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.

[a]AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan; [b]Center for AIDS Research, Kumamoto University, Kumamoto, Japan; and [c]Department 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: higatana@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-naive 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-naive 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-naive 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-naive 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.

INTERNAL MEDICINE

CASE REPORT

Idiopathic Oropharyngeal and Esophageal Ulcers Related to HIV Infection Successfully Treated with Antiretroviral Therapy Alone

Yohei Hamada[1], Naoyoshi Nagata[2], Haruhito Honda[1], Katsuji Teruya[1], Hiroyuki Gatanaga[1], Yoshimi Kikuchi[1] and Shinichi Oka[1]

Abstract

We herein report the case of an HIV-positive man who was diagnosed with idiopathic esophageal and oropharyngeal ulceration. The esophageal and oropharyngeal ulcers were considered to be idiopathic and related to HIV infection after excluding the possibility of infection with known pathogens. Both the esophageal and oropharyngeal ulcers showed significant improvements following antiretroviral therapy alone. Idiopathic esophageal ulcers are a well-known complication of late-stage HIV infection. However, involvement of both the esophagus and pharynx is rare. Furthermore, antiretroviral therapy without concomitant steroids is effective against idiopathic esophageal and oropharyngeal ulcers related to HIV infection.

Key words: HIV infection, idiopathic esophageal ulcer, pharyngeal ulcer, antiretroviral therapy, gastrointestinal diseases

(Intern Med 52: 393-395, 2013)
(DOI: 10.2169/internalmedicine.52.8709)

Introduction

Esophageal ulceration is a common complication in patients with human immunodeficiency virus-1 (HIV) infection, especially in the late stage. Although esophageal ulcerations can be caused by various infectious agents, such as *Candida* species, cytomegalovirus (CMV) and herpes simplex virus (HSV), a large proportion of patients are diagnosed with idiopathic esophageal ulcerations (1, 2) with no detectable etiology. Oropharyngeal ulcers are also an important comorbidity that can become progressive in HIV-infected patients (3, 4). The common infectious agents of esophageal ulcerations are known to also cause oropharyngeal ulcerations, although some cases are considered idiopathic with no identifiable etiology (5, 6). However, simultaneous involvement of the esophagus and oropharynx is uncommon outside of HSV esophagitis (5). We herein report a case of unusual discrete ulcers of the oropharynx and esophagus in a patient with HIV infection that showed a rapid improvement following treatment with antiretroviral therapy alone.

Case Report

A previously healthy 60-year-old Japanese homosexual man presented with severe odynophagia. He was diagnosed with oral candidiasis and HIV infection and therefore had been referred to our hospital (day-1). Laboratory tests showed a low CD4+ cell count (49/μL), a high HIV-RNA titer (1.0×10^6 copies/mL) and a low serum albumin level (Alb 2.9 g/dL). Whole-blood polymerase chain reaction (PCR) was negative for both CMV and HSV. The patient was treated with fluconazole for seven days for suspected esophageal candidiasis. Despite this treatment, the odynophagia did not improve. Since oral ulcers were noticed, treatment with oral valaciclovir at a dose of 1,000 mg/day was initiated based on a presumptive diagnosis of HSV infection. However, the odynophagia persisted, and the oral ulcers did not show any improvement despite a 3-week

[1]AIDS Clinical Center, National Center for Global Health and Medicine, Japan and [2]Department of Gastroenterology, National Center for Global Health and Medicine, Japan
Received for publication July 30, 2012; Accepted for publication October 30, 2012
Correspondence to Dr. Naoyoshi Nagata, nnagata_ncgm@yahoo.co.jp

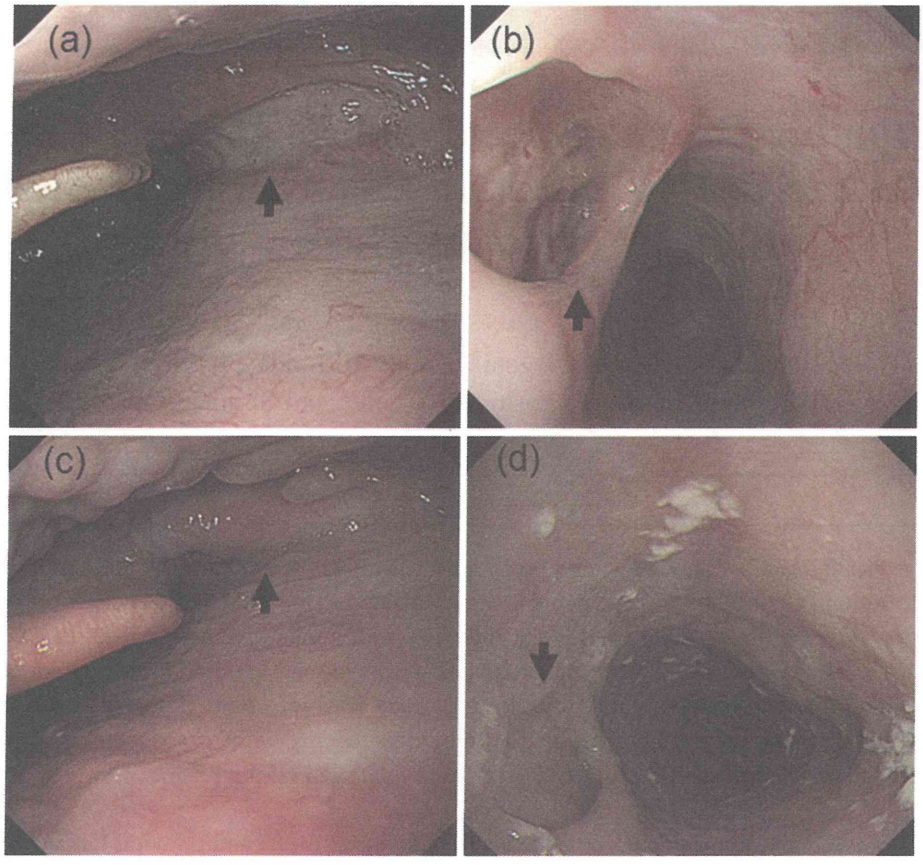

Figure. Endoscopic findings of the pharynx and esophagus. The pharyngeal (a) and esophageal (b) ulcers before the administration of antiretroviral therapy. The endoscopic appearance of the pharynx (c) and esophagus (d) on day 22 of antiretroviral therapy. Black arrows: ulcers.

course of anti-HSV therapy; thus, upper gastrointestinal endoscopy was performed. Endoscopy showed large, discrete and well-circumscribed esophageal and pharyngeal ulcers (Figure a, b). Because a diagnosis of CMV esophagitis was suspected based on the endoscopic appearance of the ulcers, treatment with intravenous gancicrovir at a dose of 5 mg/kg every 12 hours was initiated and the valaciclovir was discontinued. However, a histopathological examination of the biopsy specimen obtained from the base and edge of an ulcer before the initiation of gancicrovir therapy revealed lymphocytic infiltration without intranuclear or intracytoplasmic inclusion bodies. Immunohistochemical staining for CMV and HSV was negative. PCR assays of both pharyngeal and esophageal biopsies were negative for CMV-DNA and HSV-DNA (≤40 copies/μg DNA). Furthermore, repeat endoscopy performed after two weeks of gancicrovir therapy showed exacerbation of the ulcers. Based on these findings, we administered antiretroviral therapy consisting of ritonavir-boosted darunavir with abacavir/lamivudine. The gancicrovir therapy was discontinued after the completion of a 3-week course of treatment. The odynophagia gradually improved and ultimately disappeared two weeks later, while the CD4 count increased to 91/μL and the HIV-RNA titer decreased to 4×10^4 copies/mL. Endoscopy performed on day 22 of antiretroviral therapy demonstrated significant reductions in the size and depth of the pharyngeal and esophageal ulcers (Figure c, d). Additionally, resolution of the oral ulcers was noticed.

Discussion

To our knowledge, this is the first report of idiopathic esophageal and oropharyngeal ulcers successfully treated with antiretroviral therapy alone in a patient with late-stage HIV infection. Steroids are commonly used as the standard treatment for idiopathic esophageal ulcers (2, 7). However, steroids can lead to serious opportunistic infections due to their immunosuppressive effects. The efficacy of steroids is mostly based on reports from the pre-highly active antiretroviral therapy era, and the efficacy of antiretroviral therapy has not been examined. As described above, steroid therapy may not be necessary when a potent combination of antiretroviral therapy is administered. The etiology of idiopathic esophageal ulcers is still not fully understood. Although such ulcers are considered to be associated with HIV infection, they have been referred to as idiopathic when no identifiable etiologic agent other than HIV infection is present (8, 9). The potential pathogenesis of these ulcers includes apoptosis of the esophageal mucosa induced by HIV infection (10). Based on this probable pathogenesis, it is therefore considered to be rational to administer antiretroviral therapy to treat idiopathic esophageal ulcers.

