

Research Letter

AIDS 2012, 24:000–000

Once-daily darunavir/ritonavir plus abacavir/lamivudine versus tenofovir/emtricitabine for treatment-naïve patients with baseline viral load >100,000 copies/ml

Takeshi Nishijima^{a,b}, Hirokazu Komatsu^c, Katsuji Teruya^a, Junko Tanuma^a, Kunihisa Tsukada^a, Hiroyuki Gatanaga^{a,b}, Yoshimi Kikuchi^a and Shinichi Oka^{a,b}

The efficacy and safety of fixed-dose abacavir/lamivudine against tenofovir/emtricitabine, both with once-daily darunavir/ritonavir, was examined in 80 treatment-naïve patients with baseline HIV-1 viral load >100,000 copies/ml. The time to virologic failure by 48 weeks was not different between the two groups. The percentage of patients with viral suppression was not significantly different with per protocol population. Tenofovir/emtricitabine showed better tolerability; more patients on abacavir/lamivudine changed regimen than those on tenofovir/emtricitabine. A randomized trial to elucidate the efficacy and safety of these two regimens is warranted.

Little information is available on the efficacy and safety of antiretroviral therapy (ART) of ritonavir-boosted darunavir (DRV/r) plus fixed-dose abacavir/lamivudine (ABC/3TC) [1,2]. DRV/r is a protease inhibitor with proven efficacy and safety, and with high barrier to drug resistance [3,4]. ABC/3TC is an alternative choice of nucleoside reverse transcriptase inhibitors (NRTI) in the American Department of Health and Human Services Guidelines [5]. Here we conducted a single-center, observational pilot study to compare the efficacy and safety of DRV/r plus ABC/3TC versus TDF/FTC in patients with baseline HIV-1 viral load (VL) >100,000 copies/ml. Subjects with such VL were chosen because ACTG 5202 demonstrated that the time to virologic failure (VF) was significantly shorter with ABC/3TC than with TDF/FTC in patients with VL >100,000 copies/ml on efavirenz or ritonavir-boosted atazanavir [6]. All subjects were treatment-naïve who commenced once-daily DRV/r plus either fixed-dose ABC/3TC or TDF/FTC from November 2009 to August 2011 at the AIDS Clinical Center, Tokyo. Baseline data (basic demographics, CD4 count, and VL) were collected. VL was measured by Cobas TaqMan HIV-1 real-time PCR version 1.0 assay (Roche Diagnostics, NJ) to the end of November 2011, and later by Cobas TaqMan version 2.0 assay. It was the decision of the attending physician to start ART with either TDF/FTC or ABC/3TC, because the Japanese

guidelines consider both TDF/FTC and ABC/3TC the preferred NRTIs [7].

The efficacy outcomes were the time from commencing ART to VF (defined as VL >1,000 copies/ml at or after 16 weeks and before 24 weeks, or >200 copies/ml at or after 24 weeks) [6], and the proportion of patients with VL < 50 copies/ml at 48 weeks regardless of previous VF. The tolerability outcome was the time to any regimen modification. Intent-to-treat (ITT) population, comprising all subjects, was used for all efficacy and tolerability analyses, while per protocol population was used in the efficacy analysis of the suppressed VL. Censored cases represented those who dropped out, referred to other facilities, or reached 48 weeks. Time-to-event distributions were estimated using the Kaplan–Meier method. Uni- and multivariate Cox hazards models estimated the impact of ABC/3TC use over TDF/FTC on the incidence of VF.

The study included 80 patients [ABC/3TC: 21, TDF/FTC: 59, median age: 37.9 years, males: 74 (92.5%), East Asian origin: 72 (90%)], of whom 66 (82.5%) were infected with HIV-1 through homosexual contact. Patients on ABC/3TC had lower baseline CD4 count (46/μl versus 100, $P=0.031$), higher VL (5.75 log₁₀/ml versus 5.58, $P=0.044$), and more likely to have history of AIDS (71.4% versus 37.3, $P=0.010$), than patients with TDF/FTC. All subjects were HLA-B*5701-negative, and all underwent HIV-1 drug-resistance tests before commencement of ART and none had resistant mutations.

The time to VF with ABC/3TC [3 patients (14.3%)] was not significantly different from that with TDF/FTC [4 (6.8%)] by 48 weeks (Fig. 1a), by univariate and multivariate analyses adjusted by CD4 count and VL (HR, 2.651; 95% CI, 0.592–11.88; $P=0.203$, adjusted HR, 1.589; 95% CI, 0.341–7.401; $P=0.555$). At week 48, ITT analysis showed more patients with TDF/FTC had VL of <50 copies/ml (ABC/3TC: 38.1%, TDF/FTC: 64.4%, $P=0.043$) (Fig. 1c), whereas with per protocol analysis, no difference was noted (ABC/3TC: 57.1%, TDF/FTC: 73.1%, $P=0.328$) (Fig. 1d).

Among the seven patients with VF, three (ABC/3TC: 1, TDF/FTC: 2) achieved sustained VL suppression after week 60 of the initial regimen. The other four underwent drug-resistance tests. One on ABC/3TC was switched to TDF/FTC at week 41; however, viral suppression was not achieved until raltegravir was added at week 74. The

DOI:10.1097/QAD.0b013e32835cadb7

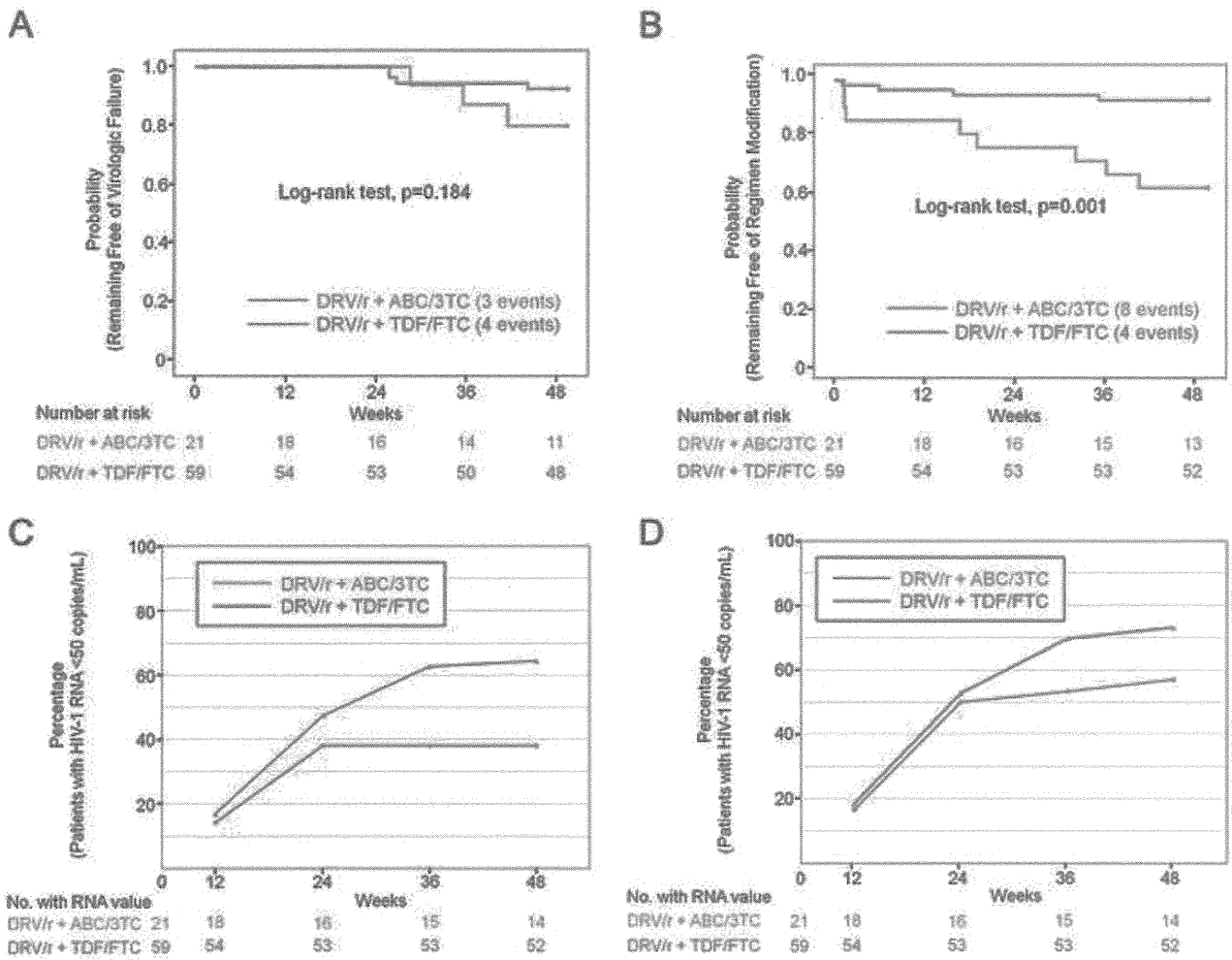


Fig. 1. Efficacy and tolerability results over 48 weeks. (a) Time to protocol-defined virologic failure. (b) Time to tolerability endpoint, defined as first change in treatment regimen. Percent of patients with HIV-1 RNA <50 copies/ml at week 12, 24, 36, and 48, regardless of previous virologic failure, with (c) intention-to-treat population, and with (d) per protocol population.

other with ABC/3TC was switched to TDF/FTC at week 49 and achieved viral suppression despite emergence of protease mutation M46I. Another patient on TDF/FTC had persistent viremia (100–200 copies/ml) without mutation. Another patient on TDF/FTC showed emergence of reverse transcriptase mutation V75L and viremia persisted with 200–500 copies/ml. Reverse transcriptase mutation M184I/T/V did not emerge in any patients.

More patients on ABC/3TC changed or discontinued the initial regimen during the research period [ABC/3TC: 8 (38.1%), TDF/FTC: 4 (6.8%), $P=0.001$] (Fig. 1b). Six [ABC/3TC: 4 (19%), TDF/FTC: 2 (3.4%)] changed ART due to adverse events or VF [ABC/3TC: VF ($n=1$), limb paresthesia ($n=1$), and nausea ($n=2$); TDF/FTC: tenofovir nephrotoxicity ($n=2$)]. None developed ABC-associated hypersensitivity.

This is the first comparison report of the efficacy and safety of ABC/3TC against TDF/FTC with DRV/r in treatment-naïve patients with VL >100,000 copies/ml. The time to VF by 48 weeks was not different between the two groups. Although a higher percentage of patients on TDF/FTC showed viral suppression than those on ABC/3TC at week 48 with ITT population, the difference was not significant with per protocol population. TDF/FTC showed better tolerability, as more patients on ABC/3TC changed regimen than those on TDF/FTC.

