B*5101-restricted CTLs (see Fig. S1 and Table S2 in the supplemental material). Three of the 8 VSP patients had VLs below 1,000 copies at all time points tested and were classified as LTNPs. We found that only 3 of the 108 HIV-1-infected hemophiliacs (KI-021, KI-051, and KI-124) were LTNPs for approximately 25 years and that all 3 of these LTNPs carried HLA-B*5101. We generated 4 HLA-B*5101 tetramers carrying Pol283-8, Pol743-9, Gag327-9, or Rev71-11, and we used them to determine the frequencies of HIV-1-specific CD8$^+$ T cells among PBMCs from these 3 LTNPs (Table 1 and Fig. 2). KI-021 had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells but neither Gag327-9- nor Rev71-11-specific CD8$^+$ T cells during the years 1997 to 2005 (Fig. 2A). KI-051 also had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells, whereas this patient had no Rev71-11-specific CD8$^+$ T cells and a low number of Gag327-9-specific CD8$^+$ T cells during the years 1999 to 2005 (Fig. 2B). KI-124 had Pol283-8-, Pol743-9-, and Gag327-9-specific CD8$^+$ T cells (Table 1). These results suggest that the 2 Pol-specific CD8$^+$ T cells may play an important role in the control of HIV-1 in these LTNPs carrying HLA-B*5101.

Selection of escape mutations of the Pol283-8 epitope in very slow progressors. Of the 8 HLA-B*5101$^+$ VSP hemophiliacs, KI-127 had Pol283-8-specific CD8$^+$ T cells at a low frequency in 1998, when the plasma viral load (pVL) was very low, whereas later this patient lost the response, and the pVL increased from an undetectable level to more than 10^3 copies (Fig. 2C). The other 4 VSPs, excluding 3 LTNBs, either had a low number of Pol283-8-specific CD8$^+$ T cells or did not have any of these cells at any time points studied. These results suggest that Pol283-8-specific CD8$^+$ T cells rather than Pol743-9-specific CD8$^+$ T cells may control HIV-1 *in vivo*.

To clarify the role of these HLA-B*5101-restricted CD8$^+$ T cells in the control of HIV-1 *in vivo*, we analyzed the correlation between the frequency of the HLA-B*5101-restricted CD8$^+$ T cells and the pVL in 10 HLA-B*5101$^+$ hemophiliacs. The frequency of Pol283-8-specific CD8$^+$ T cells was negatively correlated with the pVL (P, 5.6×10^{-8}), whereas the frequency of the other T cells was positively correlated with the pVL (Fig. 3). These results support the idea that Pol283-8-specific CD8$^+$ T cells drive the suppression of HIV-1 replication *in vivo*.

We speculated, therefore, that escape mutants within Pol283-8 epitopes were selected in slow progressors over a 25-year period, because these epitope-specific CTLs are thought to provide strong immune pressure on HIV-1. Two of the LTNPs had the Pol283-8V mutant, whereas the third had wild-type Pol283 in July 2002 but the 8V mutant in October

TABLE 1. Numbers of 4 types of HLA-B*5101-restricted CD8$^+$ T cells among HLA-B*5101$^+$ HIV-1-infected hemophiliacs

Patient	Median VL (copies/ml)[a]	Median no. of CD4 cells/μl[a]	Median no. (frequency) of HLA-B*5101-restricted CD8$^+$ T cells[b]				No. of times PBMCs were tested (dates)[c]
			Pol743	Pol283	Gag327	Rev71	
KI-021	50	618	1,910 (0.39)	1,900 (0.40)	<100 (0)	<100 (0)	10 (8/1997–11/2005)
KI-051	50	737	3,222 (0.53)	5,186 (0.87)	1,082 (0.16)	<100 (0)	5 (10/1999–9/2005)
KI-124	570	850	3,126 (0.43)	1,745 (0.24)	1,381 (0.19)	<100 (0)	8/2001
KI-386	360	459	3,164 (0.40)	554 (0.07)	5,774 (0.73)	396 (0.05)	8/2006
KI-363	1,700	676	6,696 (0.54)	1,488 (0.12)	496 (0.04)	1,116 (0.09)	11/1998
KI-127	5,500	597	8,100 (0.79)	257 (0.02)	23,411 (2.33)	<100 (0.01)	9 (2/1998–4/2006)
KI-121	16,650	327	4,853 (0.59)	134 (0.02)	<100 (0)	395 (0.04)	2 (12/1999, 8/2001)
KI-032	25,500	226	9,153 (1.80)	<100 (0)	344 (0.09)	<100 (0)	2 (10/2002, 9/2005)
KI-007	39,500	387	1,084 (0.12)	394 (0.05)	6,278 (0.68)	1,029 (0.12)	2 (6/2001, 4/2002)
KI-026	40,000	526	10,705 (1.32)	<100 (0)	6,164 (0.76)	568 (0.07)	7/2005

[a] At the time of tetramer analysis.
[b] Median number of HLA-B*5101-restricted CD8$^+$ T cells/μl among PBMCs (median frequency of HLA-B*5101-restricted T cells among CD8$^+$ T cells [expressed as a percentage]).
[c] If PBMCs were tested only once, only the date (month/year) is given.