The diagnosis of idiopathic oropharyngeal and esophageal ulcers is established by excluding other infectious agents known to cause esophageal ulceration, including CMV, HSV and *Candida* sp, by performing histopathological and immunological examinations of biopsy specimens (1, 2, 5, 6). In our case, the histopathological findings showed no evidence of any infectious pathogens, and CMV and HSV infection were also excluded by PCR assays, which have a high sensitivity (11, 12). Furthermore, the oropharyngeal and esophageal ulcers were refractory to anti-CMV and anti-HSV therapy. In addition, the ulcers showed significant improvement following the administration of antiretroviral therapy alone. Therefore, the final diagnosis was idiopathic oropharyngeal and esophageal ulcers related to HIV infection.

Involvement of both the oropharynx and esophagus in HSV-related ulcers is not uncommon (5). However, in our patient, the esophageal and oropharyngeal ulcers were considered idiopathic, which is extremely rare. In this case, the ulcers in both regions were examined endoscopically. Therefore, performing careful endoscopic examinations of not only the esophagus, but also the pharynx, is considered to be important for establishing the cause of odynophagia in HIV-infected patients.

In conclusion, a pharyngeal and esophageal biopsy obtained using upper gastrointestinal endoscopy was useful for establishing the diagnosis in this case. Furthermore, antiretroviral therapy alone resulted in a significant improvement of the idiopathic ulcers in our HIV-infected patient. The initiation of antiretroviral therapy without steroids is therefore a reasonable option for treating idiopathic oropharyngeal and esophageal ulcers in HIV-infected patients.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors thank Toru Igari for valuable help in performing the histopathological examination and the entire clinical staff at the AIDS Clinical Center. We also thank the staff of the endoscopy unit.

References

1. Bonacini M, Young T, Laine L. The causes of esophageal symptoms in human immunodeficiency virus infection. A prospective study of 110 patients. Arch Intern Med **151**: 1567-1572, 1991.
2. Wilcox CM, Schwartz DA, Clark WS. Esophageal ulceration in human immunodeficiency virus infection. Causes, response to therapy, and long-term outcome. Ann Intern Med **123**: 143-149, 1995.
3. Gorin I, Vilette B, Gehanno P, Escande JP. Thalidomide in hyperalgic pharyngeal ulceration of AIDS. Lancet **335**: 1343, 1990.
4. MacPhail LA, Greenspan D, Feigal DW, Lennette ET, Greenspan JS. Recurrent aphthous ulcers in association with HIV infection. Description of ulcer types and analysis of T-lymphocyte subsets. Oral Surg Oral Med Oral Pathol **71**: 678-683, 1991.
5. Wilcox CM, Straub RF, Clark WS. Prospective evaluation of oropharyngeal findings in human immunodeficiency virus-infected patients with esophageal ulceration. Am J Gastroenterol **90**: 1938-1941, 1995.
6. Liang GS, Daikos GL, Serfling U, et al. An evaluation of oral ulcers in patients with AIDS and AIDS-related complex. J Am Acad Dermatol **29**: 563-568, 1993.
7. Wilcox CM, Schwartz DA. Comparison of two corticosteroid regimens for the treatment of HIV-associated idiopathic esophageal ulcer. Am J Gastroenterol **89**: 2163-2167, 1994.
8. Bhaijee F, Subramony C, Tang SJ, Pepper DJ. Human immunodeficiency virus-associated gastrointestinal disease: common endoscopic biopsy diagnoses. Patholog Res Int **2011**: 247923, 2011.
9. Nishijima T, Tsukada K, Nagata N, et al. Antiretroviral therapy alone resulted in successful resolution of large idiopathic esophageal ulcers in a patient with acute retroviral syndrome. AIDS **25**: 1677-1679, 2011.
10. Houghton JM, Korah RM, Kim KH, Small MB. A role for apoptosis in the pathogenesis of AIDS-related idiopathic esophageal ulcers. J Infect Dis **175**: 1216-1219, 1997.
11. Jazeron JF, Barbe C, Frobert E, et al. Virological diagnosis of herpes simplex virus 1 esophagitis by quantitative real-time PCR assay. J Clin Microbiol **50**: 948-952, 2012.
12. Reddy N, Wilcox CM. Diagnosis & management of cytomegalovirus infections in the GI tract. Expert Rev Gastroenterol Hepatol **1**: 287-294, 2007.

JVI
Journals.ASM.org

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

Yuichi Yagita,[a] Nozomi Kuse,[a] Kimiko Kuroki,[b] Hiroyuki Gatanaga,[a,c] Jonathan M. Carlson,[d] Takayuki Chikata,[a] Zabrina L. Brumme,[e,f] Hayato Murakoshi,[a] Tomohiro Akahoshi,[a] Nico Pfeifer,[d] Simon Mallal,[g] Mina John,[g] Toyoyuki Ose,[b] Haruki Matsubara,[b] Ryo Kanda,[b] Yuko Fukunaga,[b] Kazutaka Honda,[a] Yuka Kawashima,[a] Yasuo Ariumi,[a] Shinichi Oka,[a,c] Katsumi Maenaka,[b] Masafumi Takiguchi[a]

Center for AIDS Research, Kumamoto University, Chuo-ku, Kumamoto, Japan[a]; Laboratory of Biomolecular Science, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan[b]; AIDS Clinical Center, National Center for Global Health and Medicine, Shinjuku-ku, Tokyo, Japan[c]; eScience Group, Microsoft Research, Los Angeles, California[d]; Faculty of Health Sciences, Simon Fraser University, Burnaby BC, Canada[e]; British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada[f]; Institute for Immunology & Infectious Diseases, Murdoch University, Murdoch, Western Australia, Australia[g]

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

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

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

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

Received 20 September 2012 **Accepted** 26 November 2012
Published ahead of print 12 December 2012
Address correspondence to Masafumi Takiguchi, masafumi@kumamoto-u.ac.jp.
Y.Y., N.K., and K.K. contributed equally to this study.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.02572-12.

doi:10.1128/JVI.02572-12

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

[a] PlyloLOR, phylogenetically corrected lnOR.

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

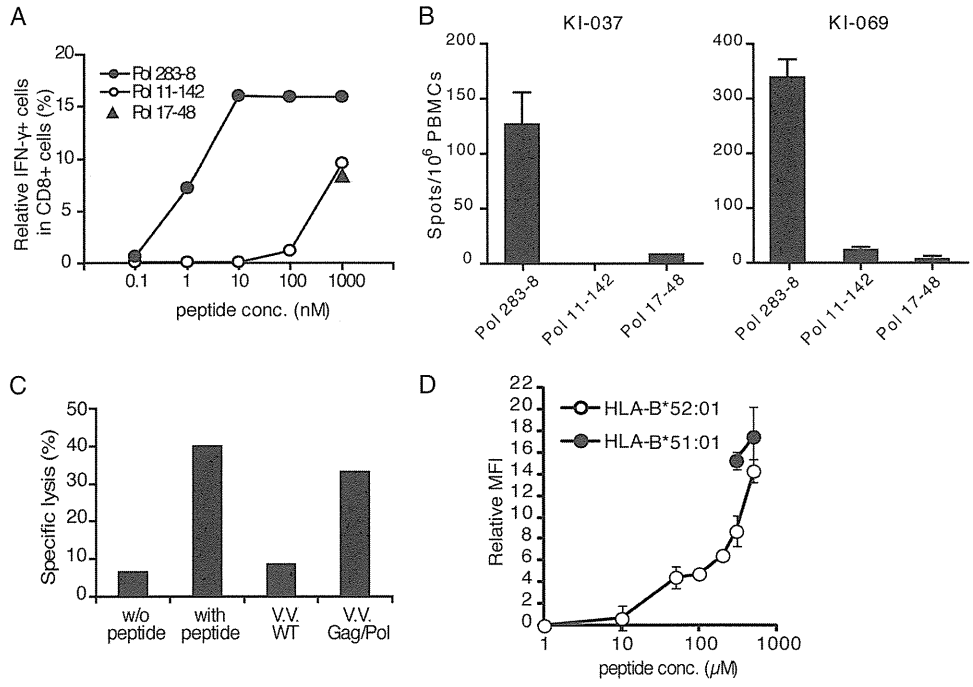

FIG 1 Identification of HLA-B*52:01-restricted Pol epitope. (A) Identification of the epitope peptide recognized by HLA-B*52:01-restricted CD8$^+$ T cells. Bulk T cells were cultured for 2 weeks after stimulation with the Pol17-48 peptide, and then the recognition of C1R-HLA-B*52:01 cells prepulsed with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

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

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

[a] More than the mean number of negative-control spots + 2 SD was defined as a positive response (positive response, >79 spots).
[b] NT, not tested.