These results need to be interpreted with caution, because the baseline characteristics of patients of the two groups were not well-matched due to the nature of the observational study, and this study did not have sufficient power due to the small number of enrolled patients. Because our patients had small stature with median body weight of 58.1 kg, a risk factor for TDF nephrotoxicity, it

is sometimes our practice to avoid TDF in patients with multiple risks, such as advanced HIV-1 infection, to prevent possible acute kidney injury [8–10]. This is presumably the reason for prescribing ABC/3TC to patients with worse disease condition in this study. This allocation bias might have worked as a disadvantage for the efficacy and tolerability results of ABC/3TC.

The usefulness of ABC/3TC has recently received higher recognition than it did in the past; the FDA meta-analysis did not confirm the association between ABC use and myocardial infarction [11], and it became clear that TDF use is associated with decreased bone mineral density and renal dysfunction, both of which might develop into serious complications with long-term TDF use [12–17]. Thus, once-daily DRV/r, a protease inhibitor with high barrier to drug resistance, plus ABC/3TC could be good alternative, especially in patients who cannot tolerate TDF. A randomized trial to elucidate the efficacy and safety of ABC/3TC and TDF/FTC with once-daily DRV/r is warranted.

Acknowledgements

The authors thank the patients and all the clinical staff at the AIDS Clinical Center.

Author contributions: All authors contributed to the concept and design of the study and/or the analyses and interpretation of the data. The manuscript was drafted by TN, HK, HG, and SO and critically reviewed and subsequently approved by all authors.

Conflicts of interest

Conflict of Interest and Source of Funding: SO received research grants from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Pfizer, Co., and Roche Diagnostics K.K. The other authors declare no conflict of interest.

This work was supported by Grants-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001), and the Global Center of Excellence Program (Global Education and Research Center Aiming at the Control of AIDS) from the Japanese Ministry of Education, Science, Sports and Culture.

^aAIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan; ^bCenter for AIDS Research, Kumamoto University, Kumamoto, Japan; and ^cDepartment of Community Care, Saku Central Hospital, Nagano, Japan.

Correspondence to Hiroyuki Gatanaga, MD, PhD, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku,

Tokyo 162-0052, Japan. Tel: +81 3 3202 7181; fax: +81 3 5273 6483; e-mail: hingatana@acc.ncgm.go.jp

Received: 23 July 2012; revised: 31 October 2012; accepted: 15 November 2012.

References

1. Trottier B, Machouf N, Thomas R, Longpré D, Vézina S, Boissonnault M, et al. (2011). **Effective and safe use of abacavir/lamivudine fixed-dose combination with ritonavir-boosted Darunavir, a novel regimen for HIV therapy** [abstract CDB333]. In: 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention; 2011. Rome, Italy.
2. Nishijima T, Tsukada K, Teruya K, Gatanaga H, Kikuchi Y, Oka S. **Efficacy and safety of once-daily ritonavir-boosted darunavir and abacavir/lamivudine for treatment-naïve patients: a pilot study.** *AIDS* 2012; **26**:649–651.
3. Clotet B, Bellos N, Molina JM, Cooper D, Goffard JC, Lazzarin A, et al. **Efficacy and safety of darunavir-ritonavir at week 48 in treatment-experienced patients with HIV-1 infection in POWER 1 and 2: a pooled subgroup analysis of data from two randomised trials.** *Lancet* 2007; **369**:1169–1178.
4. Madruga JV, Berger D, McMurchie M, Suter F, Banhegyi D, Ruxrungtham K, et al. **Efficacy and safety of darunavir-ritonavir compared with that of lopinavir-ritonavir at 48 weeks in treatment-experienced, HIV-infected patients in TITAN: a randomised controlled phase III trial.** *Lancet* 2007; **370**:49–58.
5. **Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents.** Department of Health and Human Services. 1–239. Available at <http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>. Accessed 4 July 2012.
6. Sax PE, Tierney C, Collier AC, Fischl MA, Mollan K, Peeples L, et al. **Abacavir-lamivudine versus tenofovir-emtricitabine for initial HIV-1 therapy.** *N Engl J Med* 2009; **361**:2230–2240.
7. **The Guidelines for the Treatment of HIV Infection, March 2012 version.** The Japanese Ministry of Health, Labour and Welfare. 1–154. Available at <http://www.haart-support.jp/pdf/guideline2012.pdf>. Accessed 4 July 2012.
8. Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B, Clotet B, et al. **The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years.** *AIDS* 2007; **21**:1273–1281.
9. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K, Kinai E, et al. **Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients.** *PLoS One* 2011; **6**:e22661.
10. Rodriguez-Novoa S, Alvarez E, Labarga P, Soriano V. **Renal toxicity associated with tenofovir use.** *Expert Opin Drug Saf* 2010; **9**:545–559.
11. Ding X, Andraca-Carrera E, Cooper C, Miele P, Kornegay C, Soukup M, et al. (2011). **No association of myocardial infarction with ABC use: an FDA meta-analysis.** [abstract O-1004]. In: 18th Conference on Retroviruses and Opportunistic Infections; 2011. Boston, USA.
12. Peyriere H, Reynes J, Rouanet I, Daniel N, de Boever CM, Mauboussin JM, et al. **Renal tubular dysfunction associated with tenofovir therapy: report of 7 cases.** *J Acquir Immune Defic Syndr* 2004; **35**:269–273.
13. McComsey GA, Kitch D, Daar ES, Tierney C, Jahed NC, Tebas P, et al. **Bone mineral density and fractures in antiretroviral-naïve persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202.** *J Infect Dis* 2011; **203**:1791–1801.
14. Fux CA, Rauch A, Simcock M, Bucher HC, Hirschel B, Opravil M, et al. **Tenofovir use is associated with an increase in serum alkaline phosphatase in the Swiss HIV Cohort Study.** *Antivir Ther* 2008; **13**:1077–1082.

15. Gallant JE, Winston JA, DeJesus E, Pozniak AL, Chen SS, Cheng AK, *et al.* **The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue-containing regimen in antiretroviral-naïve patients.** *AIDS* 2008; **22**:2155–2163.
16. Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T, Aoki T, *et al.* **Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naïve patients with HIV infection.** *PLoS One* 2012; **7**:e29977.
17. Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S, Tonelli M. **Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients.** *Clin Infect Dis* 2010; **51**:496–505.

Long-Term Control of HIV-1 in Hemophiliacs Carrying Slow-Progressing Allele HLA-B*5101^{∇†}

Yuka Kawashima,^{1‡} Nozomi Kuse,^{1‡} Hiroyuki Gatanaga,^{2,3‡} Takuya Naruto,¹ Mamoru Fujiwara,¹ Sachi Dohki,¹ Tomohiro Akahoshi,¹ Katsumi Maenaka,⁴ Philip Goulder,⁵ Shinichi Oka,^{2,3} and Masafumi Takiguchi^{1*}

Divisions of Viral Immunology¹ and Infectious Disease,² Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655,³ and Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Fukuoka,⁴ Japan, and Department of Paediatrics, Peter Medawar Building for Pathogen Research, Oxford University, Oxford OX1 3SY, United Kingdom⁵

Received 25 January 2010/Accepted 12 April 2010

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

* Corresponding author. Mailing address: Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Phone: 81-96-373-6529. Fax: 81-96-373-6532. E-mail: masafumi@kumamoto-u.ac.jp.

† Supplemental material for this article may be found at <http://jvi.asm.org/>.

‡ Y.K., N.K., and H.G. contributed equally to this work.

∇ Published ahead of print on 21 April 2010.

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 -4I5I) 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 μ l of cloning mixture (about 1×10^5 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-B*5101 cells prepulsed with the corresponding peptide at 1 μ M 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 ⁵¹Cr release assay as described previously (42). The infected cells were incubated with 150 μ Ci Na₂⁵¹CrO₄ 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 TTGCCCTGCTTCTGTAT-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'-CCTGATCTTACTGTGCC-3' for the first round of PCR and 5'-AAAGGTCTACTGGCATGGG-3' or 5'-TTGGAGAGCAATG GCTAGTG-3' and 5'-AGTCTACTGTCCATGCATGGC-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 μ M) 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 μ M), 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 μ g/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

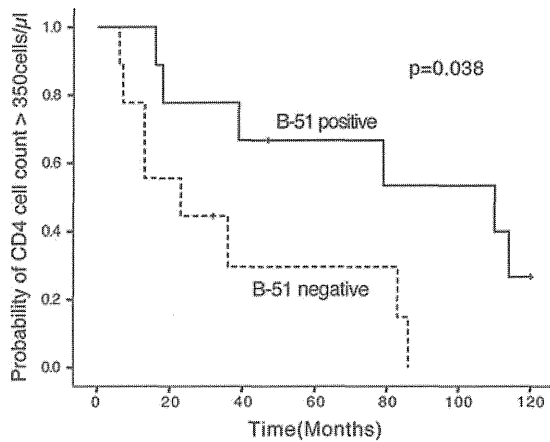


FIG. 1. Association of HLA-B*5101 with slow progression to AIDS. Kaplan-Meier survival analysis was used to estimate the time to the first CD4 cell count (24-week time-weighted average levels of CD4 cells) of $<350/\mu\text{l}^3$ for 9 HLA-B*5101-positive (solid line) and 9 HLA-B*5101-negative (dashed line) hemophiliacs who had not been treated with antiretroviral therapy (ART) and who had a CD4 count of $>350/\mu\text{l}$ 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 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 LTNPs, 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 ($P, 5.6 \times 10^{-8}$), 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.