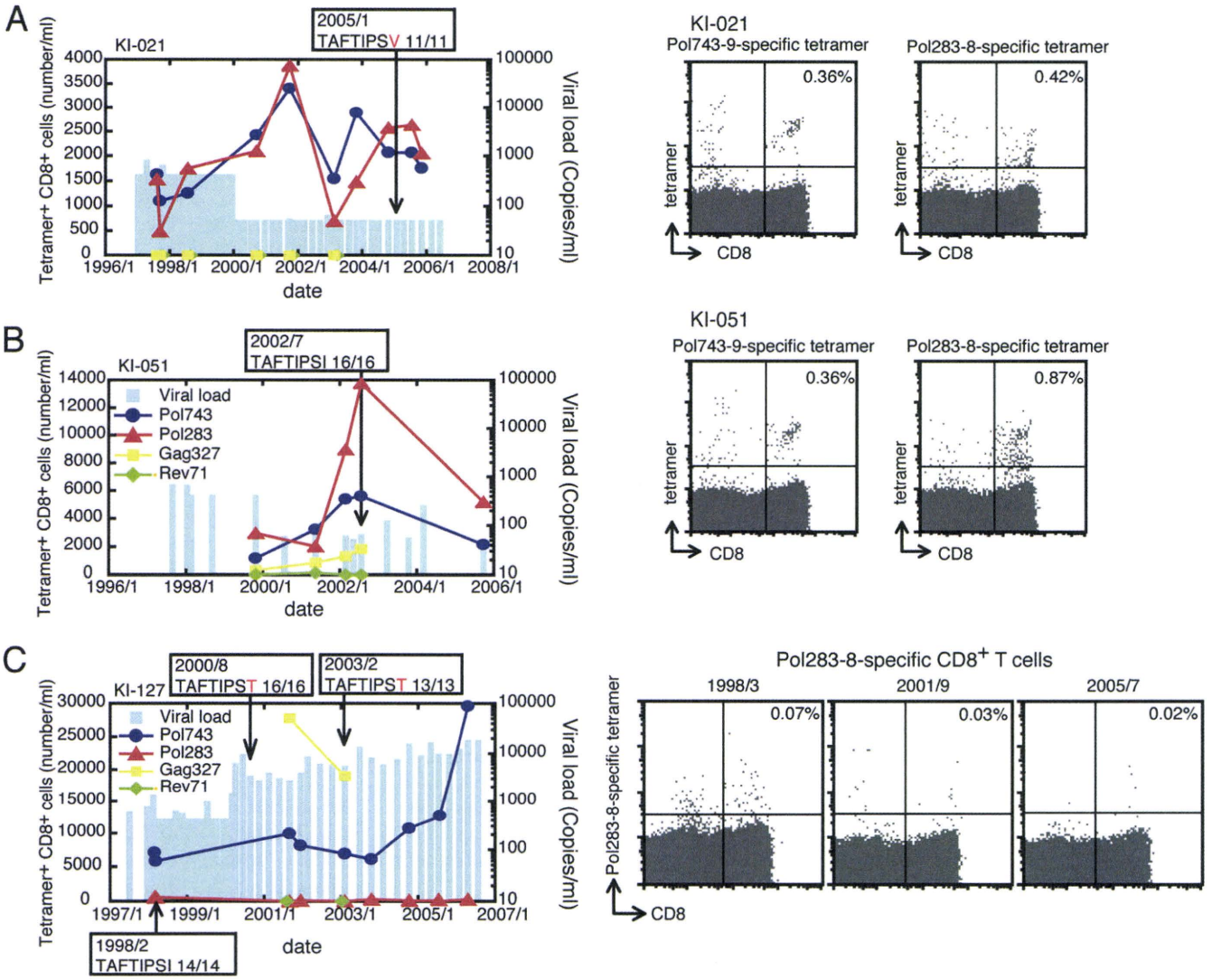

FIG. 2. Longitudinal analysis of HLA-B*5101-restricted $CD8^+$ T cells and Pol283 epitope sequences in 3 slow-progressing hemophiliacs. Four types of HIV-1-specific $CD8^+$ T cells were detected by use of specific tetramers. PBMCs from KI-021 (A), KI-051 (B), and KI-127 (C) were analyzed by using Pol743-9-specific and Pol283-8-specific tetramers. The percentage of tetramer-positive cells among the $CD8^+$ T-cell population is given in the upper right quadrant of each histogram. The sequence of the Pol283-8 epitope from each patient is shown. The detection limit of pVL was 400 copies/ml until 2000 and 50 copies/ml after 2000.

2006 (Table 2). As previously noted (34), Pol283-8-specific CTL clones showed the same killing activity toward target cells prepulsed with the Pol283-8V peptide as toward those prepulsed with the wild-type peptide. These T cells revealed similar killing activity toward 721.221-B*5101 cells infected with NL-432 carrying Pol283-8V (NL-Pol283-8V) as toward those infected with NL-432 (see Fig. S2A in the supplemental material) and only a marginally weaker ability to suppress the replication of NL-Pol283-8V (see Fig. S2B in the supplemental material). In contrast, the 5 VSPs and 2 SPs had Pol283-8T or Pol283-8R mutants (Table 2). Three Pol283-8-specific CTL clones failed to kill target cells infected with NL-432 carrying these mutants (NL-Pol283-8T and NL-Pol283-8R [see Fig. S2A in the supplemental material]) or to suppress the replication of these mutants (see Fig. S2B in the supplemental material), indicating that these were escape mutants.

Longitudinal analysis of KI-127 showed that the 8T mutant appeared in August 2000, when the VL had increased approximately 10-fold, whereas wild-type Pol283 was found in February 1998, when the VL was very low or undetectable (Fig. 2C). Previous population analysis using 9 cohorts showed strong association between HLA-B*51 and Pol283-8T (20). These observations together suggest that the 8T mutant is an escape mutant selected by Pol283-specific CTLs and implies that escape from this epitope reduces immune control of HIV-1.

***In vitro* selection of Pol283 escape mutants by Pol283-specific CTLs.** The results shown in Fig. 4 suggested that Pol283-specific CTLs selected 8T, 8R, and 8L escape mutants. To further confirm the selection of these mutants by Pol283-specific CTLs, we investigated whether Pol283-specific CTLs selected these mutant viruses *in vitro* when the CTLs were cul-

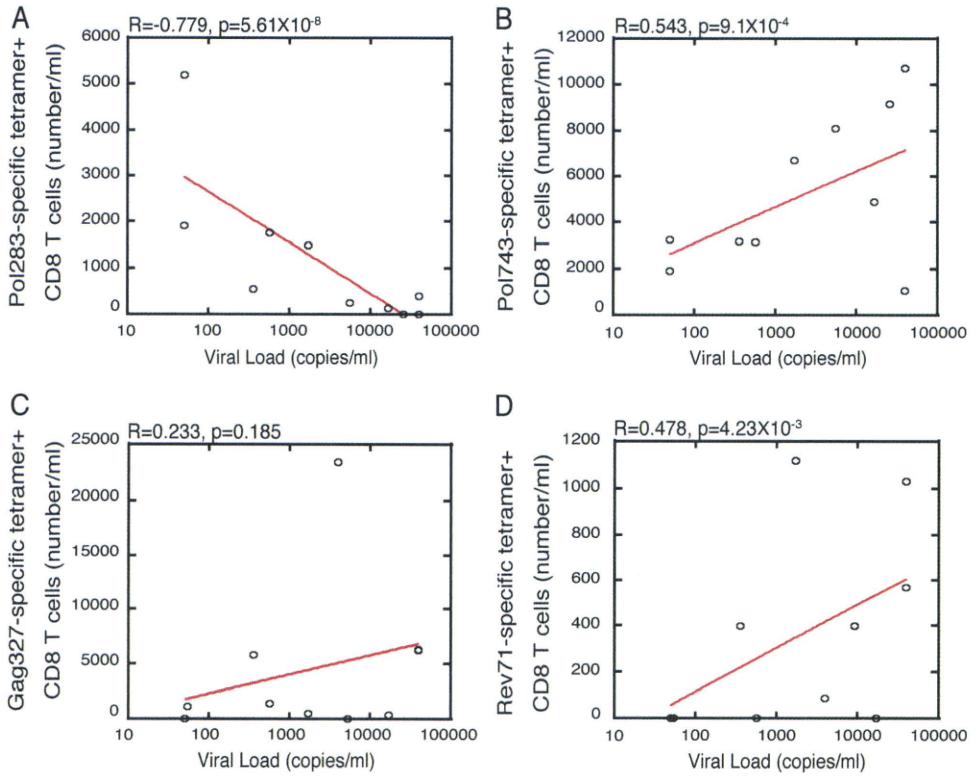

FIG. 3. Correlation of the number of HLA-B*5101-restricted CD8+ T cells with the viral load. The number of Pol283-8-specific (A), Pol743-9-specific (B), Gag327-specific (C), or Rev71-specific (D) CD8+ T cells among PBMCs from 10 HLA-B*5101+ hemophiliacs was measured at 1 time point or at 2 to 10 different time points (see Table 1) by using specific tetramers. The correlation of the median number of tetramer-positive cells with the median viral load was analyzed.