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

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

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

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

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

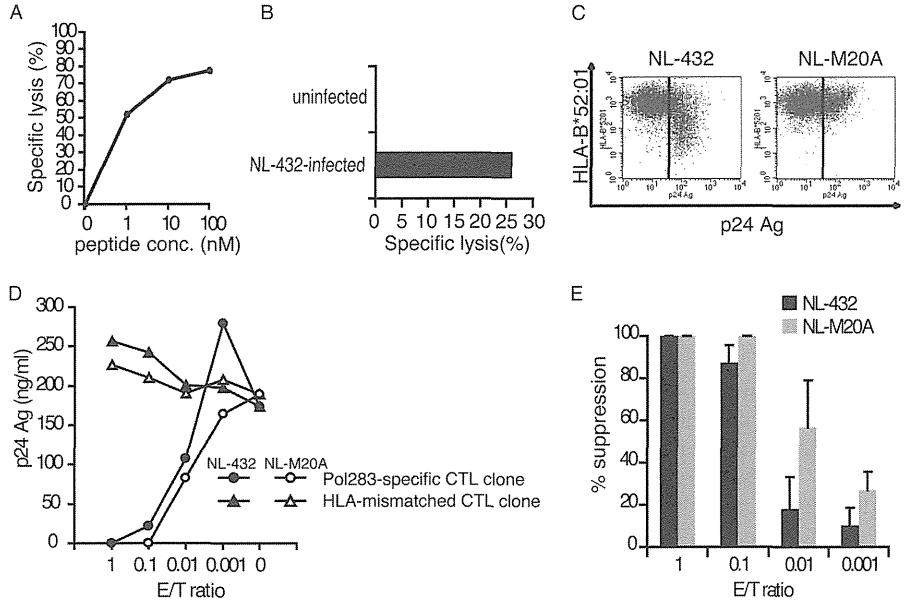

FIG 2 Abilities of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to kill HIV-1-infected CD4$^+$ T cells and to suppress HIV-1 replication. (A) Killing activity of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone against C1R-B*52:01 cells prepulsed with Pol283-8 peptides.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

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

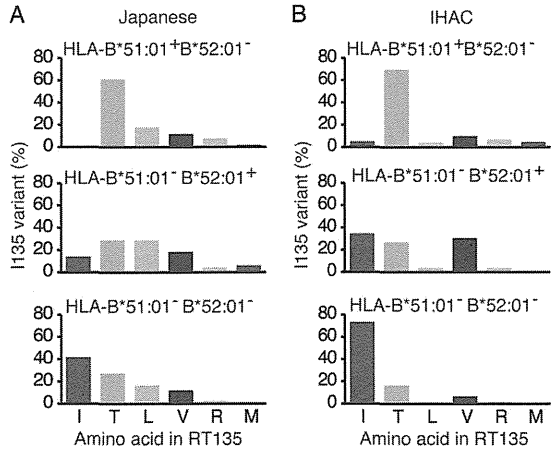

FIG 3 Amino acid variation at RT135 in Japanese individuals. (A) Frequency of the amino acid at RT135 in 51 HLA-B*51:01$^+$ HLA-B*52:01$^-$, 49 HLA-B*51:01$^-$ HLA-B*52:01$^+$, and 151 HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese subjects. (B) Frequency of the amino acid at RT135 in 131 HLA-B*51:01$^+$ HLA-B*52:01$^-$,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

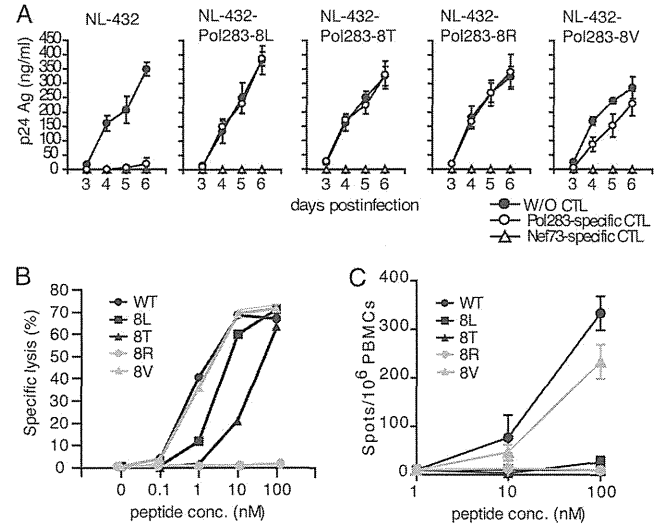

FIG 4 Ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to suppress the replication of four mutant viruses. (A) Ability of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone to suppress the replication of four (8L, 8T, 8R, and 8V) mutant viruses and NL-432. The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

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

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

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

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

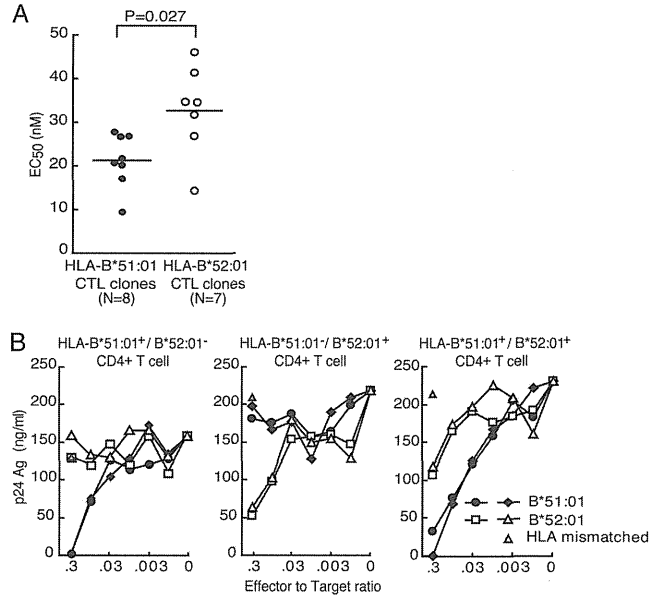

FIG 5 Differences between HLA-B*51:01-restricted and HLA-B*52:01-restricted CD8$^+$ T cell clones in TCR avidity and the ability to suppress HIV-1 replication. (A) TCR avidity of the HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones expressed as EC_{50}. The ability of the TCRs of HLA-B*51:01-restricted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

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

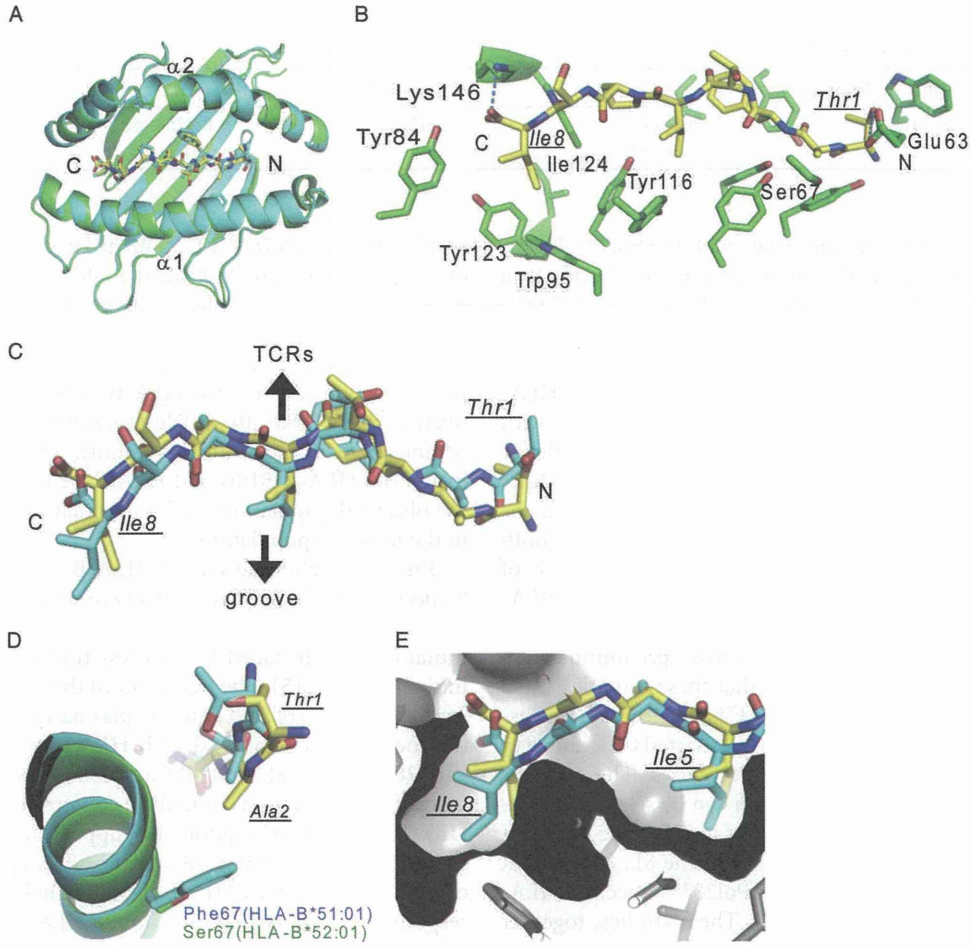

FIG 6 Structural comparison of HLA-B*52:01 and HLA-B*51:01 molecules complexed with the Pol283-8 peptide. (A) Crystal structures of HLA α1-α2 domains complexed with the Pol283-8 peptide (stick model) on the HLA-B*52:01 (green, yellow) and HLA-B*51:01 (cyan, cyan) complexes. This same coloring also applies to panels B to E. (B) Pol283-8 peptide and interacting side chains on the HLA-B*52:01 complex. Hydrogen bonds are shown as blue dotted lines. (C) Comparison of the Pol283-8 peptide conformations of HLA-B*52:01 and HLA-B*51:01 complexes. (D) N-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. (E) C-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. Surface presentation for the α1-α2 domains is shown in gray.