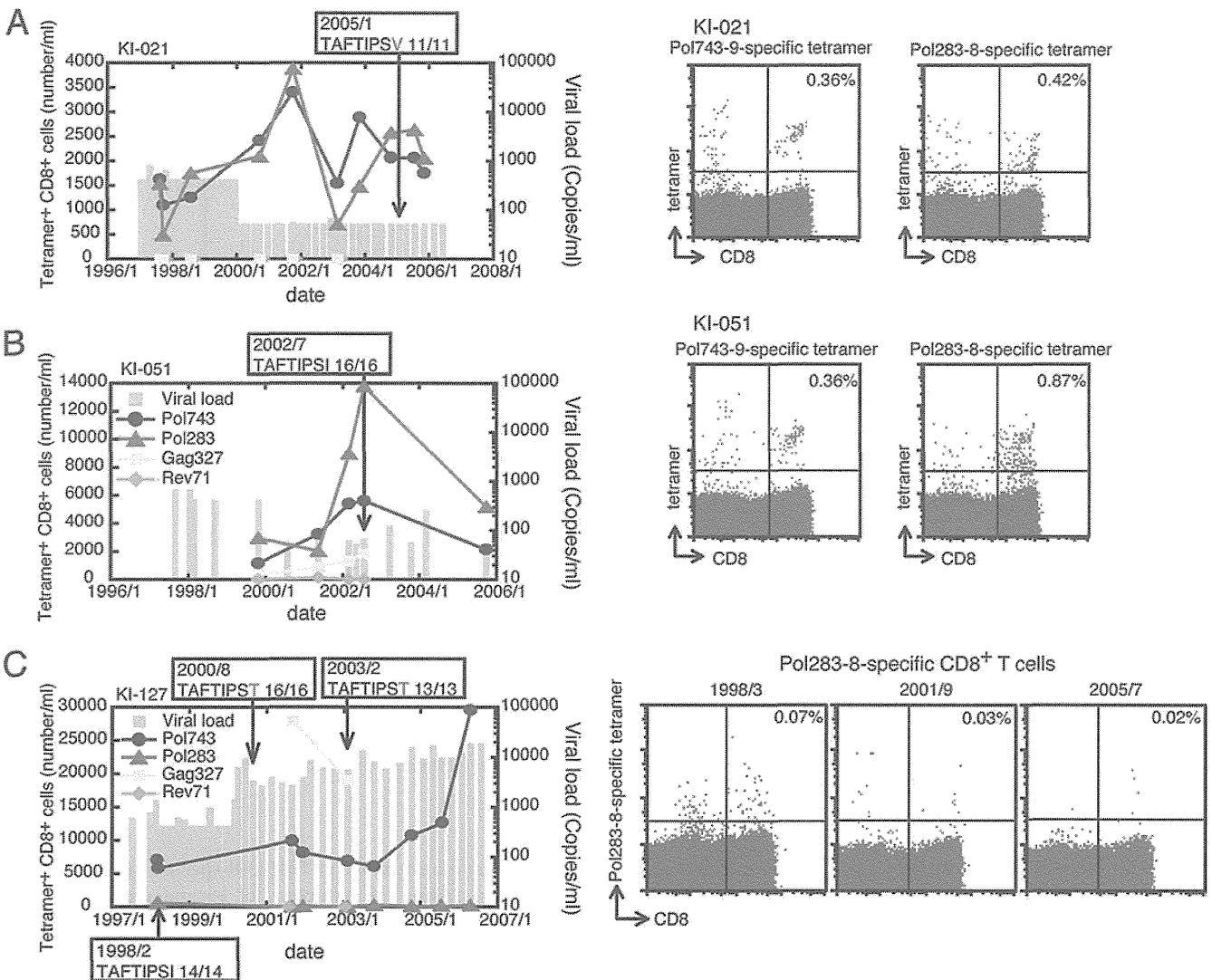


FIG. 2. Longitudinal analysis of HLA-B*5101-restricted CD8⁺ T cells and Pol283 epitope sequences in 3 slow-progressing hemophiliacs. Four types of HIV-1-specific CD8⁺ T cells were detected by use of specific tetramers. PBMCs from KI-021 (A), KI-051 (B), and KI-127 (C) were analyzed by using Pol743-9-specific 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-

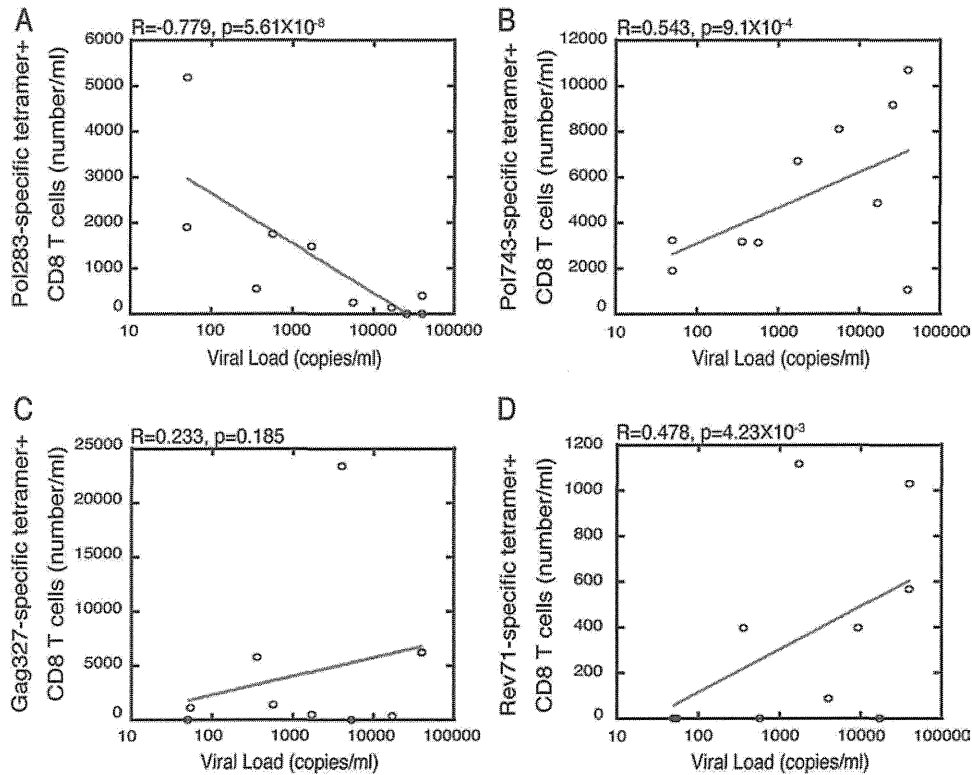


FIG. 3. Correlation of the number of HLA-B*5101-restricted CD8⁺ T cells with the viral load. The number of Pol283-8-specific (A), Pol743-9-specific (B), Gag327-specific (C), or Rev71-specific (D) CD8⁺ T cells among PBMCs from 10 HLA-B*5101⁺ hemophiliacs was measured at 1 time point or at 2 to 10 different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

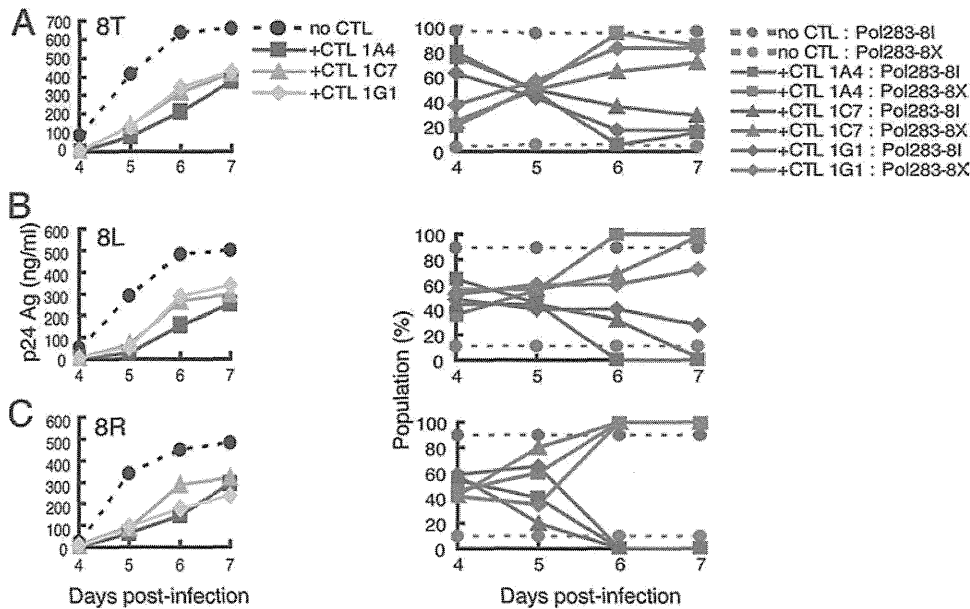


FIG. 4. *In vitro* selection of Pol283 escape mutants by a Pol283-8-specific CTL clone. T1 cells were infected with paired viruses (NL-432 [Pol283-8I] and a mutant virus [Pol283-8L, -8T, or -8R]) at a ratio of 9:1. The infected cells were incubated with Pol283-8-specific CTL clones at an E:T ratio of 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 hemophiliac 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

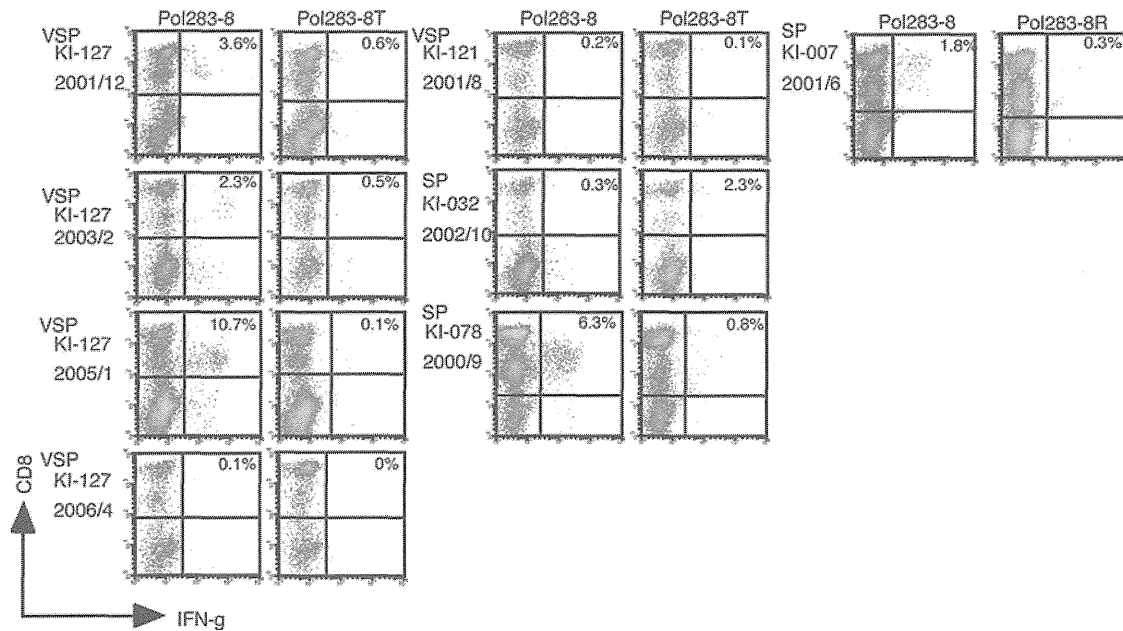


FIG. 5. Induction of Pol283-8-specific CD8⁺ T cells from PBMCs of 2 very slow progressors and 3 slow progressors. PBMCs from 2 very slow progressors (KI-127 and KI-121) and from 3 slow progressors (KI-032, KI-007, and KI-078) were stimulated with the Pol283-8 epitope peptide or the Pol283-8T or -8R peptide 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

