

21. Tominaga M, Kawakami N, Ono Y, Nakane Y, Nakamura Y et al. (2009) Prevalence and correlates of illicit and non-medical use of psychotropic drugs in Japan: findings from the World Mental Health Japan Survey 2002-2004. *Soc Psychiatry Psychiatr Epidemiol* 44: 777-783. doi:10.1007/s00127-009-0499-1. PubMed: 19190833.
22. Wada K, Greberman SB, Konuma K, Hirai S (1999) HIV and HCV infection among drug users in Japan. *Addiction* 94: 1063-1069. doi: 10.1046/j.1360-0443.1999.947106311.x. PubMed: 10707444.
23. Vandembroucke JP, von Elm E, Altman DG, Gøtzsche PC, Mulrow CD et al. (2007) Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *Epidemiology* 18: 805-835. doi:10.1097/EDE.0b013e3181577511. PubMed: 18049195.
24. Ndiaye B, Ould-Kaci K, Salleron J, Bataille P, Bonnevie F et al. (2009) Incidence rate and risk factors for loss to follow-up in HIV-infected patients from five French clinical centres in Northern France - January 1997 to December 2006. *Antivir Ther* 14: 567-575. PubMed: 19578242.
25. Rice BD, Delpech VC, Chadborn TR, Elford J (2011) Loss to follow-up among adults attending human immunodeficiency virus services in England, Wales, and Northern Ireland. *Sex Transm Dis* 38: 685-690. PubMed: 21844719.
26. Hidaka Y, Ichikawa S, Koyano J, Urao M, Yasuo T et al. (2006) Substance use and sexual behaviours of Japanese men who have sex with men: a nationwide internet survey conducted in Japan. *BMC Public Health* 6: 239. doi:10.1186/1471-2458-6-239. PubMed: 17002800.
27. Giordano TP, Hartman C, Gifford AL, Backus LI, Morgan RO (2009) Predictors of retention in HIV care among a national cohort of US veterans. *HIV Clin Trials* 10: 299-305. doi:10.1310/hct1005-299. PubMed: 19906622.
28. Qian HZ, Stinnette SE, Rebeiro PF, Kipp AM, Shepherd BE et al. (2011) The relationship between injection and noninjection drug use and HIV disease progression. *J Subst Abuse Treat* 41: 14-20. doi: 10.1016/j.jsat.2011.01.007. PubMed: 21349679.
29. Mocroft A, Kirk O, Aldins P, Chies A, Blaxhult A et al. (2008) Loss to follow-up in an international, multicentre observational study. *HIV Med* 9: 261-269. doi:10.1111/j.1468-1293.2008.00557.x. PubMed: 18400074.
30. Lebouché B, Yazdanpanah Y, Gérard Y, Sissoko D, Ajana F et al. (2006) Incidence rate and risk factors for loss to follow-up in a French clinical cohort of HIV-infected patients from January 1985 to January 1998. *HIV Med* 7: 140-145. doi:10.1111/j.1468-1293.2006.00357.x. PubMed: 16494627.
31. Latkin CA, Knowlton AR, Sherman S (2001) Routes of drug administration, differential affiliation, and lifestyle stability among cocaine and opiate users: implications to HIV prevention. *J Subst Abuse* 13: 89-102. doi:10.1016/S0899-3289(01)00070-0. PubMed: 11547627.
32. Lankenau SE, Teti M, Silva K, Jackson Bloom J, Harocopos A et al. (2012) Initiation into prescription opioid misuse amongst young injection drug users. *Int J Drug Policy* 23: 37-44. doi:10.1016/j.drugpo.2011.05.014. PubMed: 21689917.
33. Pollini RA, Banta-Green CJ, Cuevas-Mota J, Metzner M, Teshale E et al. (2011) Problematic use of prescription-type opioids prior to heroin use among young heroin injectors. *Subst Abuse Rehabil* 2: 173-180. PubMed: 23293547.
34. Wada K (2010) [Epidemiology of drug abuse and dependence]. *Nihon Rinsho* 68: 1437-1442. PubMed: 20715473.
35. Forrest DW, Metsch LR, LaLota M, Cardenas G, Beck DW et al. (2010) Crystal methamphetamine use and sexual risk behaviors among HIV-positive and HIV-negative men who have sex with men in South Florida. *J Urban Health* 87: 480-485. doi:10.1007/s11524-009-9422-z. PubMed: 20101468.
36. Kurtz SP (2005) Post-circuit blues: motivations and consequences of crystal meth use among gay men in Miami. *AIDS Behav* 9: 63-72. doi: 10.1007/s10461-005-1682-3. PubMed: 15812614.
37. Mansergh G, Shouse RL, Marks G, Guzman R, Rader M et al. (2006) Methamphetamine and sildenafil (Viagra) use are linked to unprotected receptive and insertive anal sex, respectively, in a sample of men who have sex with men. *Sex Transm Infect* 82: 131-134. doi:10.1136/sti.2005.017129. PubMed: 16581738.
38. Halkitis PN, Green KA, Mourgues P (2005) Longitudinal investigation of methamphetamine use among gay and bisexual men in New York City: findings from Project BUMPS. *J Urban Health* 82: i18-i25. doi:10.1093/jurban/jti020. PubMed: 15738324.