tured with HLA-B*5101-positive CD4+ T cells infected with NL-432 and the mutant virus together. Pol283-specific CTL clones selected these 3 mutant (8T, 8R, and 8L) viruses rapidly in this assay (Fig. 4A to C), supporting the notion that these mutants were selected as escape mutants by Pol283-specific CTLs.

Long-term maintenance of Pol283-8-specific memory CD8+ T cells and failure of induction of escape mutant-specific CD8+ T cells. If the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with HIV-1 carrying the Pol283-8 wild-type epitope, we can speculate that the donors had Pol283-8-specific memory CD8+ T cells but failed to elicit

TABLE 2. Sequences of Pol283-8 and Pol743-9 epitopes in HLA-B*5101+ HIV-1-infected hemophiliacs

Patient	Epitope				VL (copies/ml)	Date (mo/yr) of PBMC testing[b]
	Pol283-8		Pol743-9			
	Sequence	Clonal frequency[a]	Sequence	Clonal frequency		
NA[c] (wild-type sequence)	TAFTIPSI		LPPVVAKEI			
KI-021	-------V	11/11	---------	10/12	<50	1/2005
KI-051	---------	16/16	---------	15/15	63	7/2002
	-------V	DS	ND[d]	ND	<50	10/2006
KI-124	-------V	14/14	---------	14/15	600	8/2001
KI-386	-------T	DS	---------	DS	1,200	10/2006
KI-363	-------T	DS	---------	DS	1,700	11/1998
KI-127	-------T	13/13	---------	17/17	5,300	2/2003
KI-121	-------T	16/16	I--------	12/13	9,300	12/1999
KI-032	-------T	13/13	---------	15/15	17,000	10/2002
KI-007	-------R	15/16	---II----	18/18	33,000	6/2001
KI-026	-------T	DS	I--------	DS	28,000	1/2004

[a] Expressed as (number of clones carrying the indicated sequence)/(number of clones tested). DS, direct sequence.
[b] The sequence for patient KI-021 is from proviral DNA; those for all other patients are from plasma RNA.
[c] NA, not applicable.
[d] ND, not determined.

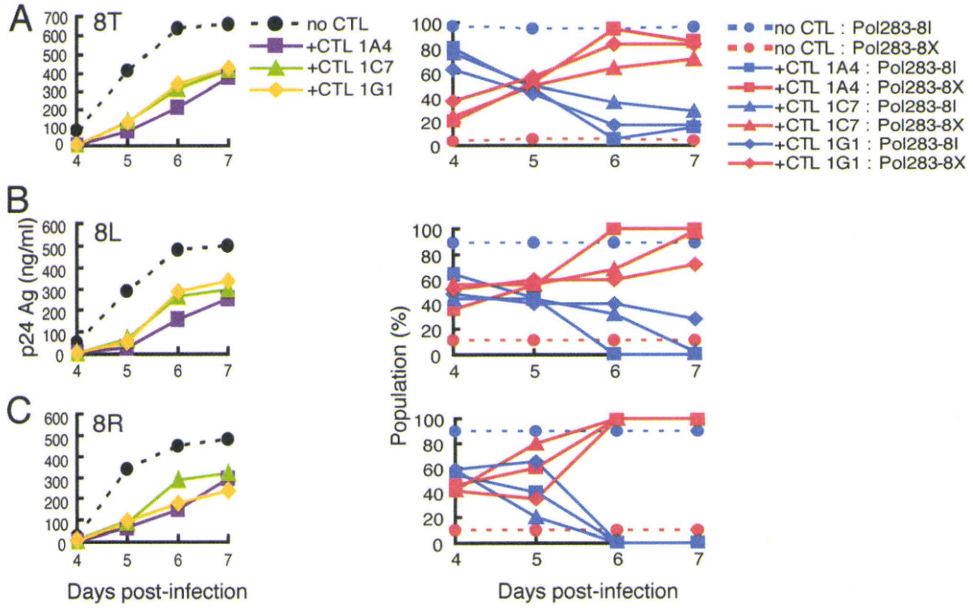

FIG. 4. *In vitro* selection of Pol283 escape mutants by a Pol283-8-specific CTL clone. T1 cells were infected with paired viruses (NL-432 [Pol283-8I] and a mutant virus [Pol283-8L, -8T, or -8R]) at a ratio of 9:1. The infected cells were incubated with Pol283-8-specific CTL clones at an E:T ratio of 1:0.05. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram. From day 4 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in these supernatants was measured by an ELISA. The data obtained by using the mixture of Pol283-8T, -8L, or -8R with Pol283-8I are shown in panels A, B, and C, respectively.

Pol283-8T-specific $CD8^+$ T cells after the Pol283-8T mutation appeared. None of 4 HLA-B^*5101^+ hemophiliac donors carrying Pol283-8T (KI-032, KI-121, and KI-127 [Table 2] and 1 ART-treated hemophilic donor, KI-078 [data not shown]) had detectable Pol283-8-specific $CD8^+$ T cells by analysis using the specific tetramers. But they may have had very small numbers of memory $CD8^+$ T cells. To induce Pol283-8-specific $CD8^+$ T cells from a possible Pol283-8-specific memory T-cell source, we stimulated PBMCs from these patients with the Pol283-8 peptide and then measured the number of Pol283-8-specific $CD8^+$ T cells in 2-week cultures. The KI-127 and KI-078 cultures indeed showed the presence of Pol283-8-specific $CD8^+$ T cells, but KI-127 lost the detectable memory response by April 2006 (Fig. 5), indicating that these 2 patients could maintain Pol283-8-specific memory $CD8^+$ T cells for more than 20 years. In contrast, Pol283-8T-specific $CD8^+$ T cells were not detected among PBMCs from any of these 4 donors after 2 weeks in culture (Fig. 5), indicating that the Pol283-8T escape mutant did not elicit specific $CD8^+$ T cells *in vivo*. These results support the idea that the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with the wild-type virus. Similarly, Pol283-8R-specific $CD8^+$ T cells were not detected in KI-007, although this patient had Pol283-8-specific memory $CD8^+$ T cells (Fig. 5), supporting the notion that the 8R mutant was an escape mutant selected by Pol283-8-specific CTLs and failed to elicit these escape mutant-specific CTLs.