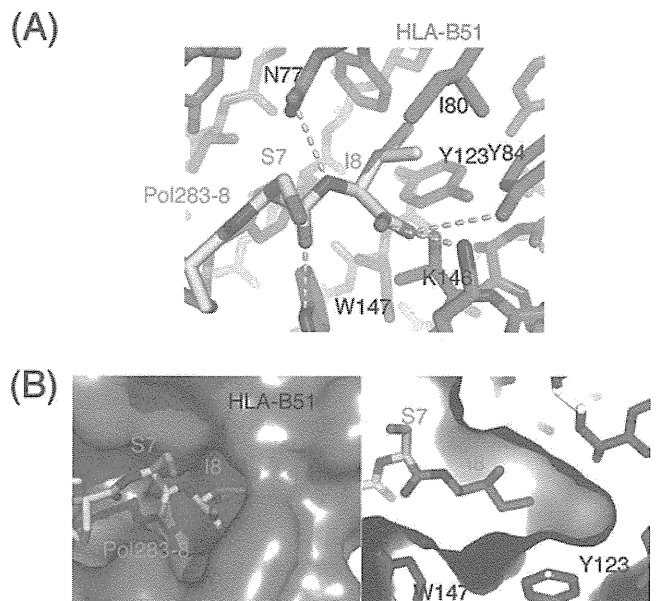


FIG. 6. Binding model of HLA-B*5101 mutant peptides. (A) Polar interactions around the PC residue in the HLA-B51–Pol283-8 complex. The Pol283-8 peptide and the HLA-B51 heavy chain are shown as yellow and cyan stick models, respectively (N and O atoms are shown as blue and red, respectively). The dotted 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.

ACKNOWLEDGMENTS

We thank Manami Satoh for technical assistance and Sachiko Sakai for secretarial assistance.

This research was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases and by the Global COE program "Global Education and Research Center Aiming at the Control of AIDS," launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture of Japan; by a grant-in-aid for scientific research from the Ministry of Health of Japan; by a grant-in-aid (1839014120390134) for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan; and by a grant from the Japan Health Science Foundation.

REFERENCES

- Akari, H., S. Arold, T. Fukumori, T. Okazaki, K. Strelbel, and A. Adachi. 2000. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. *J. Virol.* **74**:2907-2912.
- Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**:94-96.
- Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones. 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med.* **192**:63-75.
- Bailey, J. R., T. M. Williams, R. F. Siliciano, and J. N. Blankson. 2006. Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations. *J. Exp. Med.* **203**:1357-1369.
- Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* **68**:6103-6110.
- Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Pfeffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* **3**:205-211.
- Buchbinder, S. P., M. H. Katz, N. A. Hessel, P. M. O'Malley, and S. D. Holmberg. 1994. Long-term HIV-1 infection without immunologic progression. *AIDS* **8**:1123-1128.
- Collins, K. L., B. K. Chen, S. A. Kalams, B. D. Walker, and D. Baltimore. 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**:397-401.
- Crawford, H., W. Lumm, A. Leslie, M. Schaefer, D. Boeras, J. G. Prado, J. Tang, P. Farmer, T. Ndung'u, S. Lakhi, J. Gilmour, P. Goepfert, B. D. Walker, R. Kaslow, J. Mulenga, S. Allen, P. J. R. Goulder, and E. Hunter. 2009. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *J. Exp. Med.* **206**:909-921.
- Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270**:988-991.
- Feeney, M. E., K. A. Roosevelt, Y. Tang, K. J. Pfafferoth, K. McIntosh, S. K. Burchett, C. Mao, B. D. Walker, and P. J. R. Goulder. 2003. Comprehensive screening reveals strong and broadly directed human immunodeficiency virus type 1-specific CD8 responses in perinatally infected children. *J. Virol.* **77**:7492-7501.
- Frahm, N., S. Adams, P. Kiepiela, C. H. Linde, H. S. Hewitt, M. Lichtenfeld, K. Sango, N. V. Brown, E. Pae, A. G. Wurcel, M. Altfeld, M. E. Feeney, T. M. Allen, T. Roach, M. A. St. John, E. S. Daar, E. Rosenberg, B. Korber, F. Marincola, B. D. Walker, P. J. R. Goulder, and C. Brander. 2005. HLA-B63 presents HLA-B57/B58-restricted cytotoxic T-lymphocyte epitopes and is associated with low human immunodeficiency virus load. *J. Virol.* **79**:10218-10225.
- Gillespie, G. M., R. Kaul, T. Dong, H. B. Yang, T. Rostron, J. J. Bwayo, P. Kiama, T. Peto, F. A. Plummer, A. J. McMichael, and S. L. Rowland-Jones. 2002. Cross-reactive cytotoxic T lymphocytes against a HIV-1 p24 epitope in slow progressors with B*57. *AIDS* **16**:961-972.
- Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* **3**:212-217.
- Hayashi, H., P. D. Ennis, H. Ariga, R. D. Salter, P. Parham, K. Kano, and M. Takiguchi. 1989. HLA-B51 and HLA-Bw52 differ only by two amino acids which are in the helical region of α 1 domain. *J. Immunol.* **142**:306-311.
- Honeyborne, I., A. Prendergast, F. Pereyra, A. Leslie, H. Crawford, R. Payne, S. Reddy, K. Bishop, E. Moodley, K. Nair, M. van der Stok, N. McCarthy, C. M. Rousseau, M. Addo, J. I. Mullins, C. Brander, P. Kiepiela, B. D. Walker, and P. J. R. Goulder. 2007. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple gag-specific CD8+ T-cell epitopes. *J. Virol.* **81**:3667-3672.
- Huang, Y., L. Zhang, and D. D. Ho. 1995. Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. *J. Virol.* **69**:93-100.
- Itoh, Y., N. Mizuki, T. Shimada, F. Azuma, M. Itakura, K. Kashiwase, E. Kikkawa, J. K. Kulski, M. Satake, and H. Inoko. 2005. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics* **57**:717-729.
- Kaslow, R. A., M. Carrington, R. Apple, L. Park, A. Muñoz, A. J. Saah, J. J. Goedert, C. Winkler, S. J. O'Brien, C. Rinaldo, R. Detels, W. Blattner, J. Phair, H. Erlich, and D. L. Mann. 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* **2**:405-411.
- Kawashima, Y., K. Pfafferoth, J. Frater, P. Matthews, R. Payne, M. Addo, H. Gatanaga, M. Fujiwara, A. Hachiya, H. Koizumi, N. Kuse, S. Oka, A. Duda, A. Prendergast, H. Crawford, A. Leslie, Z. Brumme, C. Brumme, T. Allen, C. Brander, R. Kaslow, J. Tang, E. Hunter, S. Allen, J. Mulenga, S. Branch, T. Roach, M. John, S. Mallal, A. Ogwu, R. Shapiro, J. G. Prado, S. Fidler, J. Weber, O. G. Pybus, P. Klenerman, T. Ndung'u, R. Phillips, D. Heckerman, P. R. Harrigan, B. D. Walker, M. Takiguchi, and P. Goulder. 2009. Adaptation of HIV-1 to human leukocyte antigen class I. *Nature* **458**:641-645.
- Keet, I. P., A. Krol, M. R. Klein, P. Veugelers, J. de Wit, M. Roos, M. Koot, J. Goudsmit, F. Miedema, and R. A. Coutinho. 1994. Characteristics of long-term asymptomatic infection with human immunodeficiency virus type 1 in men with normal and low CD4+ cell counts. *J. Infect. Dis.* **169**:1236-1243.
- Kiepiela, P., A. J. Leslie, I. Honeyborne, D. Ramduth, C. Thobakgale, S. Chetty, P. Rathnavalu, C. Moore, K. J. Pfafferoth, L. Hilton, P. Zimbwa, S. Moore, T. Allen, C. Brander, M. M. Addo, M. Altfeld, I. James, S. Mallal, M. Bunce, L. D. Barber, J. Szinger, C. Day, P. Klenerman, J. Mullins, B. Korber, H. M. Coovadia, B. D. Walker, and P. J. Goulder. 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* **432**:769-775.
- Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder. 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13**:46-53.
- Kirchhoff, F., T. C. Greenough, D. B. Bretler, J. L. Sullivan, and R. C. Desrosiers. 1995. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N. Engl. J. Med.* **332**:228-232.
- Klein, M. R., C. A. van Baalen, A. M. Holwerda, S. R. K. Garde, R. J. Bende, I. P. Keet, J. K. Eeftink-Schattenkerk, A. D. Osterhaus, H. Schuitemaker, and F. Miedema. 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181**:1365-1372.
- Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**:4650-4655.
- Kwong, P. D., M. L. Doyle, D. J. Casper, C. Cicala, S. A. Leavitt, S. Majeed, T. D. Steenbeke, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P. W. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt, J. Sodroski, W. A. Hendrickson, and J. Arthos. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**:678-682.
- Leslie, A., D. Kavanagh, I. Honeyborne, K. Pfafferoth, C. Edwards, T. Pillay, L. Hilton, C. Thobakgale, D. Ramduth, R. Draenert, S. Le Gall, G. Luzzi, A. Edwards, C. Brander, A. K. Sewell, S. Moore, J. Mullins, C. Moore, S. Mallal, N. Bhardwaj, K. Yusim, R. Phillips, P. Klenerman, B. Korber, P.