we had previously reported (45). This finding explains the cross presentation of this peptide by both HLA alleles. On the other hand, there was a notable conformational difference in the N-terminal region of the peptide between the two alleles (Fig. 6C and D). The replacement of Phe67 of HLA-B*51:01 with Ser in HLA-B*52:01 makes a local space, causing the N-terminal region of the peptide (T1 and A2) to reside deeper in the peptide-binding groove. Furthermore, the Gln63Glu mutation in HLA-B*52:01 affords a new interaction with the T1 residue of the peptide. These changes would, to some extent, have hidden the side chains of T1 and A2 (flat surface) from the TCRs, which may have reduced their interactions with TCRs on the HLA-B*52:01-restricted CTLs. On the other hand, the conformation of the C-terminal region of the peptide complexed with HLA-B*51:01 or HLA-B*52:01 was similar, even though C-terminal Ile8 of the peptide exhibited shallower penetration of the hydrophobic groove in the case of HLA-B*52:01 than in that of HLA-B*51:01 (Fig. 6C and E). These results may indicate that the relatively flat surface of the N-terminal side of the peptide contributed to the lower affinity for TCRs in the case of HLA-B*52:01.

DISCUSSION

HLA-B*52:01 and HLA-B*51:01 differ by only two residues, at positions 63 and 67 (44). Substitutions at these residues affect the formation of the B pocket in the peptide-binding pocket (45), suggesting the possibility that HLA-B*52:01 has a peptide motif different from that of HLA-B*51:01. Indeed, HLA-B*52:01-binding peptides have P2 primary anchors that are different from HLA-B*51:01-binding ones (30, 46). Since the Pol283-8 epitope carries Ala at its second position and Ile at the C terminus of the peptide, it is likely that this peptide would effectively bind to HLA-B*51:01 but not to HLA-B*52:01. However, the results of the HLA stabilization assay demonstrated that the Pol283-8 peptide did effectively bind to HLA-B*52:01. Since the HLA-B*52:01-binding peptide is known to have Pro as its preferred P2 anchor residue, this peptide carrying Ala at position 2 may be capable of binding to HLA-B*52:01. A previous study showed cross-recognition of alloreactive T cells between HLA-B*51:01 and HLA-B*52:01 (47, 48), indicating that some self-peptides can be presented by both of these HLA class I molecules. The findings on the crystal structure

TABLE 3 Numbers and frequencies of individuals having I135X mutations in a Japanese cohort and a predominantly Caucasian cohort

Cohort	No./total no. (%) of individuals				
	B*51:01$^+$ B*52:01$^-$	B*51:01$^-$ B*52:01$^+$	B*51:01$^+$ B*52:01$^+$	B*51:01$^-$ B*52:01$^-$	Total
Japanese	51/51 (100)	42/49 (85.7)	5/5 (100)	88/151 (58.3)	186/256 (72.6)
Caucasian	125/131 (95.4)	17/26 (65.4)	0/0	331/1,198 (27.6)	473/1,355 (34.9)

of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide clarified that HLA-B*52:01 could bind to the peptide in a fashion similar to but slightly different from that of HLA-B*51:01. These findings support the presentation of the Pol283-8 peptide by both HLA-B*52:01 and HLA-B*51:01.

Pol283-8-specific CD8$^+$ T cells were detected in 7 of 14 HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals chronically infected with HIV-1. A previous analysis showed that CD8$^+$ T cells specific for this epitope are frequently detected in HLA-B*51:01$^+$ individuals chronically infected with HIV-1 (49). These results, taken together, indicate that this epitope is immunodominant in both HLA-B*51:01$^+$ and HLA-B*52:01$^+$ individuals. The analysis of 257 Japanese individuals revealed an association between HLA-B*52:01 and a variety of nonconsensus residues at RT codon 135 (I135X). Specifically, variants 8T, 8L, 8R, and 8V predominated in HLA-B*52:01$^+$ individuals, suggesting that these mutations had been selected by HLA-B*52:01-restricted CTLs. The viral suppression assay revealed that the HLA-B*52:01-restricted CTLs failed to suppress the replication of these mutant viruses. These results support the idea that the I135X mutation can be selected by immune pressure via Pol283-8-specific CTLs in HLA-B*52:01$^+$ individuals. Our previous studies showed that the 8L, 8T, and 8R mutations affected the recognition by Pol283-8-specific, HLA-B*51:01-restricted CTL clones (15, 28). These studies, together with the present study, indicate that accumulation of 8L, 8T, and 8R mutations in the HIV-infected Japanese population may be due to immune pressure by both HLA-B*52:01-restricted and HLA-B*51:01-restricted CTLs. Our analysis of the crystal structure of the HLA-B*52:01-peptide complex demonstrated that position 8 of the Pol283-8 peptide was deeply packed into the hydrophobic groove. Whereas the 8L, 8T, and 8R substitutions likely had a relatively large effect on the structure of the complex, the 8V mutation, resulting in only the deletion of the small methyl group, caused only very limited changes. Thus, the structural analysis supports the idea that the 8L, 8T, and 8R mutations affected the TCR recognition of the peptide and/or its binding to HLA-B*52:01.

The present study confirmed previous studies of nine worldwide cohorts (15) and a Chinese cohort (50) that showed a strong association of I135X with HLA-B*51:01. The I135X mutation was found in 58.3 and 27.6% of HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese and predominantly Caucasian individuals, respectively (Table 3), supporting greater population level accumulation of this mutation in Japanese than in other cohorts. Since the Japanese cohort included twice as many HLA-B*51:01$^+$ individuals as the IHAC cohort (21.9% of Japanese and 9.4% of Caucasians in IHAC), the difference in the I135X variant frequency between these two cohorts would be driven, to a large extent, by the higher HLA-B*51:01 prevalence in the former than in the latter. The association of HLA-B*52:01 with this mutation was much weaker than that of HLA-B*51:01 in both cohorts but still highly statistically significant (an lnOR of 11.7 [$P = 8.77 \times 10^{-4}$] versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] in the Japanese cohort and an lnOR of 3.06 [$P = 2.95 \times 10^{-5}$] versus an lnOR of 5.71 [$P = 1.58 \times 10^{-51}$] in IHAC). Because of the relatively low B*52:01$^+$ frequency (~2%) in IHAC, the effect of HLA-B*52:01 on the overall prevalence of I135X was relatively low in this cohort. In contrast, in the Japanese cohort, where the HLA-B*52:01$^+$ prevalence was relatively high (>20%), this allele represents a major driving force behind 135X selection in this cohort. Thus, selection pressure from both HLA-B*51:01 and HLA-B*52:01 likely contributed to the observed population level accumulation of I135X mutations in the Japanese population.

Previous studies showed that HLA-B*51:01-restricted, Pol283-8-specific CTLs have a strong ability to suppress HIV-1 replication *in vitro* (28) and that they suppressed the replication of the 8V mutant virus but failed to suppress that of the 8T, 8L, and 8R mutant viruses (15). The frequency of the Pol283-8-specific CTLs was inversely correlated with the plasma viral load in HLA-B*51:01$^+$ hemophiliacs infected with HIV-1 approximately 30 years ago (28). The 8T, 8L, and 8R mutations did not affect replication capacity, whereas the 8V mutation conferred a modest fitness cost (15). These findings support the suppression of the wild-type or 8V mutant virus by Pol283-8-specific CTLs as a major mechanism of slow progression to AIDS in Japanese hemophiliacs. This CTL response was also elicited in Chinese HLA-B*51:01$^+$ individuals infected with the 8V mutant virus; furthermore, a low viral load and a high CD4 count were significantly associated with the presence of at least one of three HLA-B*51:01-restricted CTL responses, including a Pol283-8-specific one (50). Thus, these findings support the idea that Pol283-8-specific CTLs play an important role in the control of HIV-1 infection.