DISCUSSION

It is well known that HLA-B*57 and -B*27 are associated with slow progression to AIDS (19, 37). HLA-B*57-mediated and HLA-B*27-mediated effects on disease progression are seen early and late, respectively, during an infection (6, 14). In the present study, we analyzed 108 HIV-1-infected Japanese hemophiliacs. In Japan, 1,439 patients had been infected with HIV-1 before 1985, mostly around 1983. At present, only 801 of these patients remain alive. Since they had not been treated with highly active antiretroviral therapy (HAART) before 1997, the survivors would seem to be slow progressors. This cohort does not include a large number of patients, because it is not easy to recruit a large number of HIV-1-infected hemophiliacs in Japan, where only 800 are still alive. We found that HLA-B*5101 had effects on the slow progression of the disease in the late phase (both in 1998 and during the years from 1998 to 2007), even when a small number of samples was analyzed. Our recent study also revealed that HLA-B^*5101^+ hemophiliacs had lower VLs and higher CD4 counts than HLA-B^*5101^- hemophiliacs but that only the CD4 count was significantly higher in HLA-B^*5101^+ than in HLA-B^*5101^- hemophiliacs (20). These findings support the idea that HLA-B*5101-restricted immune responses are associated with slow progression to AIDS.

Pol283-8, Pol743-9, and Gag327-9 are thought to be immunodominant HIV-1 epitopes, because CTLs specific for them were frequently detected in chronically HIV-1 infected HLA-B^*5101^+ individuals (45). A previous study demonstrated that Pol283-8-specific and Pol743-9-specific CTLs suppress HIV-1 replication strongly but that Gag327-9-specific CTLs suppress it only weakly *in vitro* (43), suggesting that HIV-1 replication can be suppressed *in vivo* by Pol283-8-specific and Pol743-9-specific CTLs. In the present study, we demonstrated that a higher number of Pol283-8-specific $CD8^+$ T cells was detected predominantly in LTNPs, whereas Pol743-9-specific $CD8^+$ T cells were found at higher levels in all 10 of the SP hemophiliac

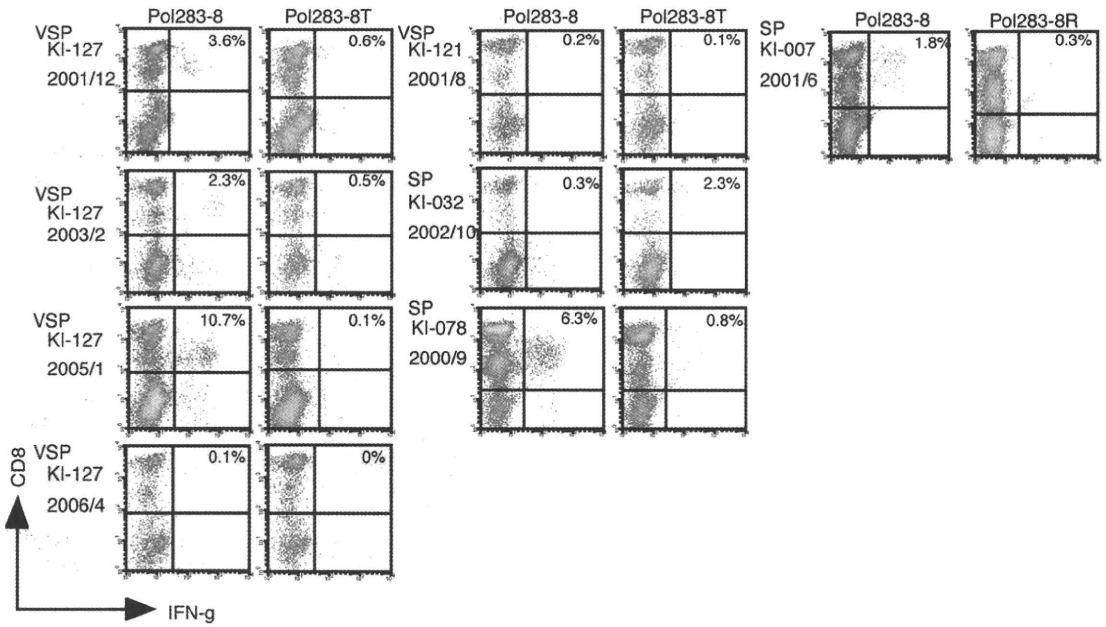

FIG. 5. Induction of Pol283-8-specific CD8+ T cells from PBMCs of 2 very slow progressors and 3 slow progressors. PBMCs from 2 very slow progressors (KI-127 and KI-121) and from 3 slow progressors (KI-032, KI-007, and KI-078) were stimulated with the Pol283-8 epitope peptide or the Pol283-8T or -8R peptide and were then cultured for 12 to 14 days. The cultured cells were stimulated with C1R-B*5101 cells prepulsed with the peptide. IFN-γ-producing CD8+ T cells were measured by using flow cytometry. The percentages of IFN-γ-producing CD8+ T cells are given in the upper right quadrants.

patients examined. ART-treated HLA-B*5101+ patients also carried Pol743-9-specific CD8+ T cells but not Pol283-8-specific CD8+ T cells (data not shown). The frequency of Pol283-specific CD8+ T cells was negatively correlated with the pVL, whereas the frequencies of the other 3 types of T cells were positively correlated with the pVL (Fig. 3). The longitudinal analysis of KI-127 showed that the VL increased after the 8T mutant appeared. This suggests that Pol283-specific CTLs may control HIV-1 in this patient, but the possibility that other CTLs also control HIV-1 cannot be excluded. These results support the notion that Pol283-8-specific CTLs play a key role in the control of HIV-1 in chronically HIV-1 infected HLA-B*5101+ hemophiliacs.

Previous studies showed that Gag-specific responses are negatively correlated with VL in chronically HIV-1 infected individuals (23, 25, 28, 49). Especially HLA-B*57/5801-, HLA-B*27-, HLA-B*13-, or HLA-B*63-restricted Gag-specific CD8+ T-cell responses are related to a low viral load (12, 16, 23, 34, 49). However, these studies had been performed with Caucasian and African cohorts. Since HLA-B*57/5801, HLA-B*27, and HLA-B*13 are very rare in Japan, Gag-specific CD8+ T-cell responses might not be related to a low pVL in Japanese patients. For the HLA-B*5101+ hemophiliacs studied here, it is striking that Pol283-specific CD8+ T-cell responses were much more effective in the control of HIV replication than Gag327-specific CD8+ T-cell responses. A previous study revealed that simian immunodeficiency virus (SIV)-infected cells are recognized earlier by Pol-specific T cells than by Nef-specific T cells (39). These results suggest that Pol-specific responses may be important in the control of HIV-1, and not only in the Japanese population. This is potentially an important result in relation to vaccine design and the specificity of the CD8+ T-cell responses that must be induced to achieve immune control of HIV.