Switching Tenofovir/Emtricitabine plus Lopinavir/r to Raltegravir plus Darunavir/r in Patients with Suppressed Viral Load Did Not Result in Improvement of Renal Function but Could Sustain Viral Suppression: A Randomized Multicenter Trial

Takeshi Nishijima^{1,2}, Hiroyuki Gatanaga^{1,2}, Takuro Shimbo³, Hirokazu Komatsu⁴, Tomoyuki Endo⁵, Masahide Horiba⁶, Michiko Koga⁷, Toshio Naito⁸, Ichiro Itoda⁹, Masanori Tei¹⁰, Teruhisa Fujii¹¹, Kiyonori Takada¹², Masahiro Yamamoto¹³, Toshikazu Miyakawa¹⁴, Yoshinari Tanabe¹⁵, Hiroaki Mitsuya^{14,16}, Shinichi Oka^{1,2*}, on behalf of the SPARE study team[†]

1 AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, 2 Center for AIDS Research, Kumamoto University School of Medicine, Kumamoto, Japan, 3 Department of Clinical Study and Informatics, Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan, 4 Department of Community Care, Saku Central Hospital, Nagano, Japan, 5 Department of Hematology, Hokkaido University Hospital, Sapporo, Japan, 6 Division of Respiratory Medicine, Higashisaitama National Hospital, Saitama, Japan, 7 Division of Infectious Diseases, Advanced Clinical Research Center, the Institute of Medical Science, University of Tokyo, Tokyo, Japan, 8 Department of General Medicine, Juntendo University School of Medicine, Tokyo, Japan, 9 Shirakaba Clinic, Tokyo, Japan, 10 Department of Integrated Medicine, Saku Central Hospital, Nagano, Japan, 11 Division of Blood Transfusion, Hiroshima University Hospital, Hiroshima, Japan, 12 Postgraduate Clinical Training Center, Ehime University Hospital, Ehime, Japan, 13 Internal Medicine, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan, 14 Department of Infectious Diseases and Hematology, Kumamoto University School of Medicine, Kumamoto, Japan, 15 Division of Infection Control and Prevention, Niigata University Medical and Dental Hospital, Niigata, Japan, 16 Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Background: Whether tenofovir nephrotoxicity is reversible after its withdrawal is unknown. Furthermore, there are no data on the viral efficacy of raltegravir (RAL) plus ritonavir-boosted Darunavir (DRV/r) in patients with suppressed viral load.

Methods: This multicenter, randomized trial compared renal function and viral efficacy in patients with suppressed viral load treated with RAL+DRV/r and ritonavir-boosted lopinavir (LPV/r) plus tenofovir/emtricitabine (TVD), who had been previously on LPV/r+TVD. The primary endpoint was the proportion of patients with >10% improvement in estimated glomerular filtration rate (eGFR) at 48 weeks calculated with Cockcroft-Gault equation.

Results: 58 randomized and treatment-exposed patients were analyzed (28 on RAL+DRV/r and 30 on LPV/r+TVD). Greater than 10% improvement in eGFR was noted in 6 (25%) out of 24 with RAL+DRV/r and 3 (11%) of 28 with LPV/r+TVD, and the difference was not statistically significant ($p=0.272$, 95% CI -0.067 to 0.354). Sensitivity analyses using three other equations for eGFR showed the same results. Urinary β_2 microglobulin, a sensitive marker of tenofovir tubulopathy, significantly improved with RAL+DRV/r than with LPV/r+TVD (-271 versus -64 $\mu\text{g}/\text{gCr}$, $p=0.026$). Per protocol analysis showed that the HIV-RNA was <50 copies/mL at week 48 in all patients of both arms (24 in RAL+DRV and 29 in LPV/r+TVD).

Conclusions: Switching LPV/r+TVD to RAL+DRV/r did not significantly increase the proportion of patients who showed >10% improvement in renal function among those with relatively preserved eGFR. However, the switch improved urinary β_2 microglobulin, suggesting that discontinuation of TDF might be beneficial in the long-term. RAL+DRV/r showed favorable viral efficacy in patients with suppressed viral load.