- Kiepiela, B. Walker, and P. Goulder. 2005. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. *J. Exp. Med.* **201**:891–902.
29. Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder. 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nat. Med.* **10**:282–289.
 30. Maenaka, K., T. Maenaka, H. Tomiyama, M. Takiguchi, D. I. Stuart, and E. Y. Jones. 2000. Nonstandard peptide binding revealed by crystal structures of HLA-B*5101 complexed with HIV immunodominant epitopes. *J. Immunol.* **165**:3260–3267.
 31. Magierowska, M., I. Theodorou, P. Debré, F. Sanson, B. Autran, Y. Riviere, D. Charron, and D. Costagliola. 1999. Combined genotypes of CCR5, CCR2, SDF1, and HLA genes can predict the long-term nonprogressor status in human immunodeficiency virus-1-infected individuals. *Blood* **93**:936–941.
 32. Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela, and P. Goulder. 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J. Virol.* **80**:3617–3623.
 33. Matsumoto, K., J. Yamamoto, M. Hiraiwa, K. Kano, and M. Takiguchi. 1990. Discrimination of HLA-B5 cross reactive group antigen by human allospecific CTL clones. *Transplantation* **49**:1164–1167.
 34. Matthews, P. C., A. Prendergast, A. Leslie, H. Crawford, R. Payne, C. Rousseau, M. Rolland, I. Honeyborne, J. Carlson, C. Kadie, C. Brander, K. Bishop, N. Mlotshwa, J. I. Mullins, H. Coovadia, T. Ndung'u, B. D. Walker, D. Heckerman, and P. J. R. Goulder. 2008. Central role of reverting mutations in HLA associations with human immunodeficiency virus set point. *J. Virol.* **82**:8548–8559.
 35. Migueles, S. A., A. C. Laborico, H. Imamichi, W. L. Shupert, C. Royce, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, C. W. Hallahan, and M. Connors. 2003. The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. *J. Virol.* **77**:6889–6898.
 36. Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors. 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc. Natl. Acad. Sci. U. S. A.* **97**:2709–2714.
 37. O'Brien, S. J., X. Gao, and M. Carrington. 2001. HLA and AIDS: a cautionary tale. *Trends Mol. Med.* **7**:379–381.
 38. Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, L. K. Schrager, J. B. Margolick, S. Buchbinder, J. V. Giorgi, and A. S. Fauci. 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N. Engl. J. Med.* **332**:209–216.
 39. Sacha, J. B., C. Chung, J. Reed, A. K. Jonas, A. T. Bean, S. P. Spencer, W. Lee, L. Vojnov, R. Rudersdorf, T. C. Friedrich, N. A. Wilson, J. D. Lifson, and D. I. Watkins. 2007. Pol-specific CD8+ T cells recognize simian immunodeficiency virus-infected cells prior to Nef-mediated major histocompatibility complex class I downregulation. *J. Virol.* **81**:11703–11712.
 40. Schneidewind, A., M. A. Brockman, J. Sidney, Y. E. Wang, H. Chen, T. J. Suscovich, B. Li, R. I. Adam, R. L. Allgaier, B. R. Mothé, T. Kuntzen, C. Oniangue-Ndza, A. Trocha, X. G. Yu, C. Brander, A. Sette, B. D. Walker, and T. M. Allen. 2008. Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid. *J. Virol.* **82**:5594–5605.
 41. Schneidewind, A., M. A. Brockman, R. Yang, R. I. Adam, B. Li, S. L. Gall, C. R. Rinaldo, S. L. Craggs, R. L. Allgaier, K. A. Power, T. Kuntzen, C. S. Tung, M. X. LaBute, S. M. Mueller, T. Harrer, A. J. McMichael, P. J. R. Goulder, C. Aiken, C. Brander, A. D. Kelleher, and T. M. Allen. 2007. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J. Virol.* **81**:12382–12393.
 42. Tomiyama, H., H. Akari, A. Adachi, and M. Takiguchi. 2002. Different effects of Nef-mediated HLA class I down-regulation on HIV-1-specific CD8+ T cell cytokine activity and cytokine production. *J. Virol.* **76**:7535–7543.
 43. Tomiyama, H., M. Fujiwara, S. Oka, and M. Takiguchi. 2005. Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication. *J. Immunol.* **174**:36–40.
 44. Tomiyama, H., N. Yamada, H. Komatsu, K. Hirayama, and M. Takiguchi. 2000. A single CTL clone can recognize a naturally processed HIV-1 epitope presented by two different HLA class I molecules. *Eur. J. Immunol.* **30**:2521–2530.
 45. Tomiyama, H., T. Sakaguchi, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, and M. Takiguchi. 1999. Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules. *Hum. Immunol.* **60**:177–186.
 46. Turnbull, E. L., A. R. Lopes, N. A. Jones, D. Cornforth, P. Newton, D. Aldam, P. Pellegrino, J. Turner, I. Williams, C. M. Wilson, P. A. Goepfert, M. K. Maini, and P. Borrow. 2006. HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently. *J. Immunol.* **176**:6130–6146.
 47. Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. *Nature* **422**:307–312.
 48. Wyatt, R., and J. Sodroski. 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**:1884–1888.
 49. Zuñiga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez, and C. Brander. 2006. Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. *J. Virol.* **80**:3122–3125.

Selection and Accumulation of an HIV-1 Escape Mutant by Three Types of HIV-1-Specific Cytotoxic T Lymphocytes Recognizing Wild-Type and/or Escape Mutant Epitopes

Tomohiro Akahoshi,^a Takayuki Chikata,^a Yoshiko Tamura,^a Hiroyuki Gatanaga,^{a,b} Shinichi Oka,^{a,b} and Masafumi Takiguchi^a

Center for AIDS Research, Kumamoto University, Kumamoto, Japan,^a and AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan^b

It is known that cytotoxic T lymphocytes (CTLs) recognizing HIV-1 escape mutants are elicited in HIV-1-infected individuals, but their role in the control of HIV-1 replication remains unclear. We investigated the antiviral ability of CTLs recognizing the HLA-A*24:02-restricted Gag28-36 (KYKCLKHIVW) epitope and/or its escape mutant (KYRLKHIVW) elicited in the early and chronic phases of the infection. Wild-type (WT)-epitope-specific CTLs, as well as cross-reactive CTLs recognizing both WT and K30R (3R) epitopes, which were predominantly elicited at early and/or chronic phases in HLA-A*24:02⁺ individuals infected with the WT virus, suppressed the replication of the WT virus but failed to suppress that of the 3R virus, indicating that the 3R virus was selected by these 2 types of CTLs. On the other hand, cross-reactive and 3R-specific CTLs, which were elicited in those infected with the 3R virus, did not suppress the replication of either WT or 3R virus, indicating that these CTLs did not contribute to the control of 3R virus replication. High accumulation of the 3R mutation was found in a Japanese population recently recruited. The selection and accumulation of this 3R mutation resulted from the antiviral ability of these Gag28-specific CTLs and high prevalence of HLA-A*24:02 in a Japanese population. The present study highlighted the mechanisms for the roles of cross-reactive and mutant-epitope-specific CTLs, as well as high accumulation of escape mutants, in an HIV-1-infected population.

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) play an important role in the control of HIV-1 during the acute and chronic phases of an HIV-1 infection (22, 40). However, HIV-1-specific CTLs cannot completely eliminate HIV-1-infected cells, because HIV-1 escapes from CTL-mediated immune pressure by various mechanisms, such as selection of escape mutations, Nef-mediated HLA class I downregulation, and skewed maturation of memory HIV-specific CD8⁺ T lymphocytes (5, 8, 9). The most documented escape mechanism is acquisition of amino acid mutations within the CTL epitope and/or its flanking regions. These mutations lead to reduced ability of peptide to bind to HLA class I molecules, impaired T cell receptor (TCR) recognition, and defective epitope generation (21, 31). These escape mechanisms are involved in impaired activities of HIV-1-specific CTLs to kill target cells infected with escape mutant virus and to suppress HIV-1 replication, contributing to the selection of escape mutant viruses (5, 10, 13, 20, 29, 35, 41).

There is growing evidence that escape mutations selected by HLA class I-restricted CTLs accumulate at the population level (7, 28, 36). The accumulation of escape mutants may affect the clinical outcomes for HIV-1-infected individuals (11, 37, 38). On the other hand, it is known that CTLs recognizing escape mutants are elicited after the emergence of the escape mutant selected by wild-type (WT) epitope-specific CTLs (2, 4, 12, 15, 33, 39). The escape mutant-specific CTLs were also elicited in new hosts carrying the same restricted HLA allele when they were infected with the mutant (15). Several studies showed that CTLs cross-recognizing the WT and its escape mutant epitopes are elicited before or after the emergence of the escape mutant in the same hosts (18, 25, 26, 33, 34). However, the antiviral abilities of these cross-reactive CTLs remain unknown, since the recognition of cross-reactive CTLs for synthesized epitope peptides

was characterized by using the enzyme-linked immunosorbent spot assay (ELISPOT) or ⁵¹Cr cytotoxic assay in those studies. We previously showed that HLA-A*24:02-restricted Nef 138-specific CTLs recognizing an escape mutant had weaker ability to suppress the replication of the mutant virus than that of the WT virus (15). However, it still remains unclear whether cross-reactive or escape mutant-specific CTLs contribute to the control of HIV-1, since the CTLs have not been analyzed in detail.

To clarify the abilities of cross-reactive and escape mutant-specific CTLs to recognize HIV-1-infected cells, we analyzed CTLs specific for HLA-A*24:02-restricted HIV-1 Gag28-36 (KYKCLKHIVW; Gag28), which is the only immunodominant Gag epitope presented by this HLA class I allele (24). Since HLA-A*24:02 is found in approximately 70% of the Japanese population (42), the mutants of HLA-A*24:02-restricted epitopes may accumulate in HIV-1-infected Japanese individuals. We previously suggested that K30R (3R) in the Gag28 epitope is an escape mutation from HLA-A*24:02-restricted Gag28-specific CTLs (30) and that CTLs recognizing 3R are elicited in HIV-1-infected HLA-A*24:02⁺ individuals (46). From these studies, we hypothesized that cross-reactive CTLs recognizing WT and 3R mutant epitopes and/or 3R-specific CTLs are elicited in HLA-A*24:02⁺ HIV-1-infected individuals after the 3R mutant is selected and in new 3R virus-infected hosts carrying HLA-A*24:02. Here, we investigated the elicitation of Gag28-specific CTLs in 12 HLA-A*24:02⁺ HIV-1-

Received 3 October 2011 Accepted 23 November 2011

Published ahead of print 7 December 2011

Address correspondence to Masafumi Takiguchi, masafumi@kumamoto-u.ac.jp.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.06470-11

infected Japanese individuals who could be monitored from the early phase to the chronic phase of an HIV-1 infection, as well as the abilities of cross-reactive, 3R mutant-specific, and WT-specific CTLs to kill WT or 3R virus-infected cells and to suppress the replication of the WT or 3R virus. In addition, we investigated the accumulation of the 3R mutation in HIV-1-infected nonhemophiliac Japanese individuals, as well as in Japanese hemophiliacs who had been infected around 1983. The results clarified the role of CTLs recognizing the WT and/or 3R epitope in high accumulation of the 3R mutant in HIV-1-infected Japanese individuals.