Our recent study using 9 cohorts showed that there are 4 mutations (8T, 8R, 8L, and 8V) at position 8 of the Pol283 epitope, that the frequency of the 8T variant is significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors, and that some acutely infected HLA-B*5101+ subjects who had been infected with the wild-type virus had the 8T virus at only 6 or 12 months after the first test (20), indicating that the 8T mutant is selected by Pol283-specific CTLs. In the present study, we revealed that the Pol283-8T escape mutation was detected for the first time approximately 20 years post-HIV-1 infection in KI-127, indicating that this mutation had been slowly selected by Pol283-8-specific CTLs in this donor. Pol283-8R and Pol283-8L were also apparently escape mutants, because Pol283-8-specific CTLs failed to suppress the replication of HIV-1 carrying these mutants. However, the frequency of these mutations is not significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors (20), suggesting that other, non-HLA-B*5101-restricted CTLs may also select these particular mutants. Nonetheless, it is clear that the HLA-B*5101-restricted Pol283-specific CTLs select the 8R mutant, because KI-007, who had the 8R mutant virus, possessed Pol283-specific memory T cells (Fig. 5), and one HLA-B*5101+ subject with an acute HIV infection who had been infected with the wild-type virus had the 8R mutant 12 months after the first test (20).

The Pol283-8V mutant was found in only 6 of 60 HLA-B*5101+ donors, including 3 LTNP hemophiliacs (data not shown). Of the 3 nonhemophiliacs, 2 were progressors and 1 was a slow progressor. Since this mutation is rare and it is speculated that the mutations had not accumulated 25 years

ago, it is unlikely that the 3 LTNP hemophiliacs had been infected with this mutant virus. On the other hand, the 3 nonhemophiliacs may have been infected with the 8V mutant. The 8V mutation did not influence the killing activity of Pol283-8-specific CTLs toward target cells infected with the HIV-1 mutant, whereas the ability of CTLs to suppress replication was significantly weaker for the Pol283-8V mutant than for the wild-type virus. Previous studies showed that HIV-1-specific CTL clones can partially suppress HIV-1 replication but fail to kill HIV-1-infected $CD4^+$ T cells (42, 45), indicating that the replication suppression assay is more sensitive than the CTL assay. Since Pol283-8-specific CTLs cannot completely suppress the replication of the 8V mutant virus, and since the 8V virus has a higher fitness cost than the wild-type virus, the donors selecting this mutant virus can be LTNP hemophiliacs. However, it still remains unclear why the 8V virus appears in both LTNPs and progressors. We are now analyzing the HLA-B^*5101^+ nonhemophiliacs carrying the 8V mutants in order to compare them with the LTNPs carrying the 8V mutant.

Our previous study on the crystal structure of the HLA-B*5101–Pol283-8 peptide complex showed that the C-terminal anchor (PC) pocket is hydrophobic and relatively small compared with those of the serologically close alleles, HLA-B*3501 and -B*5301, whose C-terminal preferential amino acids include aromatic amino acids (30). Those findings explain why the PC residues for HLA-B*5101 are preferably aliphatic amino acids and not bulky aromatic amino acids. The PC residue is tethered with well-ordered polar and hydrophobic interactions, as observed in other major histocompatibility complex (MHC) class I molecules (Fig. 6A). Thus, the amino acid substitutions of the PC residue did not likely lead to large rearrangements of this network, and so the orientations of the side chains were presumably maintained. In the case of the 8R mutation, the PC pocket was not large enough to accommodate the Arg residue (Fig. 6B), conferring structural changes around the PC pocket that could possibly result in a lack of binding activity toward HLA-B*5101 (2). The 8L mutant exhibited slightly reduced binding activity toward HLA-B*5101 and CTL recognition for 8L peptide-pulsed target cells but no CTL response to 8L mutant-infected cells, suggesting that the mutation had a deleterious effect on antigen presentation in the system for export to the cell surface. The 8V mutation would delete only one methylene group from the Ile residue and thus would presumably have only a small influence on the binding to HLA-B*5101 as well as on its specific T-cell receptor (TCR) recognition. On the other hand, the Pol283-8T mutation likely introduces a hydrophilic OH group that probably is not appropriate for the hydrophobic pocket, resulting in diminished binding activity (43). Furthermore, the Pol283-8T mutation was detrimental to the CTL response and thus may also have induced a structural rearrangement that had a negative effect on TCR recognition.

A higher accumulation of Pol283-8 escape mutations is found in the Japanese population than in other populations, because the frequency of HLA-B*51 is much higher in Japan than in other countries (20). The fitness of the 8T, 8R, and 8L viruses is similar to that of the wild-type virus, and these escape mutants do not revert to wild-type viruses in HLA-B^*5101^- donors (20). The donors with escape mutant viruses failed to elicit escape mutant-specific CTLs. These findings suggest a

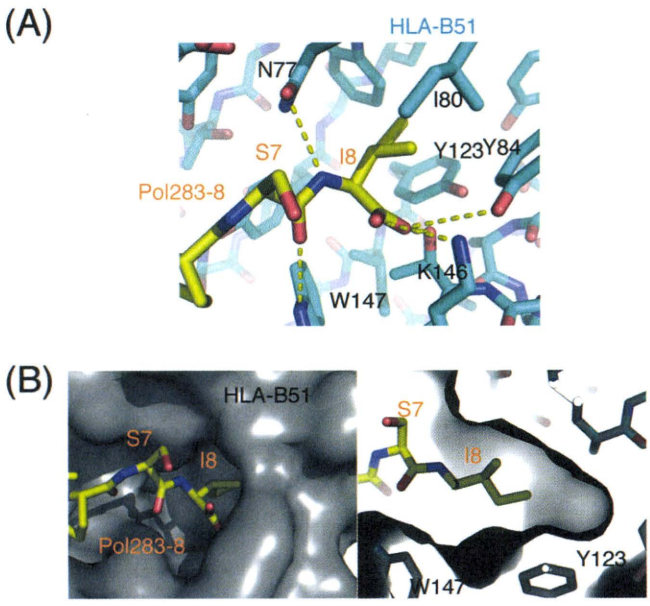

FIG. 6. Binding model of HLA-B*5101 mutant peptides. (A) Polar interactions around the PC residue in the HLA-B51–Pol283-8 complex. The Pol283-8 peptide and the HLA-B51 heavy chain are shown as yellow and cyan stick models, respectively (N and O atoms are shown as blue and red, respectively). The dotted lines indicate hydrogen bonds or salt bridges. (B) (Left) Surface representation (gray) of the HLA-B51 heavy chain with the stick model of the Pol283-8 peptide (with the same coloring as in panel A). 8I (PC) penetrates into the small pocket. (Right) The sliced image of the small PC pocket (right) explains why bulky and long amino acids are not preferential.

difficulty in controlling the replication of these mutant viruses in HLA-B^*5101^+ individuals initially infected with the mutant virus. We showed previously that recently infected HLA-B^*5101^+ donors have no advantage in the control of HIV-1 (20). Thus, the association between HLA-B*5101 and slow progression to AIDS may disappear in newly HIV-1 infected Japanese donors.