Trial Registration: ClinicalTrials.gov NCT01294761 <http://clinicaltrials.gov/ct2/show/NCT01294761?term=SPARE&rank=2>, Umin Clinical Trials Registry UMIN000005116 <http://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000006083&language=J>

Citation: Nishijima T, Gatanaga H, Shimbo T, Komatsu H, Endo T, et al. (2013) Switching Tenofovir/Emtricitabine plus Lopinavir/r to Raltegravir plus Darunavir/r in Patients with Suppressed Viral Load Did Not Result in Improvement of Renal Function but Could Sustain Viral Suppression: A Randomized Multicenter Trial. PLoS ONE 8(8): e73639. doi:10.1371/journal.pone.0073639

Editor: D William Cameron, University of Ottawa, Canada

Received: April 18, 2013; **Accepted:** July 19, 2013; **Published:** August 8, 2013

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: This work was supported by a Grant-in-Aid for the research on HIV/AIDS (H22-AIDS-001) from the Ministry of Health, Labor, and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: T. Naito received research grants from MSD K.K., Janssen Pharmaceutical K.K., and ViiV Healthcare. TF received research grants from ViiV Healthcare and MSD K.K. HM is a co-inventor on a government patent for darunavir as an employee of the U.S. Government under the terms of the Federal Technology Transfer Act. All rights, title, and interest to the patent have been assigned to the Government. SO received research grants from MSD K.K., Abbott Japan, Co., Janssen Pharmaceutical K.K., Pfizer, Co., and Roche Diagnostics K.K. All other authors declare no conflict of interest. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: oka@acc.ncgm.go.jp

¶ Membership of the SPARE study team is provided in the Acknowledgments.

Introduction

Tenofovir disoproxil fumarate (TDF) is one of the most widely used nucleotide reverse transcriptase inhibitors (NRTI) for patients with HIV infection, with proven efficacy and safety [1-6]. However, tenofovir is excreted by both glomerular filtration and tubular secretion, and is known to cause renal proximal tubular dysfunction. Moreover, long-term TDF use reduces glomerular filtration rate more than other NRTIs [7-10]. Although the mechanism of tenofovir-induced kidney damage is not fully understood, mitochondria toxicity, a well-known adverse event of NRTIs [11,12], in the proximal renal tubular cells is considered to be the main mechanism [13,14]. In addition to renal dysfunction, TDF also reduces bone mineral density, and both complications might lead to serious outcomes with long-term use of TDF [9,15-19]. The concurrent use of ritonavir-boosted protease inhibitors (PI/r) is a risk factor for TDF-associated nephrotoxicity, since PI/r modifies tenofovir clearance and thus increases the severity of tenofovir nephrotoxicity [20,21].

Clinical manifestations such as lipoatrophy and neuropathy caused by NRTI-induced mitochondria toxicity are difficult to reverse [22,23], but whether TDF nephrotoxicity is reversible after discontinuation of TDF remains unknown at present. Unfortunately, the results of few small studies that have examined this issue are contradictory [24-26]. Of note, there is no randomized controlled study that has examined the reversibility of TDF-associated nephrotoxicity.

Recently, antiretroviral therapy (ART) not containing NRTIs (NRTI sparing regimens) has gained a wide attention, since these combinations can avoid NRTI toxicity. Despite high expectations, the results of studies on the efficacy and safety of NRTI sparing regimens for treatment-naïve patients showed dismal results. A small single arm study of CCR5 inhibitor maraviroc plus ritonavir-boosted Darunavir (DRV/r) showed a high rate of virologic failure, especially in patients with high baseline viral load of >100,000 copies/mL [27]. Raltegravir (RAL) plus unboosted atazanavir in a small randomized trial showed frequent grade 4 hyperbilirubinemia and emergence of raltegravir resistance [28]. Even the combination of RAL, a well-tolerated integrase inhibitor, and DRV/r, a protease inhibitor with high barrier to drug resistance and favorable lipid profile [29,30], showed a high prevalence of virological failure for patients with high baseline viral load in a single arm study [31].

At this stage, it is important to elucidate the effectiveness of NRTI sparing regimen for patients with suppressed HIV-1 viral load, because longer exposure with NRTIs tends to result in

clinically overt NRTI-associated mitochondrial toxicity [22,32], and NRTI sparing regimens may avoid such long-term NRTI toxicity. Of note, the viral efficacy of NRTI-sparing regimen of RAL plus DRV/r has not been evaluated in patients with suppressed viral load [31].

Based on the above background, this multicenter randomized trial was conducted to elucidate 1) the reversibility of tenofovir nephrotoxicity, and 2) efficacy and safety of RAL +DRV/r for patients with suppressed viral load.

Methods

This clinical trial was designed and reported according to the recommendations of the Consolidated Standard of Reporting Trials (CONSORT) statement [33]. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and protocol S1.