MATERIALS AND METHODS

Samples from HIV-1-infected individuals. This study was approved by the ethics committee of Kumamoto University and the National Center for Global Health and Medicine. Informed consent was obtained from all individuals according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types were determined by standard sequence-based genotyping. Twelve HLA-A*24:02⁺ individuals who could be monitored from the early to the chronic phase of an HIV-1 infection were recruited for CTL analysis. Early HIV-1 infection was confirmed by seroconversion within 6 months or by an increasing number and density of bands on Western blots. Four-hundred fifty-one chronically HIV-1-infected individuals were also recruited for sequence analysis.

Cells. C1R cells expressing HLA-A*24:02 (C1R-A2402) and 721.221 cells expressing CD4 and HLA-A*24:02 (721.221-CD4-A2402) were previously generated (27, 30). These cells were cultured in RPMI 1640 medium containing 5 to 10% fetal bovine serum (FBS) and 0.15 mg/ml hygromycin B. MAGIC-5 cells (CCR5-transfected HeLa-CD4/long terminal repeat- β -galactosidase [LTR- β -Gal] cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS as described previously (17).

Induction of Gag28-specific T cells. PBMCs from HIV-1-infected HLA-A*24:02⁺ individuals were stimulated with WT or 3R peptide (1 μ M) in culture medium (RPMI 1640 containing 10% FBS and 200 U/ml human recombinant interleukin-2 [rIL-2]). After 14 days, the cultured PBMCs were tested for gamma interferon (IFN- γ) production by performing an intracellular cytokine staining (ICC) assay.

ICC assay. C1R-A2402 cells were prepulsed or not with the WT or 3R peptide at concentrations from 0.1 to 1,000 nM at 37°C for 1 h and then were washed twice with RPMI 1640 containing 10% FBS. PBMCs cultured for 2 weeks after peptide stimulation were incubated with the C1R-A2402 cells in a 96-U plate (Nunc) at 37°C. Brefeldin A (10 μ g/ml) was added after a 2-h incubation, and then the cells were incubated for an additional 4 h. Subsequently, the cells were stained with Pacific-blue-conjugated anti-CD8 monoclonal antibody (MAb) (BD Biosciences) and 7-aminocoumarin D (7-AAD) (BD Biosciences) at 4°C for 30 min, after which the cells were fixed with 4% paraformaldehyde solution and rendered permeable with permeabilization buffer (0.1% saponin and 10% FBS in phosphate-buffered saline) at 4°C for 10 min. Thereafter the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ MAb (BD Biosciences) at 4°C for 30 min and then washed twice with the permeabilization buffer. The percentage of CD8⁺ cells producing IFN- γ was analyzed by flow cytometry (FACSCanto II).

Generation of Gag28-specific CTL clones. Gag28-specific CTL clones were generated from Gag28-specific bulk-cultured T cells by limiting dilution in 96-U plates, together with 200 μ l of cloning mixture (1 \times 10⁶ irradiated allogeneic PBMCs from healthy donors and 1 \times 10⁵ irradiated C1R-A2402 cells prepulsed with the WT or 3R peptide at a concentration of 1 μ M in RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% phytohemagglutinin [PHA] soup). After 14 to 21 days in culture, the growing cells were tested for cytotoxic activity by performing the standard

chromium release assay. Since TCRs on these CTL clones were not sequenced, it is still possible that they were oligonucleotide clones.

HIV-1 clones. An infectious provirus, HIV-1 pNL-432, was reported previously (1). NL-432gagSF2 and NL-432gagSF2-3R were previously generated (30).

Assay of cytotoxicity of CTL clones toward target cells prepulsed with the epitope peptide. The cytotoxic activities of Gag28-specific CTL clones were determined by use of the standard chromium release assay, as described previously (15). Briefly, 721.221-CD4-A2402 cells were incubated with 100 μ Ci of Na₂⁵¹CrO₄ in saline for 1 h and then washed 3 times with RPMI 1640 containing 10% newborn calf serum. The labeled target cells (2 \times 10³/well) were prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM for 1 h and then cocultured at 37°C for 4 h with effector cells at an effector-to-target (E:T) ratio of 1:1 in 96-U plates (Nunc). The supernatants were collected and analyzed with a gamma counter. Spontaneous ⁵¹Cr release was determined by measuring the counts per minute in supernatants from wells containing only target cells (cpm spn). Maximum ⁵¹Cr 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) \times 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells.

Assay of cytotoxicity of CTL clones toward target cells infected with HIV-1. 721.221-CD4-A2402 cells were infected with WT or 3R virus, and then the infection rates were determined by detecting intracellular p24 antigen (Ag)-positive cells stained with FITC-conjugated anti-p24 Ag MAb (KC57-FITC; BD Biosciences). When approximately 50% of the total cells were p24 Ag-positive cells, they were used as target cells. The ⁵¹Cr-labeled target cells (2 \times 10³/well) were cocultured with effector cells at E:T ratios of 0:1 to 2:1 in 96-U plates at 37°C for 6 h. The supernatants were collected and analyzed with a gamma counter.

Generation of HLA-peptide tetrameric complexes. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as previously described (3). The WT or 3R peptide was added to the refolding solution containing the biotinylation sequence-tagged extracellular domain of the HLA-A*24:02 molecule and β 2 microglobulin. The purified monomer complexes were mixed with phycoerythrin (PE)-labeled streptavidin (Molecular Probes) at a molar ratio of 4:1.

Tetramer binding assay. CTL clones were stained with PE-conjugated tetramer at concentrations of 1 to 100 nM at 37°C for 30 min. After 2 washes with RPMI 1640 containing 10% FBS (R10), the cells were stained with FITC-conjugated anti-CD8 MAb and 7-AAD at 4°C for 30 min. Thereafter, the cells were washed twice with R10 and then analyzed by flow cytometry (FACSCanto II). The mean fluorescence intensity (MFI) of tetramer-positive cells among CD8-positive cells was calculated.

Replication suppression assay. The ability of Gag28-specific CTLs to suppress HIV-1 replication was examined as previously described (43). CD4⁺ T cells were isolated from PBMCs of healthy HLA-A*24:02⁺ donors and incubated with a given HIV-1 clone at 37°C for 6 h. After 3 washes with R10, the cells (3 \times 10⁴/well) were cocultured with Gag28-specific CTL clones at E:T ratios of 0.1:1 to 1:1 in R10 containing 1% nonessential amino acid solution and, 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. From day 3 to day 7 postinfection, a 30- μ l volume of culture supernatant was collected, and the volume removed was replaced with fresh medium. The concentration of p24 Ag was measured by using an enzyme-linked immunosorbent assay (ELISA) (HIV-1-p24-Ag ELISA kit; ZeptoMetrix).

Replication kinetics assay. The replication kinetics of the WT and 3R viruses were examined as previously described (17). After CD4⁺ T cells (2 \times 10⁶) had been exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 2 h and washed twice with R10, they were cultured in 1 ml of R10 containing 1% nonessential amino acid solution and 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. Then, 0.1 ml of the culture supernatant was collected from

day 2 to day 10 postinfection, and the volume removed was replaced with fresh medium. The concentration of p24 Ag in the supernatant was measured by using ELISA. Replication kinetics assays were performed in triplicate.

Sequence of autologous virus. Viral RNA was extracted from plasma samples from HIV-1-infected individuals by using a QIAamp MinElute virus spin kit (Qiagen). For clone sequencing, cDNA was synthesized from the RNA with SuperScript III and Random Primers (Invitrogen), and the Gag region was amplified by nested PCR with *Taq* DNA polymerase (Promega). Then, the PCR products were gel purified and cloned with a TOPO TA cloning kit (Invitrogen). For bulk sequencing, the Gag region was amplified from the RNA by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen) and Gag-specific primers, and then the second PCR was done. We prepared the Gag-specific primer sets shown below. For clone sequencing, 5'-TTTTT GACTAGCGGAGGCTAGAA-3' and 5'-CACAAATAGAGGGTTGCTAC TGT-3' were used for the first PCR and 5'-GGGTGCGAGAGCGTCGG TATTAAGC-3' and 5'-TAAGTTCTTCTGATCCTGTCTG-3' for the second PCR. For bulk sequence, 5'-TCTCTCGACGCAGGACTC-3' and 5'-AGGGTTCCCTTGGTCCTTGT-3' were employed for the reverse transcription (RT)-PCR and 5'-TCTCTCGACGCAGGACTC-3' and 5'-TCTCCTACTGGGATAGGTG-3' for the second PCR. All DNA sequencing was performed by using a BigDye Terminator cycle-sequencing kit (Applied Biosystems) and an ABI Prism 310 or 3100 genetic analyzer.

RESULTS

Selection of the 3R mutation by WT epitope-specific CD8⁺ T cells in individuals infected with WT virus. We investigated 12 HIV-1-infected HLA-A*24:02⁺ individuals who could be monitored from the early to the chronic phases of their infections. We first analyzed the sequence of the Gag28 epitope at an early phase in the 12 HIV-1-infected HLA-A*24:02⁺ individuals. The WT sequence of the Gag28 epitope was detected in 4 of these individuals, whereas 3R was found in the other 8, suggesting that the former and the latter individuals had been infected with WT and 3R viruses, respectively (Table 1). This is consistent with a previous finding that the 3R mutant is found in approximately 70% of HIV-1-infected HLA-A*24:02⁺ individuals (30). We investigated the elicitation of Gag28-specific CD8⁺ T cells in the individuals infected with WT virus. PBMCs from these individuals at early and chronic phases were stimulated with WT or 3R peptide and then cultured for 2 weeks. The frequency of Gag28-specific CD8⁺ T cells among the cultured cells was measured by performing the ICC assay using WT and 3R peptides. Gag28-specific CD8⁺ T cells were detected at the early phase in 3 of the 4 individuals when the PBMCs were stimulated with WT peptide (Table 2). In 2 individuals, i.e., KI-092 and KI-161, Gag28-specific CD8⁺ T cells were much more WT specific than 3R mutant specific, whereas in KI-158 they recognized both peptides, but especially the WT peptide (Fig. 1). On the other hand, cross-reactive CD8⁺ T cells were induced in KI-092 and KI-161 when their PBMCs had been stimulated with 3R peptide, although the frequency of cross-reactive CD8⁺ T cells induced by stimulation with 3R peptide was lower than that of WT-specific cells induced by stimulation with WT peptide. The 3R peptide failed to induce Gag28-specific CD8⁺ T cells in PBMCs from KI-158. Thus, WT-specific CD8⁺ T cells were predominantly elicited at an early phase in the individuals infected with WT virus, although a small but significant number of cross-reactive T cells were also elicited in them.