HLA-B*57-mediated immune pressure early selects an escape mutant of the TW10 epitope, which has a low viral fitness (29, 32). Escape mutations (K, G, Q, and T at position 242) of the KK10 epitope selected by HLA-B*27-mediated immune pressure impair viral replication, but the compensatory S173A mutation restores viral replication (40, 41). Pol283-8 escape mutations (T, L, and R) are different from those escape mutations, because these Pol283-8 mutations do not influence viral fitness (43). HLA-B*5701 is highly associated with LTNPs, but the mechanism of suppression of HIV-1 replication by epitope-specific CTLs still remains unknown (35, 36). On the other hand, several reports indicate that epitope-specific CTLs in HLA-B^*57^+ LTNPs have the ability to cross-recognize variant epitopes (4, 13, 46), suggesting the control of escape mutants by these CTLs. In the present study, we demonstrated the selection of escape mutations by HLA-B*5101-mediated immune pressure and showed that 2 kinds of mutations, escape mutations for slow progressors and a mutation reducing viral fitness and weakly affecting T-cell recognition for LTNPs, were selected in slow-progressing and LTNP hemophiliacs.

In the present study, we showed that HLA-B^*5101^+ hemo-

philiacs exhibited significantly slow progression during the years 1998 to 2007. Furthermore, we demonstrated that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs was associated with a Pol283-8-specific $CD8^+$ T-cell response. This is the first study finding that a Pol-specific CTL response is more effective in the control of HIV-1 than a Gag-specific CTL response. Our findings provide a novel mechanism for understanding the long-term control of HIV-1 in LTNPs and slow progressors.

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Introduction of TaqMan HIV-1 Assay Increased Unnecessary Drug Resistance Testing

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Dear Editor:

In early 2008, the Roche COBAS TaqMan HIV-1 assay (the TaqMan assay; Roche Diagnostics, Pleasanton, CA) replaced the Roche COBAS Amplicor HIV-1 Monitor version 1.5 (the Amplicor Monitor) for measuring HIV-1 viral load. Although the Amplicor Monitor and the TaqMan assay perform comparably over their respective overall dynamic ranges, there is an increasing concern about poor agreement in measuring low HIV-1 viral load. After the introduction of the TaqMan assay, detectable plasma HIV-1 RNA levels have been reported in a substantial number of infected individuals whose HIV-1 was previously suppressed below the detection limit of the Amplicor Monitor.[1,2] Because reemergence of previously undetectable HIV-1 load while on antiretroviral therapy is a sign of possible virologic failure, the need for therapeutic regimen modification seems necessary and drug resistance testing is reasonable.[3] Therefore, replacement of the Amplicor Monitor by the TaqMan assay might have resulted in unnecessary drug resistance testing. To determine the change in frequency of drug resistance testing in our center, we counted the number of such tests conducted before and after the introduction of the TaqMan assay.

Approximately 1400 HIV-1–infected patients regularly visit our outpatient clinic and approximately two thirds of them are on antiretroviral treatment. The TaqMan assay has been used in our clinic since early March 2008 instead of the Amplicor Monitor.[2] In our clinic, a regular genotypic drug resistance test is normally ordered, unless the HIV-1 load is less than 1000 copies per milliliter, in which case a sensitive genotypic resistance test (preceded by virion concentration with ultracentrifugation) is requested. The frequency of regular genotypic test was 15–52 tests per month between April 2007 and March 2009, and this frequency was similar before and after the introduction of the TaqMan assay (Fig. 1). In comparison, the sensitive genotypic test was requested 0–9 tests per month between April 2007 and April 2008. However, this frequency increased sharply to 37–86 tests per month from May through August 2008. Most of the patients with consistently undetectable HIV-1 load regularly visit the clinic every 2–3 months. It is most likely that their HIV-1 loads were measured by the TaqMan assay for the first time during their visits between March and May 2008. The next visit was probably between May and August 2008, after the results of the first TaqMan assay became available and HIV-1 load was unexpectedly detected in a substantial proportion of the patients. The detected HIV-1 levels should have been less than 1000 copies per milliliter in most cases to warrant requesting the sensitive genotypic resistance test, resulting in the unusually high number of such requests during that period, though the frequency of regular resistance test did not change. After the physicians were made aware that the detection of low-level HIV-1 was common by the TaqMan assay during successful treatment, the frequency of requests for the

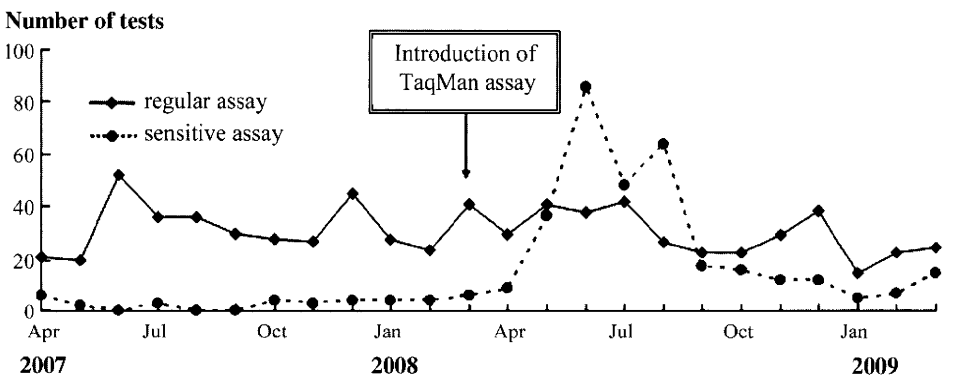

FIG. 1. The frequency of HIV-1 drug resistance tests. The monthly numbers of genotypic resistance tests conducted at the AIDS Clinical Center, International Medical Center of Japan, are shown. Usually, when the HIV-1 load is less than 1000 copies per milliliter, the sensitive resistance test is ordered. Otherwise, a regular resistance test is ordered.