The present study demonstrated that HLA-B*52:01-restricted, Pol283-8-specific CTLs also had a strong ability to suppress HIV-1 replication *in vitro* (80% suppression at an E/T cell ratio of 0.3:1). However, the ability of HLA-B*52:01-restricted CTLs to suppress the replication of HIV-1 was weaker than that of HLA-B*51:01-restricted CTLs (Fig. 5B). Inspection of the crystal structures of both HLA molecules complexed with the Pol283-8 peptide suggests that the relatively shallow penetration of the hydrophobic groove of HLA-B*52:01 by the C-terminal side of the peptide, in contrast to the tightly packed binding with HLA-B*51:01, may have resulted in an unstable conformation of the complex. Furthermore, Ser67 of HLA-B*52:01 would have provided more space and loose interactions with the peptide than in the case of the Phe of HLA-B*51:01. Interestingly, the Pol283-8 peptide would have displayed only side chains of Thr1 and Ser7, and some part of the main chains, to CTLs. Therefore, these results suggest that the unstable backbone conformation and side chain positions in the case of HLA-B*52:01 largely contributed to the lower TCR affinity than that afforded by HLA-B*51:01. These results support that selection pressure *in vivo* via the HLA-B*52:01-restricted CTLs would be weaker than that via the HLA-B*51:01-restricted CTLs. Indeed, the prevalence of I135X mutations in HLA-B*51:

01^+ individuals was higher than that in HLA-B*52:01^+ individuals. The difference in the pattern of escape mutant selection by these CTLs between the HLA-B*51:01^+ and HLA-B*52:01^+ individuals might also have been due to the difference in their abilities to suppress HIV-1 replication. However, it still remains unclear why the 8T mutant was predominantly selected in the HLA-B*51:01^+ but not in the HLA-B*52:01^+ individuals. Further studies are expected to clarify the mechanism to explain how these CTLs selected different patterns of mutations at RT135.

Previous studies showed that the T242N mutant was selected by HLA-B*58:01-restricted and HLA-B*57-restricted CTLs specific for TW10 epitope in HIV-1 clade B-infected and clade C-infected individuals (25–27). Herein we also showed that I135X was selected by Pol283-8-specific CTLs restricted by two different HLA class I molecules. However, the strength and the pattern of the selection of I135X was different between HLA-B*51:01 and HLA-B*52:01. The present study suggests that this difference in the selection pattern was associated with that between the HLA-B*51:01^+ and HLA-B*52:01^+ individuals in terms of the ability of Pol283-specific CTLs to suppress HIV-1 replication. Thus, we characterized and experimentally validated distinct HIV-1 escape patterns of CTLs with the same epitope specificity and provided evidence that the extremely high prevalence of 135X in circulating Japanese sequences is likely driven not by one but by two HLA-B alleles.

ACKNOWLEDGMENTS

We thank Sachiko Sakai for her secretarial assistance, as well as Richard Harrigan and the BC Centre for Excellence in HIV/AIDS for data access. The assistance of Jennifer Listgarten and Carl Kadie (Microsoft Research) with HLA allele interpretation is also gratefully acknowledged.

N.K. is a JSPS Research Fellow. Z.L.B. is a recipient of a Canadian Institutes of Health Research (CIHR) New Investigator Award and a Michael Smith Foundation for Health Research (MSFHR) Scholar award. This research was supported 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, Japan, and by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan (18390141 and 20390134).

We have no financial conflicts of interest.

REFERENCES

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. 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.
2. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. 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.
3. Rinaldo C, Huang XL, Fan ZF, Ding M, Beltz L, Logar A, Panicali D, Mazzara G, Liebmann J, Cottrill M. 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. J. Virol. 69:5838–5842.
4. Matano T, Shibata R, Siemon C, Connors M, Lane HC, Martin MA. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. 72:164–169.
5. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 279:2103–2106.
6. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL, Reimann KA. 1999. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science 283:857–860.
7. Gandhi RT, Walker BD. 2002. Immunologic control of HIV-1. Annu. Rev. Med. 53:149–172.
8. Goulder PJ, Watkins DI. 2008. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. 8:619–630.
9. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, Nelson JA, Gairin JE, Hahn BH, Oldstone MB, Shaw GM. 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.
10. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, Giangrande P, Luzzi G, Morgan B, Edwards A, McMichael AJ, Rowland-Jones S. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. 3:212–217.
11. Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, Addo MM, Rosenberg ES, Nguyen T, Allen R, Trocha A, Altfeld M, He S, Bunce M, Funkhouser R, Pelton SI, Burchett SK, McIntosh K, Korber BT, Walker BD. 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature 412:334–338.
12. Moore CB, John M, James IR, Christiansen FT, Witt CS, Mallal SA. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science 296:1439–1443.
13. Draenert R, Le Gall S, Pfafferott KJ, Leslie AJ, Chetty P, Brander C, Holmes EC, Chang SC, Feeney ME, Addo MM, Ruiz L, Ramduth D, Jeena P, Altfeld M, Thomas S, Tang Y, Verrill CL, Dixon C, Prado JG, Kiepiela P, Martinez-Picado J, Walker BD, Goulder PJ. 2004. Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection. J. Exp. Med. 199:905–915.
14. Leslie A, Kavanagh D, Honeyborne I, Pfafferott K, Edwards C, Pillay T, Hilton L, Thobakgale C, Ramduth D, Draenert R, Le Gall S, Luzzi G, Edwards A, Brander C, Sewell AK, Moore S, Mullins J, Moore C, Mallal S, Bhardwaj N, Yusim K, Phillips R, Klenerman P, Korber B, Kiepiela P, Walker B, Goulder P. 2005. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. J. Exp. Med. 201:891–902.
15. Kawashima Y, Pfafferott K, Frater J, Matthews P, Payne R, Addo M, Gatanaga H, Fujiwara M, Hachiya A, Koizumi H, Kuse N, Oka S, Duda A, Prendergast A, Crawford H, Leslie A, Brumme Z, Brumme C, Allen T, Brander C, Kaslow R, Tang J, Hunter E, Allen S, Mulenga J, Branch S, Roach T, John M, Mallal S, Ogwu A, Shapiro R, Prado JG, Fidler S, Weber J, Pybus OG, Klenerman P, Ndung'u T, Phillips R, Heckerman D, Harrigan PR, Walker BD, Takiguchi M, Goulder P. 2009. Adaptation of HIV-1 to human leukocyte antigen class I. Nature 458:641–645.
16. Avila-Rios S, Ormsby CE, Carlson JM, Valenzuela-Ponce H, Blanco-Heredia J, Garrido-Rodriguez D, Garcia-Morales C, Heckerman D, Brumme ZL, Mallal S, John M, Espinosa E, Reyes-Teran G. 2009. Unique features of HLA-mediated HIV evolution in a Mexican cohort: a comparative study. Retrovirology 6:72. doi:10.1186/1742-4690-6-72.
17. Boutwell CL, Essex M. 2007. Identification of HLA class I-associated amino acid polymorphisms in the HIV-1C proteome. AIDS Res. Hum. Retroviruses 23:165–174.
18. Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, Kadie C, Bhattacharya T, Chui C, Szinger J, Mo T, Hogg RS, Montaner JS, Frahm N, Brander C, Walker BD, Harrigan PR. 2007. Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. PLoS Pathog. 3:e94. doi:10.1371/journal.ppat.0030094.
19. Carlson JM, Listgarten J, Pfeifer N, Tan V, Kadie C, Walker BD, Ndung'u T, Shapiro R, Frater J, Brumme ZL, Goulder PJ, Heckerman D. 2012. Widespread impact of HLA restriction on immune control and escape pathways of HIV-1. J. Virol. 86:5230–5243.
20. Carlson JM, Brumme ZL, Rousseau CM, Brumme CJ, Matthews P, Kadie C, Mullins JI, Walker BD, Harrigan PR, Goulder PJ, Heckerman D. 2008. Phylogenetic dependency networks: inferring patterns of CTL escape and codon covariation in HIV-1 Gag. PLoS Comput. Biol. 4:e1000225. doi:10.1371/journal.pcbi.1000225.
21. Rousseau CM, Daniels MG, Carlson JM, Kadie C, Crawford H, Pren-

dergast A, Matthews P, Payne R, Rolland M, Raugi DN, Maust BS, Learn GH, Nickle DC, Coovadia H, Ndung'u T, Frahm N, Brander C, Walker BD, Goulder PJ, Bhattacharya T, Heckerman DE, Korber BT, Mullins JI. 2008. HLA class I-driven evolution of human immunodeficiency virus type 1 subtype C proteome: immune escape and viral load. J. Virol. 82:6434–6446.