To clarify the specificity of Gag28-specific CD8⁺ T cells at the early phase in KI-092 and KI-161, we generated Gag28-specific CTL clones by stimulating early-phase PBMCs from KI-092 and

TABLE 1 Sequence at Gag30 in 12 HLA-A*24:02⁺ individuals with an early-phase HIV-1 infection

Patient ID ^a	Sampling date (mo/day/yr)	Gag30 sequence	Method
KI-091	12/13/2000	3R	Cloning
	12/27/2000	3R	Direct
	1/7/2002	3R	Direct
	7/9/2003	3R	Cloning
	9/29/2004	3R	Cloning
	8/4/2005	3R	Cloning
KI-092	1/22/2001	WT	Cloning
	11/21/2001	WT	Cloning
	12/10/2002	WT/3R	Cloning
	8/14/2003	3R	Cloning
KI-102	5/11/2001	WT	Direct
	7/5/2004	WT	Direct
	3/28/2005	WT	Direct
KI-126	7/19/2001	3R	Direct
	1/18/2002	3R	Direct
	11/15/2004	3R	Direct
	9/12/2005	3R	Direct
	10/25/2001	3R	Direct
KI-134	6/30/2004	3R	Direct
	10/29/2001	3R	Direct
KI-136	7/10/2003	3R	Direct
	11/08/2001	3R	Direct
KI-140	5/2/2001	3R	Direct
	8/28/2003	3R	Direct
KI-151	4/12/2002	3R	Direct
	6/14/2002	WT	Direct
KI-154	10/11/2002	WT	Direct
	8/25/2003	WT	Direct
KI-158	11/14/2003	WT/3R	Direct
	2/23/2004	3R/WT	Direct
	11/1/2004	3R	Direct
	4/4/2005	3R	Direct
	2/15/2002	WT	Direct
KI-161	9/12/2002	WT	Direct
	3/4/2003	WT	Direct
	9/30/2003	WT/3R	Direct
	5/6/2004	3R	Direct
	1/27/2005	3R	Direct
KI-163	6/16/2005	3R	Cloning
	8/30/2002	3R	Direct
	9/27/2004	3R	Direct

^a ID, identifier.

KI-161 with the WT peptide. The CTL clones from KI-092 showed a much greater ability to kill cells prepulsed with WT peptide than to kill those prepulsed with the 3R peptide (Fig. 2A), suggesting that they were WT-specific CTLs. To further clarify the specificity of these T cell clones, we investigated the binding affinity of the clones for WT peptide-binding HLA-A*24:02 tetramer (WT tetramer) and 3R peptide-binding HLA-A*24:02 tetramer (3R tetramer). These clones exhibited much greater binding ability to the WT tetramer than to the 3R tetramer (Fig. 2B). These results together indicate that these were WT-specific CTL clones. We further analyzed the abilities of these clones to recognize HIV-1-infected cells. These CTL clones effectively killed WT-virus-infected cells, but not the 3R virus-infected cells (Fig. 2C), and showed the ability to suppress the replication of WT virus, but not to suppress that of the 3R virus (Fig. 2D). WT-specific CD8⁺ T cell

TABLE 2 Responses of CD8⁺ T cells from individuals infected with WT virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN- γ -producing cells specific for each peptide among CD8 ⁺ T cells ^a		
	Early phase	Chronic phase			Without	WT	3R
KI-092	1/22/2001 (WT)	8/14/2003 (3R)	5/24/2001	WT	0.2	34.4	13.7
				3R	0.1	12.1	16.8
			2/3/2003	WT	0.2	5.8	4.2
KI-102	5/11/2001 (WT)	3/28/2005 (WT)	7/11/2001	WT	0.6	0.3	0.3
				3R	1.0	0.6	1.1
			7/5/2004	WT	1.1	1.5	2.0
KI-158	6/14/2002 (WT)	4/4/2005 (3R)	10/11/2002	WT	0.2	28.7	9.3
				3R	0.6	0.7	0.6
			4/4/2005	WT	1.4	19.3	24.6
KI-161	2/15/2002 (WT)	6/16/2005 (3R)	7/26/2002	WT	0.1	0.5	0.4
				3R	0.3	23.3	23.8
			5/6/2004	WT	0.4	18.8	20.9
			3R	0.0	74.5	8.0	
				WT	0.2	55.1	41.8
				3R	0.1	21.4	4.9
				3R	0.2	42.5	43.9

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

clones established from early-phase PBMCs of KI-161 also showed a similar ability to kill WT virus-infected and 3R virus-infected cells (Fig. 3). In these individuals, the 3R mutant virus became dominant 1 to 2 years after the early phase (Table 1). Taken together, these findings suggest that the 3R mutation was selected by WT-specific CTLs.

The 3R virus was not detected by approximately 4 years postinfection in KI-102, who had been infected with the WT virus (Table 1). This individual did not have Gag28-specific CD8⁺ T cells at an early phase of the HIV-1 infection (Fig. 1). Interestingly, only WT-specific CD8⁺ T cells were induced from PBMCs of this patient 2.5 year later. Thus, WT-specific CD8⁺ T cells did not select 3R within about 2 years after the WT-specific CD8⁺ T cells had been elicited in the patient.

Cross-reactive CD8⁺ T cells in individuals who had been infected with WT virus and had selected 3R virus. We investigated whether the 3R-specific or cross-reactive CD8⁺ T cells were elicited after the 3R mutant had been selected in individuals who had been infected with the WT virus. In KI-158, no Gag28-specific CD8⁺ T cells were induced from early-phase PBMCs stimulated with the 3R peptide, whereas cross-reactive CD8⁺ T cells were induced from chronic-phase PBMCs stimulated with WT peptide or 3R peptide (Fig. 1). In KI-161, Gag28-specific CD8⁺ T cells recognizing WT peptide more than the 3R peptide were induced from early-phase PBMCs stimulated with WT peptide or the 3R peptide, whereas cross-reactive CD8⁺ T cells were predominantly induced from chronic-phase PBMCs stimulated with the 3R peptide (Fig. 1). These results indicate that cross-reactive CD8⁺ T cells became dominant in the Gag28-specific CD8⁺ T cell population after the emergence of the 3R virus in these 2 individuals.

To investigate the function of these cross-reactive CD8⁺ T cells, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-161 by stimulating them with the 3R peptide. The CTL clones evenly recognized both WT and the 3R peptides (Fig. 3A) and showed the same binding affinity to the 2 tetramers (Fig. 3B). These results suggest that the two peptides had the same

binding affinity for HLA-A*24:02. They effectively killed WT-virus-infected cells and weakly killed the 3R virus-infected cells (Fig. 3C), whereas they suppressed the replication of the WT virus but not that of the 3R virus (Fig. 3D). These results indicate that these cross-reactive CTLs contributed to the selection of the 3R virus. In addition, the results strongly suggest weak presentation of the 3R peptide in the cells infected with 3R virus, because the cross-reactive CTL clones had TCR with the same binding affinity for both HLA-A*24:02-WT peptide and HLA-A*24:02-3R peptide complexes and because WT and 3R peptides had the same binding affinity for HLA-A*24:02. This reduced presentation may have affected the control of 3R virus by the cross-reactive CTLs.

Gag28-specific T cell repertoire in an individual infected with WT virus. The results in Fig. 1 suggest that both WT-specific and cross-reactive CD8⁺ T cells were elicited at an early phase of HIV-1 infection in 3 individuals infected with WT virus (KI-092, KI-158, and KI-161). To characterize Gag28-specific CTLs elicited at that time, we established Gag28-specific CTL clones from PBMCs at an early phase in KI-161 by stimulating them with the WT peptide. We found 3 types of CTL clones among the 8 clones analyzed. As shown in Fig. 3A, 3 clones effectively recognized the WT peptide but not the 3R peptide (WT specific), 3 clones recognized the WT peptide more than the 3R peptide (WT dominant), and 2 clones evenly recognized both peptides (cross-reactive). We next investigated the binding affinity of TCRs on these clones to WT tetramer and 3R tetramer. The results confirmed the specificity of these 3 types of CTL clones (Fig. 3B). These results together indicate that KI-161 had a multiple T cell repertoire for the Gag28 epitope before the 3R virus had been selected.

Next, we analyzed the abilities of these T cell clones to kill HIV-1-infected cells. The WT-specific and WT-dominant CTL clones effectively killed the target cells infected with WT virus but failed to kill those infected with the 3R virus (Fig. 3C, left and right graphs under early phase). On the other hand, cross-reactive CTL clones weakly killed the target cells infected with the 3R virus and effectively killed those infected with the WT virus (Fig. 3C, middle