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sensitive resistance test decreased since September 2008 (5–18 tests per month), although it was still higher compared to that before April 2008. Thus, the introduction of the TaqMan assay resulted in an increase in unnecessary resistance testing. In fact, no emergence of resistance mutations was identified by the sensitive resistance test between March and August 2008. Therefore, clinicians should be notified about which assay is being used to measure their patients' HIV-1 viral load, and they should be aware of the properties of the assays in order to lessen the number of unnecessary resistance tests.

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Autoimmune Diabetes in HIV-Infected Patients on Highly Active Antiretroviral Therapy

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Context: Various autoimmune diseases, especially autoimmune thyroid disease, are known to occur in HIV-infected patients on highly active antiretroviral therapy (HAART). However, no reports have described the development of autoimmune diabetes during HAART.

Objective: Our objective was to investigate the clinical course of the development of autoantibodies and diabetes during HAART.

Patients and Methods: Based on their high antiislet autoantibody titers and requirement for insulin therapy, we diagnosed three HIV-infected patients with autoimmune diabetes. To clarify the relationship between the development of an autoimmune reaction against pancreatic β-cells and recovery of $CD4^+$ T lymphocyte (CD4) counts, we retrospectively assayed stored samples of the patients' plasma for antiglutamic acid decarboxylase antibody (GAD-Ab).

Results: No GAD-Ab was detected in the plasma samples of any of the three patients prior to HAART, and their CD4 counts were below 20 cells/μl at their nadir. The GAD-Ab tests became positive from 6 to 38 months after the start of HAART, and their conversion to positive followed a dramatic increase in the patients' CD4 count. Two patients developed diabetes after testing positive for GAD-Ab. Although one patient had mild diabetes prior to testing positive for GAD-Ab, the rapid worsening of glycemic control and introduction of insulin therapy almost coincided with the detection of GAD-Ab. The high magnitude of the CD4 increase during HAART and the timing of the detection of autoantibody were similar to the magnitude and timing reported in HAART-associated autoimmune thyroid disease.

Conclusions: Autoimmune diabetes develops in some HIV-infected patients after immune restoration during HAART. (*J Clin Endocrinol Metab* **95: 4056–4060, 2010**)

Autoimmune diabetes is characterized by the presence of antiislet autoantibodies and is caused by autoimmune-mediated destruction of pancreatic β-cells (1). Although a high prevalence of diabetes has been reported in HIV-infected patients, most cases are considered attributable to insulin resistance induced by antiretroviral drugs (2, 3). The immunodeficiency of HIV-infected patients is characterized by a low $CD4^+$ T-lymphocyte (CD4) count, but highly active antiretroviral therapy (HAART) can reduce the HIV plasma viral load (pVL), and the CD4 count sometimes increases dramatically (immune restoration). As a result, some patients experience clinical deterioration

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Abbreviations: AITD, Autoimmune thyroid disease; CD4, $CD4^+$ T lymphocyte; GAD-Ab, glutamic acid decarboxylase antibody; HAART, highly active antiretroviral therapy; HbA_{1c}, glycosylated hemoglobin; HLA, human leukocyte antigen; IA2-Ab, insulinoma-associated antigen-2 antibody; pVL, plasma viral load; T1D, type 1 diabetes; T2D, type 2 diabetes.

due to restoration of an inflammatory immune response against both infectious and noninfectious antigens (4). Various autoimmune diseases have been reported after immune restoration (5, 6), especially autoimmune thyroid disease (AITD) (7–11), but none have described the development of autoimmune diabetes during HAART. We report the cases of three HIV-infected patients who developed autoimmune diabetes after immune restoration during HAART.

Ethics

This report was approved by the local ethics committee of the National Center for Global Health and Medicine. We obtained written informed consent from all three patients.

Case reports

The characteristics and laboratory findings of the three patients are indicated in Table 1. All of the three patients are currently on intensive insulin therapy, and recent doses of insulin are shown. The diagnosis of diabetes was made on the basis of repeated measurements of fasting and/or casual plasma glucose levels. To investigate the clinical course of immune restoration and development of autoimmunity, we retrospectively measured the titers of several autoantibodies sequentially by RIA of plasma samples that had been stored frozen.

Patient 1 was a 30-yr-old Japanese man who had been diagnosed with HIV infection and hepatitis C virus infection at 19 yr of age. Although HAART had been started then, the HAART regimen had often been suspended and switched to other regimens because of adverse effects, and his CD4 count and pVL had been uncontrolled for years. Although the patient was overweight and had a family history of type 2 diabetes (T2D), he had never been diagnosed with diabetes until he was 29 yr old. At 29 yr of age, HAART was resumed with a new regimen, which the pa-

TABLE 1. Characteristics and laboratory findings at the diagnosis of autoimmune diabetes

	Patient 1	Patient 2	Patient 3
Age (yr)	30	31	68
Sex	Male	Male	Female
Body mass index (kg/m^2)	24.2	20.0	19.1
Weight loss within 3–6 months (%)	20	11	27
Family history of diabetes	+	–	–
Regimen of the tolerated HAART	3TC TDF LPVr	3TC d4T LPVr	3TC, ETR RTV, DRV RAL
Duration of the tolerated HAART (months)[a]	18	10	55
Duration of HIV infection (yr)[a]	11	17	5
Recent dose of insulin injection (U/kg)	0.9	0.7	0.9
Data related to HIV infection			
pVL	Undetectable	Undetectable	Undetectable
CD4 count (cells/μl)	311	172	316
CD4 count at nadir (cells/μl)	12	14	19
Data related to diabetes			
Casual plasma glucose (mmol/liter)	26.6	9.2	8.1
HbA_{1c} (%)	10.8	10.9	12.2
HbA_{1c} increment within 3–6 months (%)	5.3	5.5	5.8
Fasting serum insulin (pmol/liter)	20.1	15.3	16.0
Fasting serum C-peptide (nmol/liter)	0.33	0.22	0.13
Urinary C-peptide (nmol per 24 h)	40.5	3.8	9.3
Urine ketone body	+	–	–
Autoantibody tests [positive test after the start of HAART regimen (months)][b]			
GAD-Ab (U/ml)	606 (6)	26000 (7)	1023 (38)
IA2-Ab (U/ml)	22.5 (9)	<0.4	5.9 (38)
TSHR-Ab (IU/liter)	12.6 (13)	<1.0	<1.0 (64)[c]
TPO-Ab (U/ml)	33.4 (6)	29.3 (4)	>60.0 (26)
Tg-Ab (U/ml)	1.6 (6)	<0.3	13.2 (38)
Antiadrenal cortex antibody (fold)	<10	<10	<10

3TC, Lamivudine; TDF, tenofovir; LPVr, lopinavir/ritonavir; d4T, sanilvudine; ETR, etravirine; RTV, ritonavir; DRV, darunavir; RAL, raltegravir; TSHR-Ab, anti-TSH receptor antibody; TPO-Ab, antithyroid peroxydase antibody; Tg-Ab, antithyroglobulin antibody.