22. Crawford H, Lumm W, Leslie A, Schaefer M, Boeras D, Prado JG, Tang J, Farmer P, Ndung'u T, Lakhi S, Gilmour J, Goepfert P, Walker BD, Kaslow R, Mulenga J, Allen S, Goulder PJ, Hunter E. 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.
23. Honeyborne I, Prendergast A, Pereyra F, Leslie A, Crawford H, Payne R, Reddy S, Bishop K, Moodley E, Nair K, van der Stok M, McCarthy N, Rousseau CM, Addo M, Mullins JI, Brander C, Kiepiela P, Walker BD, Goulder PJ. 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.
24. Naruto T, Murakoshi H, Chikata T, Koyanagi M, Kawashima Y, Gatanaga H, Oka S, Takiguchi M. 2011. Selection of HLA-B57-associated Gag A146P mutant by HLA-B*48:01-restricted Gag140-147-specific CTLs in chronically HIV-1-infected Japanese. Microbes Infect. 13:766–770.
25. Goulder PJ, Bunce M, Krausa P, McIntyre K, Crowley S, Morgan B, Edwards A, Giangrande P, Phillips RE, McMichael AJ. 1996. Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. AIDS Res. Hum. Retroviruses 12:1691–1698.
26. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, Tang Y, Holmes EC, Allen T, Prado JG, Altfeld M, Brander C, Dixon C, Ramduth D, Jeena P, Thomas SA, St John A, Roach TA, Kupfer B, Luzzi G, Edwards A, Taylor G, Lyall H, Tudor-Williams G, Novelli V, Martinez-Picado J, Kiepiela P, Walker BD, Goulder PJ. 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. 10:282–289.
27. Miura T, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, Rathod A, Block BL, Brumme ZL, Brumme CJ, Baker B, Rothchild AC, Li B, Trocha A, Cutrell E, Frahm N, Brander C, Toth I, Arts EJ, Allen TM, Walker BD. 2009. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J. Virol. 83:2743–2755.
28. Kawashima Y, Kuse N, Gatanaga H, Naruto T, Fujiwara M, Dohki S, Akahoshi T, Maenaka K, Goulder P, Oka S, Takiguchi M. 2010. Long-term control of HIV-1 in hemophiliacs carrying slow-progressing allele HLA-B*5101. J. Virol. 84:7151–7160.
29. Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. 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.
30. Falk K, Rotzschke O, Takiguchi M, Gnau V, Stevanovic S, Jung G, Rammensee HG. 1995. Peptide motifs of HLA-B51, -B52 and -B78 molecules, and implications for Behcet's disease. Int. Immunol. 7:223–228.
31. Tomiyama H, Fujiwara M, Oka S, Takiguchi M. 2005. Cutting edge: 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.
32. Tomiyama H, Akari H, Adachi A, Takiguchi M. 2002. Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8(+) T-cell cytolytic activity and cytokine production. J. Virol. 76:7535–7543.
33. Takamiya Y, Schonbach C, Nokihara K, Yamaguchi M, Ferrone S, Kano K, Egawa K, Takiguchi M. 1994. HLA-B*3501-peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. Int. Immunol. 6:255–261.
34. Huang KH, Goedhals D, Carlson JM, Brockman MA, Mishra S, Brumme ZL, Hickling S, Tang CS, Miura T, Seebregts C, Heckerman D, Ndung'u T, Walker B, Klenerman P, Steyn D, Goulder P, Phillips R, Bloemfontein-Oxford Collaborative Group, van Vuuren C, Frater J. 2011. Progression to AIDS in South Africa is associated with both reverting and compensatory viral mutations. PLoS One 6:e19018. doi:10.1371/journal.pone.0019018.
35. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94–96.
36. Kabsch W. 2010. XDS. Acta Crystallogr. D Biol. Crystallogr. 66(Pt 2):125–132.
37. Evans PR. 1993. Proceedings of the CCP4 Study Weekend on Data Collection & Processing. Daresbury Laboratory, Warrington, United Kingdom.
38. Matthews BW. 1968. Solvent content of protein crystals. J. Mol. Biol. 33:491–497.
39. Vagin A, Teplyakov A. 1997. MOLREP: an automated program for molecular replacement. J. Appl. Crystallogr. 30:1022–1025.
40. Murshudov GN, Vagin AA, Dodson EJ. 1997. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53:240–255.
41. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66:213–221.
42. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26:283–291.
43. Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66:486–501.
44. Sakaguchi T, Ibe M, Miwa K, Yokota S, Tanaka K, Schonbach C, Takiguchi M. 1997. Predominant role of N-terminal residue of nonamer peptides in their binding to HLA-B* 5101 molecules. Immunogenetics 46:245–248.
45. Maenaka K, Maenaka T, Tomiyama H, Takiguchi M, Stuart DI, Jones EY. 2000. Nonstandard peptide binding revealed by crystal structures of HLA-B*5101 complexed with HIV immunodominant epitopes. J. Immunol. 165:3260–3267.
46. Sakaguchi T, Ibe M, Miwa K, Kaneko Y, Yokota S, Tanaka K, Takiguchi M. 1997. Binding of 8-mer to 11-mer peptides carrying the anchor residues to slow assembling HLA class I molecules (HLA-B*5101). Immunogenetics 45:259–265.
47. Hiraiwa M, Yamamoto J, Matsumoto K, Karaki S, Nagao T, Kano K, Takiguchi M. 1991. T cell can recognize the allospecificities formed by the substitution of amino acids associated with HLA-Bw4/Bw6 public epitopes. Hum. Immunol. 32:41–45.
48. Yamamoto J, Hiraiwa M, Hayashi H, Tanabe M, Kano K, Takiguchi M. 1991. Two amino acid substitutions at residues 63 and 67 between HLA-B51 and HLA-Bw52 form multiple epitopes recognized by allogeneic T cells. Immunogenetics 33:286–289.
49. Tomiyama H, Sakaguchi T, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M. 1999. Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules. Hum. Immunol. 60:177–186.
50. Zhang Y, Peng Y, Yan H, Xu K, Saito M, Wu H, Chen X, Ranasinghe S, Kuse N, Powell T, Zhao Y, Li W, Zhang X, Feng X, Li N, Leligdowicz A, Xu X, John M, Takiguchi M, McMichael A, Rowland-Jones S, Dong T. 2011. Multilayered defense in HLA-B51-associated HIV control. J. Immunol. 187:684–691.

Hindawi Publishing Corporation
AIDS Research and Treatment
Volume 2012, Article ID 246280, 9 pages
doi:10.1155/2012/246280

Clinical Study

Differences in Lipid Measurements by Antiretroviral Regimen Exposure in Cohorts from Asia and Australia

Amit C. Achhra,[1] Janaki Amin,[1] Jennifer Hoy,[2] Junko Tanuma,[3] Thira Sirisanthana,[4] David Nolan,[5] Tuti Merati,[6] and Michelle Giles[2]

[1] *The Kirby Institute for Infection and Immunity in Society (Formerly the National Centre in HIV Epidemiology and Clinical Research), Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia*
[2] *Infectious Diseases Unit, Alfred Hospital and Monash University, Melbourne, VIC 3004, Australia*
[3] *AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo 162-8655, Japan*
[4] *Research Institute for Health Sciences, Chiang Mai University, Chiang Mai 50200, Thailand*
[5] *Centre for Clinical Immunology and Biomedical Statistics, Murdoch University and Royal Perth Hospital, Perth, Australia*
[6] *Faculty of Medicine, Udayana University and Sanglah Hospital, Bali 80233, Indonesia*

Correspondence should be addressed to Amit C. Achhra, aachhra@kirby.unsw.edu.au

Received 24 October 2011; Accepted 11 March 2012

Academic Editor: Ann Duerr

We explored the mean differences in routinely measured lipids (total cholesterol, triglycerides, and high-density lipoprotein cholesterol) according to exposure to different combination antiretroviral regimens in Asian (n = 2051) and Australian (predominantly Caucasian, n = 794) cohorts. The regimen was defined as at least 3 antiretroviral drugs with at least 2 nucleoside-reverse transcriptases (NRTIs) and either of at least one protease inhibitor (PI) or non-nucleoside-reverse transcriptases (NNRTIs). We categorised cART regimens as: NRTIs as tenofovir based or not; NNRTIs as nevirapine or efavirenz (but not both); and PI as atazanavir based or not. We found that the impact of various antiretroviral regimens on lipids in Asian and Australian cohorts was only different by cohort for total cholesterol (P for interaction between regimen and cohort: <0.001) but not in case of other lipids (P for interaction: >0.05). The differences in total cholesterol were however small and unlikely to be of clinical significance. Overall, tenofovir with nevirapine or atazanavir was associated with the most favorable lipids, while the PI regimens without tenofovir and atazanavir were associated with least favorable lipids. We conclude that the impact of various ART regimens on lipids is largely similar in Asian and Australian cohorts and that the newer drugs such as tenofovir and atazanavir are likely to provide similar benefit in terms of lipid profiles in both populations.