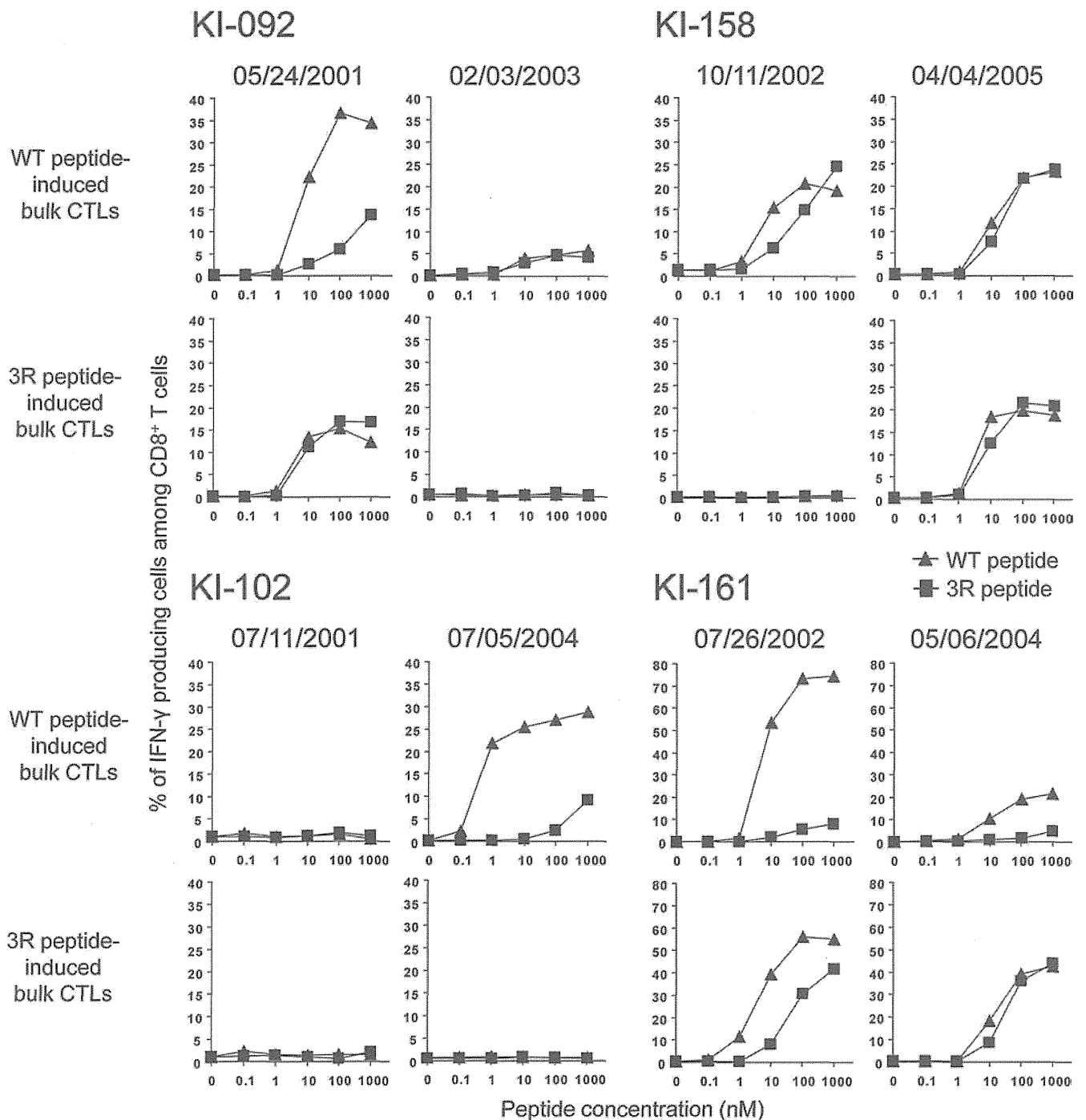


FIG 1 Gag28-specific CD8⁺ T cells from individuals infected with WT virus at early and chronic phases. Gag28-specific CD8⁺ T cells were induced by stimulating PBMCs from early and chronic phases in 4 WT-virus-infected HLA-A*24:02⁺ individuals with WT or 3R peptide. The responses of these bulk-cultured cells to C1R-A2402 cells prepulsed with WT or 3R peptide at concentrations of 0.1 to 1,000 nM were analyzed by using the ICC assay.

graphs under early phase). Then, we analyzed the abilities of these CTL clones to suppress HIV-1 replication. Both WT-specific and cross-reactive CTL clones effectively suppressed the replication of the WT virus, whereas WT-specific and cross-reactive CTL clones exhibited no and weak ability, respectively, to suppress that of the 3R virus (Fig. 3D). These results indicate that WT-specific and cross-reactive CTLs could suppress the replication of the WT virus

but that the former CTLs could not suppress the 3R virus *in vivo*. The latter CTLs may weakly suppress 3R virus *in vivo*. Interestingly, the WT-dominant CTL clones exhibited much weaker ability to suppress the replication of WT virus than did the WT-specific and cross-reactive CTLs (Fig. 3D), although no difference in killing activity against WT-virus-infected cells was found among these 3 CTL clones. Overall, KI-161 had a multiple Gag28-

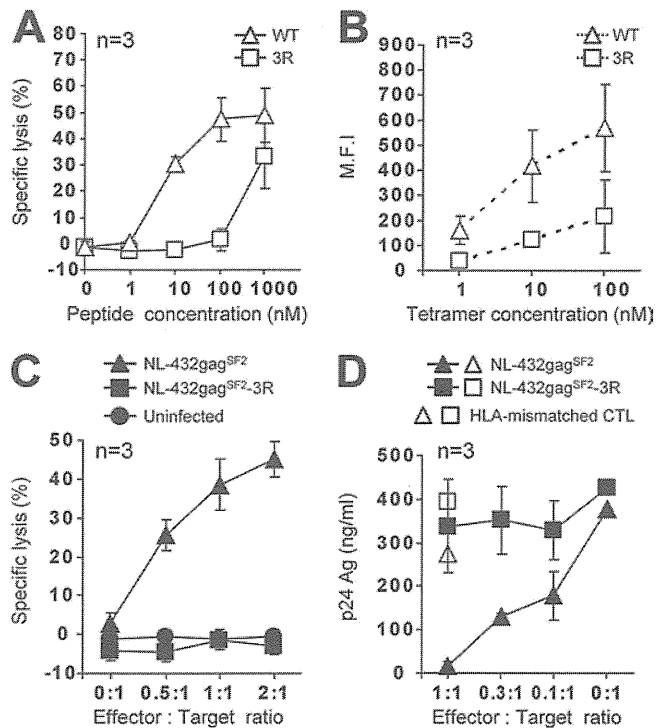


FIG 2 Antiviral activity of Gag28-specific CTL clones generated from early-phase PBMCs from patient KI-092, infected with WT virus. Gag28-specific CTL clones were generated from early-phase PBMCs from KI-092 by stimulating them with WT peptide. The activities of 3 CTL clones ($n = 3$) were analyzed. (A) Cytotoxic activity toward 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity to WT and 3R tetramers at concentrations of 1 to 100 nM. The MFI values of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with NL-432gag^{SF2} (WT virus) or NL-432gag^{SF2}-3R (3R virus). WT-virus-infected (49.1% of total cells were p24 Ag⁺) and 3R virus-infected (48.6% of total cells were p24 Ag⁺) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R viruses. The ability was tested at different E:T ratios. The error bars indicate standard deviations.

specific CTL repertoire at an early phase of HIV-1 infection, but only 2 types of Gag28-specific CTLs, which were the majority among the Gag28-specific CTLs, contributed to the suppression of WT virus replication.

Cross-reactive CD8⁺ T cells and 3R-specific CD8⁺ T cells in individuals who were infected with 3R virus. Next, we analyzed the elicitation of Gag28-specific CD8⁺ T cells in 5 individuals infected with the 3R virus. Gag28-specific CD8⁺ T cells were detected at both early and chronic phases in 3 individuals, whereas they were found at only the chronic phase in the other 2 (Table 3). Cross-reactive CD8⁺ T cells were induced by stimulating KI-091 PBMCs from both early and chronic phases, not only with 3R peptide, but also with WT peptide. To characterize Gag28-specific CD8⁺ T cells in KI-091, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-091 by stimulating them with 3R peptide. We investigated the recognition of 3 CTL clones for WT and 3R peptides. These CTL clones evenly recognized both peptides (Fig. 4A) and revealed the same binding affinity for the 2 tetramers (Fig. 4B), indicating that they were cross-reactive CTLs. They moderately killed target cells infected with either WT or 3R

virus (Fig. 4C) but did not suppress the replication of the WT and 3R viruses (Fig. 4D). Thus, Gag28-specific CD8⁺ T cells elicited in KI-091 had no ability to suppress the replication of WT and 3R viruses. Further analysis of 13 other clones revealed similar characteristics (data not shown), supporting the data indicating that cross-reactive CTLs were predominantly elicited in KI-091.

In the chronic phase, KI-091 had cross-reactive CD8⁺ T cells, whereas 3R-specific CD8⁺ T cells were found in 4 other individuals (Table 3). To characterize these 3R-specific CD8⁺ T cells, we generated 3R-specific CTL clones from KI-163 PBMCs at the chronic phase by stimulating them with 3R peptide. All 3 clones recognized the 3R peptide much more effectively than the WT peptide (Fig. 4A). These CTL clones bound to 3R tetramer, but not to WT tetramer (Fig. 4B), indicating that these CTL clones carried a 3R-specific TCR. In addition, we analyzed the abilities of these CTL clones to recognize virus-infected cells and found that they effectively killed target cells infected with 3R virus, but not those infected with WT virus (Fig. 4C). However, they failed to suppress the replication of either 3R or WT virus (Fig. 4D). These results indicate that Gag28-specific CD8⁺ T cells elicited in all individuals infected with 3R virus had no ability to suppress the replication of WT or 3R virus. Thus, Gag28-specific CD8⁺ T cells seem to have failed to control the 3R virus, although they were elicited in individuals infected with the 3R virus.

High accumulation of the 3R variant in the Japanese population. The results described above strongly suggest that WT-specific and cross-reactive CD8⁺ T cells selected the 3R mutation in the individuals infected with the WT virus and that 3R-specific and cross-reactive CD8⁺ T cells failed to control the 3R virus in the individuals infected with it. Therefore, we assume that this 3R mutation has accumulated in the HLA-A*24:02⁺ individuals. In addition, since HLA-A*24:02 is found in approximately 70% of Japanese, we speculate that the mutation has accumulated to high levels in the Japanese population.

A previous study analyzed the frequency of 3R in only 32 HLA-A*24:02⁺ and 26 HLA-A*24:02⁻ individuals chronically infected with HIV-1 and showed that the frequency of 3R was significantly higher in HLA-A*24:02⁺ individuals than in the HLA-A*24:02⁻ individuals (30). To confirm the association of this mutation with HLA-A*24:02, we analyzed a large number of chronically HIV-1-infected nonhemophiliac individuals (220 HLA-A*24:02⁺ and 154 HLA-A*24:02⁻ individuals) recruited from April 2008 to March 2011 (2008 to 2011 cohort). The results confirmed that the frequency of 3R was significantly higher in HLA-A*24:02⁺ individuals than in the HLA-A*24:02⁻ individuals ($P < 0.0005$) (Fig. 5). Since 3R was found in 74.7% of the HLA-A*24:02⁻ individuals in this cohort, we speculate that the mutation has been accumulating in the Japanese population. Therefore, we analyzed HIV-1-infected nonhemophiliac Japanese individuals who had been recruited from 1996 to 2002 (1996 to 2002 cohort), as well as Japanese hemophiliacs who had been infected around 1983 (hemophiliac cohort), and then compared them to the 2008 to 2011 cohort (Fig. 5). The association of this mutation with HLA-A*24:02 was also found in both the 1996 to 2002 cohort and the hemophiliac cohort ($P < 0.01$ and $P = 7.4 \times 10^{-7}$, respectively). The frequency of this mutation in HLA-A*24:02⁻ individuals significantly increased from 0% in the hemophiliac cohort to 50.0% in the 1996 to 2002 cohort ($P = 0.0084$) and to 74.7% in the 2008 to 2011 cohort ($P = 2.6 \times 10^{-7}$). These results indicate that the 3R mutation was strongly selected by Gag28-specific CTLs and has