[a] Interval before the diagnosis of autoimmune diabetes.

[b] Normal ranges include the following: GAD-Ab, less than 1.4 U/ml; IA2-Ab, less than 0.4 U/ml; TSHR-Ab, less than 1.0 IU/liter; TPO-Ab, less than 0.3 U/ml; Tg-Ab, less than 0.3 U/ml; antiadrenal cortex antibody, less than 10-fold.

[c] TSHR-Ab test became positive after the diagnosis of autoimmune diabetes.

tient tolerated, and his CD4 count gradually rose, but about 9 months later he was diagnosed with diabetes. At first, he was thought to have antiretroviral drug-induced diabetes, and his glycosylated hemoglobin (HbA_{1c}) levels (standardized by the Japan Diabetes Society) remained less than 5.5% in the absence of treatment with any antidiabetic agents. However, 18 months after resuming HAART, the patient's HbA_{1c} level began to increase, sometimes reaching as high as 10.8%. Insulin secretion gradually decreased, and the patient required intensive insulin therapy. He also had a high antiglutamic acid decarboxylase antibody (GAD-Ab) titer and a high insulinoma-associated antigen-2 antibody (IA2-Ab) titer (Table 1). A retrospective GAD-Ab test revealed that the patient had become GAD-Ab-positive in the period between the recovery of his CD4 count and the diagnosis of diabetes (Fig. 1A). Thus, the autoimmune response against β-cells actually began before the diagnosis of antiretroviral drug-induced diabetes, and we concluded that the patient's diabetes was caused by autoimmune mechanism. At 34 yr of age, the patient was admitted to our hospital because of ketoacidosis after omitting insulin on a sick day.

Patient 2 was a 31-yr-old Japanese man who had been diagnosed with HIV infection and hepatitis C virus infection at 13 yr of age. HAART was instituted then, but the HAART regimen had often been suspended and switched to other regimens because of the emergence of drug-resistant HIV and the occurrence of adverse effects, and his CD4 count and pVL had been uncontrolled for years. At 19 yr of age, the patient was diagnosed with diabetes, which was thought to be antiretroviral drug-induced diabetes or T2D. Good glycemic control had been maintained ($HbA_{1c} < 5.5\%$) for more than 10 yr with an α-glucosidase inhibitor. At 30 yr of age, HAART was resumed with a regimen that was tolerated, and his CD4 count gradually rose. Nine months later, however, his glycemic control rapidly deteriorated (HbA_{1c} 10.9%), and the GAD-Ab test became positive at that time (Table 1). The diabetes was not insulin dependent, but an iv glucagon challenge test demonstrated severely impaired insulin secretion. The patient required intensive insulin therapy, and the insulin dose was gradually increased. A retrospective GAD-Ab test revealed that the patient had become GAD-Ab positive in the period between the recovery of his CD4 count and the rapid deterioration of his glycemic control (Fig. 1B). We therefore concluded that his diabetes had worsened because of autoimmune destruction of β-cells, although he basically had antiretroviral drug-induced diabetes or T2D.

Patient 3 was a 68-yr-old Japanese woman who had been diagnosed with HIV infection at 63 yr of age, and HAART had been started then. She had tolerated the regimen from the beginning, and her CD4 count and pVL remained well controlled. About 36 months after the start

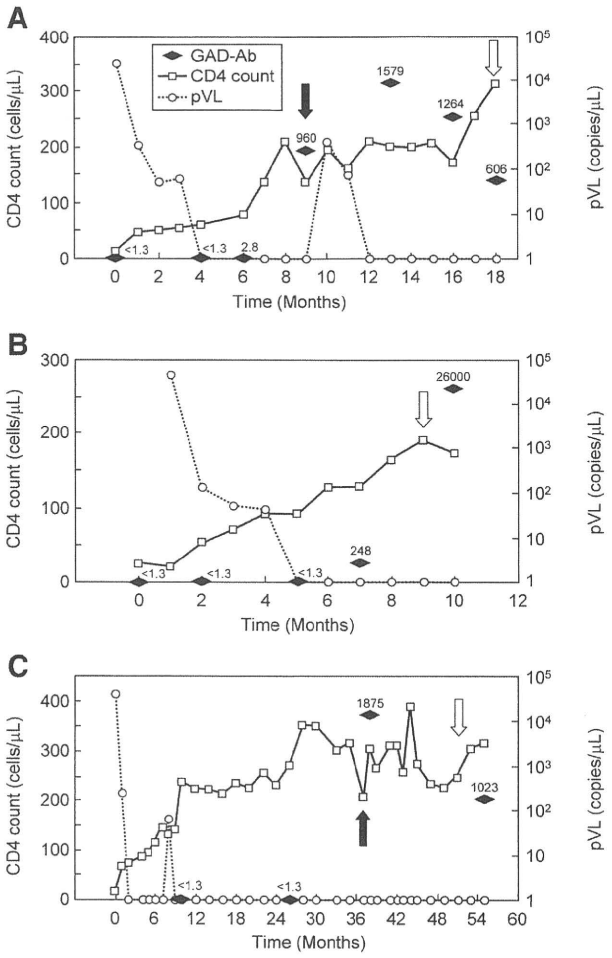

FIG. 1. Clinical courses of three patients before autoimmune diabetes diagnosis. Course of CD4 counts, pVL values, and GAD-Ab titers (units per milliliter) in patient 1 (A), patient 2 (B), and patient 3 (C) after the introduction of HAART. *Black arrows* indicate the time of the initial diagnosis of diabetes. *White arrows* indicate the beginning of progressive worsening of glycemic control. Because the initial diagnosis of diabetes in patient 2 was made several years before the introduction of HAART, the time of the diagnosis is not indicated.

of HAART, however, the patient was diagnosed with diabetes, which was thought to be antiretroviral drug-induced diabetes or T2D. She was treated with a sulfonylurea, and her glycemic control improved for approximately 5 months (representative HbA_{1c} value 6.1%). However, 55 months after the start of HAART, her HbA_{1c} level rapidly increased to as high as 12.2% and was accompanied by a marked decrease in insulin secretion, and she required intensive insulin therapy. Both GAD-Ab test and IA2-Ab test were positive (Table 1), and retrospective examination showed that GAD-Ab had appeared between the recovery of her CD4 count and the diagnosis of diabetes (Fig. 1C). Based on these findings, the diagnosis was changed to autoimmune diabetes.

Follow-up examination revealed a fasting plasma glucagon level in patients 1, 2, and 3 of 57, 82, and 61 pg/ml,