1. Introduction

Combination antiretroviral therapy (cART) for HIV infection is associated with adverse changes in lipid profiles and can include elevation in total cholesterol and triglycerides, which may increase the risk of coronary heart disease (CHD) [1–4]. Moreover, different classes of cART and drugs within each class have differential impacts on lipids [2]. Protease-inhibitors (PIs) are associated with more significant changes in lipid profile than nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, resp.) [2, 3, 5]. And within NNRTI class, efavirenz (EFV) is associated with greater changes in the lipid profile than nevirapine (NVP) [2, 5, 6]. Also tenofovir (TDF) and atazanavir (ATV) are known to have a favorable impact on lipids [5, 7, 8].

Drugs such as TDF, EFV, and ATV are becoming increasingly available in low-middle-income countries, including Asia [9, 10]. However, much of our knowledge about the relative impact of different cART regimens on lipids comes mainly from clinical trials and cohort studies from European or North-American settings [2, 4, 7, 8]. The impact of cART on lipids may vary in Asian settings due to differences in race/ethnicity, dietary, environmental, and lifestyle factors [11–13]. This has been demonstrated in other settings

where the magnitude of change in total cholesterol and triglycerides due to PIs differed between African Americans and Caucasians, highlighting the possible role of race [11, 12]. These findings illustrate the need for verifying our assumptions about the relative impact of different cART regimens on diverse populations, including Asian populations.

Observational cohort studies can complement information from clinical trials, and allow us to examine the effects of art medications in the context of combination regimens, as opposed to head-to-head comparisons of selected drugs in clinical trials. In the present study, we aim to compare the relative impact of various cart regimens on lipid profiles in Asian and Australian cohorts using data from the treat Asia and the Australian HIV observational databases (TAHOD and AHOD, resp.), which are formed on similar methodology and are known to be predominantly Asian and Caucasian, respectively [14].

2. Methods

2.1. The TAHOD and AHOD Cohorts. TAHOD and AHOD are clinical cohort studies of HIV-infected patients in Asia and Australia, respectively, and are part of the International Epidemiologic Databases to evaluate AIDS initiative. Both cohorts have similar methodologies, which have been previously published [15, 16]. Briefly, prospective data collection was commenced in 2003 for TAHOD and in 1999 for AHOD, with retrospective data being provided where available. In TAHOD, data are collected from 17 clinical sites in the Asian region, whereas for AHOD, data are collected from 27 clinical sites throughout Australia. Written informed consent was not a requirement of sites in TAHOD unless required by the site's local ethics committee because data are collected in an anonymous form, while in AHOD consent was obtained from all patients recruited at the time of enrolment. The TAHOD and AHOD cohorts are known to be predominantly of Asian and Caucasian ethnic composition, respectively [14].

Ethical approval for both the cohorts was obtained from the University of New South Wales, Sydney, Australia, and all other relevant institutional review boards. Data for both TAHOD and AHOD are transferred electronically to the Kirby Institute twice per year and include the same set of core variables. All data are subject to standardized quality control procedures.

2.2. Outcome. The outcomes of interest were mean (i) total cholesterol, (ii) triglycerides, (iii) high-density lipoprotein cholesterol (HDL-C) measured in mmol/L, and (iv) total cholesterol : HDL-C ratio. Lipid values are measured according to the local sites' standard of care in each cohort, and when measured, are captured during routine data transfer. TAHOD only records fasting lipids. In AHOD, both fasting and nonfasting lipids along with the fasting status are recorded. Further details of laboratory standards and methods at each site were not available.

Data collection on lipid profiles started later in AHOD (median date: January, 2007), compared to TAHOD (median date: March, 2006). The lipid values before starting cART (i.e., while patients were ART naive) were not available in most patients, and therefore changes from pre- to post-cART were not analysed. Mean lipid measurements were compared by different regimens and cohort.

2.3. Definition and Classification of Antiretroviral Regimens. The cART regimen variable was defined as a regimen containing at least 3 antiretroviral drugs, including at least two NRTIs and either of at least one PI or an NNRTI. In order to evaluate the net effect of a combination regimen, rather than that of a single drug or a class, we defined eight mutually exclusive regimens. We categorised cART regimens as: NRTIs as TDF based (NRTIs + TDF) or not (NRTIs); NNRTIs as NVP or EFV (but not both); and PI as ATV based (PI + ATV) or not (PI).

Based on these categories, the following mutually exclusive regimens were defined: (i) NRTIs (+TDF) + NVP; (ii) NRTIs (+TDF) + EFV; (iii) NRTIs + NVP; (iv) NRTIs + EFV; (v) NRTIs (+TDF) + PIs (+ATV); (vi) NRTIs (+TDF) + PI; (vii) NRTIs + PI (+ATV); (viii) NRTIs + PI. In all analyses, regimen (i) NRTIs (+TDF) + NVP was used as the reference group, as this regimen was thought to have the most favourable impact on lipids.

2.4. Inclusion Criteria and Time-Points Analysed. Patients from TAHOD and AHOD were eligible for inclusion in the analysis if they started cART and had at least one lipid measurement within the first 24 months of cART commencement. Time at risk was defined as time spent on any of the regimens described previously and risk time started from the commencement of that regimen. Follow-up was censored at first of 24-month exposure to regimen of interest, date of death, loss to follow-up, or 31 March, 2010. Lipids values measured at the 6-monthly intervals in the first 24 months of start of cART were used. Thus each patient on each regimen could have up to 4 measurements (1 in each interval). If more than one measurement was available in a given interval, one measured earliest in the given interval was used in the analysis. Intermittent changes in therapy including stopping part or all of a regimen for less than 14 days were not considered a stop in time at risk for that regimen. Each patient could contribute data to more than one regimen.

2.5. Variables and Statistical Analysis. The following *a priori* confounders were included in all models:

(1) fixed variables: cohort (TAHOD/AHOD), gender, HIV transmission group (homosexual contact ± intravenous drug user (IDU), IDU ± heterosexual, heterosexual, and other), and hepatitis B and C coinfection (defined as HBV surface antigen and HCV antibody positive, resp.);

(2) variables measured closest to the start of each cART regimen within past 6 months to 1 month after the

start of the regimen of interest: CD4+ T-cell count (categorised as <200, 200–350, and >350 cells/μL); HIV RNA viral load (categorised as <500, >500–<10,000, and >10,000 copies/mL); and body mass index (BMI) (categorised as <18.5, 18.5–25, 25–30, and >30 kg/m^2);

(3) variables recalculated at the start of each cART regimen: cumulative cART exposure and age.

We performed longitudinal data analysis using random effects models to take into account repeated lipid measures (defined previously). Since all lipid parameters were normally distributed with minimal skewness, data were not transformed. Separate models were fitted for each outcome. All models included time on regimen with lipid data, categorised as 6 monthly intervals. The interaction between the regimen and the cohort variables was assessed for each outcome. We also conducted the following sensitivity analyses: (i) restricting AHOD data to only lipid values which were documented to be taken as fasting in AHOD, (ii) excluding patients with missing BMI data, (iii) including only those with known Caucasian ethnicity in AHOD, and (iv) additional adjustment for stavudine (d4t) use in the multivariable model, as it was more common in TAHOD than AHOD.

Data were analysed using STATA version 10 (STATA Corporation, College Station, TX, USA).

3. Results

There were 2845 participants (2051 in TAHOD and 794 in AHOD) who met the inclusion criteria. In TAHOD, 736 (35.9%) were Chinese, 654 (31.9%) were Thai, 152 (7.4%) were Cambodian (Khmer), 100 (4.9%) were Japanese and 62 (3%) were Indian. Table 1 describes the patient characteristics at study entry for each cohort.

There were a total of 7897 total cholesterol values (5602 in TAHOD and 2295 in AHOD), 7293 triglyceride values (5002 in TAHOD and 2291 in AHOD), and 4669 HDL-C values (2949 in TAHOD and 1720 in AHOD). The frequency of total cholesterol measurements by regimen and cohort is shown in Table 2. The most common NRTI combinations for which total cholesterol measurements were available in TAHOD were zidovudine (AZT)/lamivudine (3TC) (39% of all measurements) and d4t/3TC (30% of all measurements) and in AHOD TDF/emtricitabine (FTC) (29% of all measurements) and abacavir (ABC)/3TC (18% of all measurements). The distribution was similar for triglycerides and HDL-C. In TAHOD and AHOD, 52% and 46% of measurements were taken while on NNRTI-based regimens, respectively. Of all the measurements on PI-based regimens, greater than 95% were on ritonavir-boosted regimens.

Patients contributed data to the median of 1 regimen (range: 1 to 4) with a median of 2 lipid measurements (IQR: 1–4) per patient. All measures of CD4 cell count, HIV viral load, and BMI were collected within 35 days of commencing the different ART regimens. Participants from TAHOD were more likely to be younger and female and have heterosexually acquired infection, hepatitis B coinfection, detectable HIV VL, lower median CD4+ count, shorter time spent on cART, lower BMI, lower mean total cholesterol, and higher HDL-C than those from AHOD (Table 1). Also a higher proportion of TAHOD participants had missing BMI and HIV viral load values compared with those in AHOD (Table 1).

3.1. Total Cholesterol. The relationship between mean total cholesterol and cART regimen differed by cohort (TAHOD/AHOD) ($P < 0.001$, test for interaction). Overall, the mean total cholesterol was slightly lower for TAHOD participants, compared to AHOD participants, after adjustment for demographic and HIV-related characteristics, in most of the regimens (Figure 1(a)). When compared to the NRTIs (+TDF) + NVP regimen (reference group), the NRTIs + PI regimen was associated with greater mean total cholesterol in both cohorts, with a slightly greater difference in AHOD participants (mean difference: +0.78 mmol/L, 95% CI: 0.57 to 1.00) compared to TAHOD participants (mean difference: +0.23 mmol/L, 95% CI: 0.02 to 0.44); NRTIs (+TDF) + PI (+ATV) regimen was associated with greater mean total cholesterol in AHOD (mean difference: −0.20 mmol/L, 95% CI: −0.43 to 0.02) as compared to TAHOD (mean difference: −0.62 mmol/L, 95% CI: −1.23 to −0.02).

3.2. Triglycerides, HDL-C, and Total Cholesterol: HDL-C Ratio. There was no significant interaction between cART regimen and the cohort type ($P > 0.05$, test for interaction) for triglycerides, HDL-C, and total cholesterol: HDL-C ratio. Table 3 provides adjusted analyses for each of these outcomes. As compared to the NRTIs (+TDF) + NVP regimen (reference group), the NRTIs + PI regimen was associated with the highest mean triglycerides (mean difference: 1.13 mmol/L, 95% CI: 0.83 to 1.43) and total cholesterol : HDL-C ratio (mean difference: 0.75, 95% CI: 0.47 to 1.03), followed by the NRTIs (+TDF) + PI regimen (mean difference in triglycerides: 1.06 mmol/L, 95% CI: 0.73 to 1.38, and mean difference in total cholesterol : HDl-C ratio: 0.66, 95% CI: 0.37 to 0.95), while NRTIs (+TDF) + PI (+ATV) regimen was not associated with a significant difference in triglycerides (mean difference: 0.15 mmol/L, 95% CI: −0.22 to 0.52) and total cholesterol : HDL-C ratio (mean difference: 0.29, 95% CI: −0.04 to 0.62). Also, the NRTIs + EFV regimen was associated with increase in triglycerides (mean difference: 0.64 mmol/L, 95% CI: 0.34 to 0.95) and total cholesterol : HDL-C ratio (mean difference: 0.29, 95% CI 0.01 to 0.56). The TAHOD cohort, as compared to AHOD, had higher mean triglycerides, but not total cholesterol : HDL-C ratio. Figures 1(b) and 1(c) provide the graphical representation of the adjusted mean triglycerides and total cholesterol : HDL-C ratio for each regimen and cohort, respectively.

When compared to the reference group, the NRTIs + NVP regimen and the NRTIs + EFV regimen were associated with higher mean HDL-C (mean difference of: 0.15 mmol/L, 95% CI: 0.08 to 0.21 and 0.09 mmol/L, 95% CI: 0.03 to 0.16,

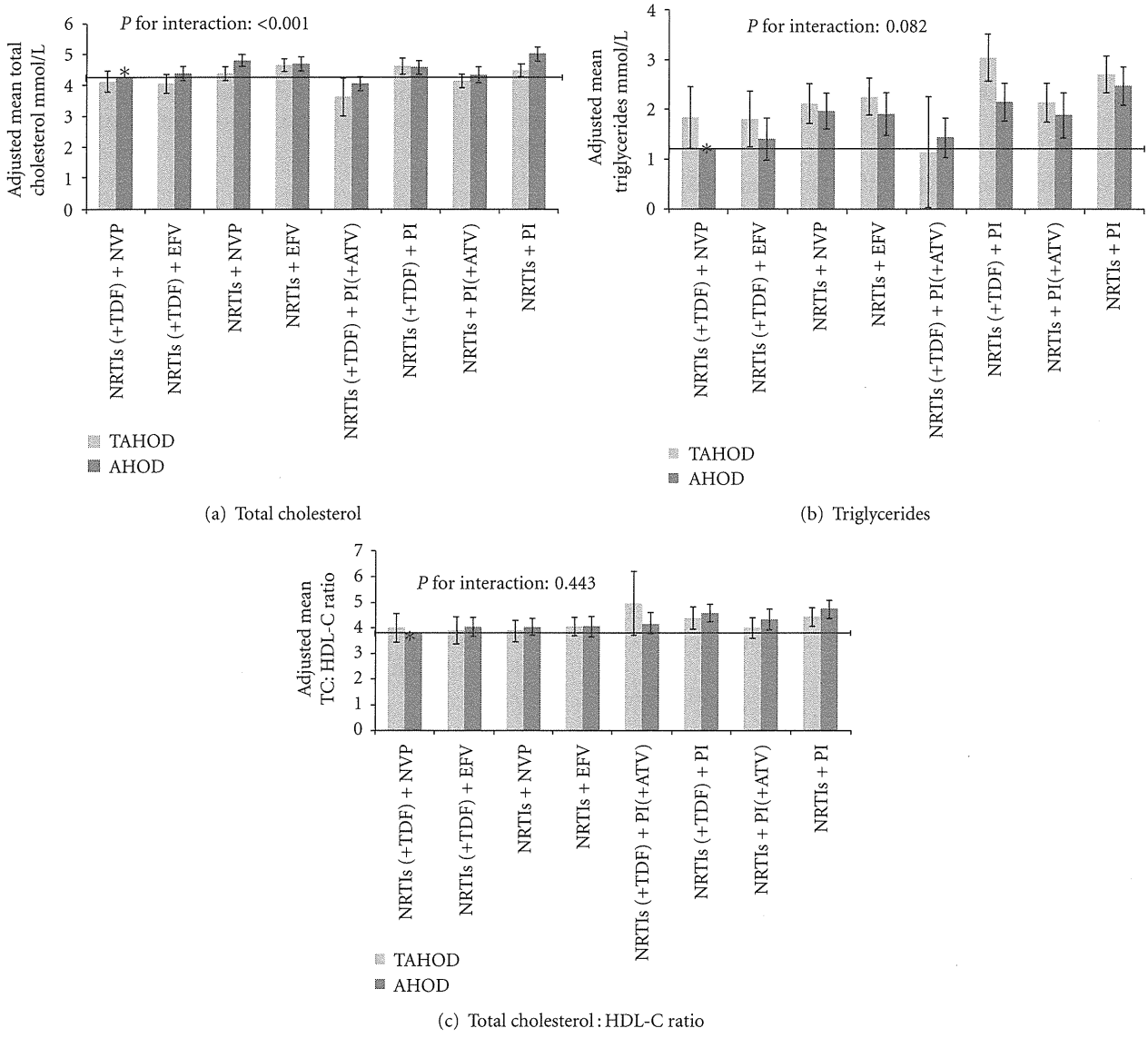

FIGURE 1: Adjusted Mean Lipids by regimen and cohort. Interaction between impact of regimen and cohort variables on lipids: (a) mean total cholesterol, (b) mean triglycerides, and (c) mean total cholesterol : HDL-C ratio. A statistically significant interaction suggests impact of regimen on lipids differed in magnitude by the cohort. Triglycerides were higher for TAHOD as compared to AHOD irrespective of the regimen, but the interaction between regimen and cohort was not significant. Means were *a priori* adjusted for time on given regimen, HBV and HCV confections, age, gender, HIV RNA viral load copies/mL, CD4+ T-cell count, BMI, cumulative exposure to cART at baseline, and HIV exposure category. *Reference category. Horizontal line shows the value of constant (mean lipid value in reference category). Error bars indicate 95% confidence interval. AHOD: Australian HIV Observational Database; ATV: Atazanavir; EFV: Efavirenz; NNRTIs: nonnucleoside reverse transcriptase inhibitors; NRTI: Nucleoside reverse transcriptase inhibitors; NVP: Nevirapine; PI: protease inhibitor; TAHOD: TREAT Asia HIV Observational Database; TDF: Tenofovir. *Key*. NRTIs are TDF based (NRTIs + TDF) or not (NRTIs) and PI as ATV based (PI + ATV) or not (PI).

resp.). Other regimens were not significantly different to the reference regimen.

3.3. Sensitivity Analyses. Forty-five percent of all of the AHOD measurements were taken fasting. Ethnicity was known in AHOD in 80% of participants, of whom greater than 80% were Caucasian. All of the sensitivity analyses, except for exclusion of missing BMI data, yielded very similar results, in terms of direction of effect, magnitude, and significance, as those from full analyses (data not presented). Since BMI was missing in a significant proportion of participants in both cohorts (Table 1), restriction of analysis to only patients with known BMI provided results that were of similar direction and magnitude of the effect, however in some cases less statistically significant, because of loss of power.