Ethics Statement

The Research Ethics Committees of Hokkaido University Hospital, Higashisaitama National Hospital, Niigata University Medical and Dental Hospital, the Institute of Medical Science, the University of Tokyo, Juntendo University School of Medicine, Shirakaba Clinic, Saku Central Hospital, Hiroshima University Hospital, Ehime University Hospital, National Hospital Organization Kyushu Medical Center, Kumamoto University Graduate School of Medical Sciences and National Center for Global Health and Medicine approved the study protocol. All patients enrolled in this study provided a written informed consent. The study was conducted according to the principles expressed in the [Declaration of Helsinki](#).

Study Design

The SPARE trial is an on-going phase 3B, multi-center, randomized, open-label, parallel group study conducted in Japan to compare renal function and viral efficacy of NRTI-sparing regimen of RAL+DRV/r and a standard regimen of PI/r + 2NRTIs [(lopinavir/ritonavir (LPV/r) plus fixed dose of tenofovir/emtricitabine (TVD))] for 96 weeks, randomly allocated to patients on LPV/r+TVD with suppressed viral load. With one to one ratio, patients with suppressed viral load on LPV/r (800 mg/200 mg) plus fixed dose of TDF (300 mg)/emtricitabine (200 mg) were randomly assigned to either RAL (800 mg) plus DRV/r (800 mg/100 mg) or to continue LPV/r+TVD. Patient enrollment remained open between February 21, 2011 and December 2011, and the follow-up period is scheduled to end in December 2013. This report summarizes the findings after 48 weeks of treatment, including the primary endpoint.

Randomization was stratified based on baseline body weight of 60 kg because low body weight, especially body weight of <60 kg, is an important risk for tenofovir nephrotoxicity [4,18,34]. Randomization was conducted at the data center with independent data managers, using a computer-generated randomization list prepared by a statistician with no clinical involvement in the trial.

Study Patients

The study population included Japanese patients with HIV-1 infection, aged ≥ 20 years, who were on LPV/r plus TVD and with suppressed HIV-1 RNA viral load of <50 copies/ml over a period of more than 15 weeks. Patients were screened and excluded if found positive for hepatitis B surface antigen, or had history of virologic failure with regimens including protease inhibitor or integrase inhibitor, or if they were considered inappropriate for the study by the attending physicians. Candidates were also excluded if the level of alanine aminotransferase was 2.5 times the upper limit of normal, estimated glomerular filtration rate (eGFR) calculated by Cockcroft-Gault equation (CG equation) was <60 ml/min, $\{[(140 - \text{age}) \times \text{weight (kg)}] / (\text{serum creatinine} \times 72)] \times 0.85$ for females} [35], or on treatment for opportunistic infection. Actual body weight was used for the calculation of eGFR. Patients who provided written informed consent started the allocated regimens within 4 weeks of enrollment.

Study Procedure

Visits for clinical and laboratory assessments were required within 15 weeks before registration for screening, at registration, and every 12 weeks for the duration of the study. Patients of the RAL+DRV/r arm were required to visit within 4 weeks after commencement of the allocated regimen to screen for adverse events. Baseline evaluation and evaluations at each visit covered medical history, including history of AIDS-defining illness and other comorbidities, concurrent medications, concurrent smoking, physical examination, CD4 cell count, HIV-1 RNA viral load, complete blood cell count, blood chemistries (albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, creatine kinase, blood urea nitrogen, serum creatinine, sodium, potassium, calcium, phosphate, triglyceride, total cholesterol, low-density lipoprotein cholesterol, high density lipoprotein cholesterol, glucose), and urine examination (urine dipstick, phosphate, creatinine, $\beta 2$ microglobulin, N-acetyl- β -D-glucosaminidase (NAG), and albumin). The values of urinary $\beta 2$ microglobulin, NAG, and albumin were expressed relative to urinary creatinine of 1 g/L (/g Cr). Percent tubular resorption of phosphate was calculated by the following formula: $\{1 - [(\text{urine phosphate} \times \text{serum creatinine}) / (\text{urine creatinine} \times \text{serum phosphate})]\} \times 100$ [36]. All data, including HIV-1 RNA viral load, were collected at each participating site and then transferred to a central data center. Grade 3 or 4 serious adverse events were reported to the independent data and safety monitoring board and analyzed for their relation to the study drugs. The grade of adverse events was classified according to the Division of AIDS Table for grading the severity of adult and pediatric events, version 2004

(URL:<http://www.mtnstopshiv.org/sites/default/files/attachments/>

Table_for_Grading_Severity_of_Adult_Pediatric_Adverse_Events.pdf). Independent monitors visited all facilities to conduct source document verification to ensure the accuracy of all submitted data by week 48 and compliance to the protocol. All authors participated in the trial design, data analysis, and preparation of the manuscript, and vouch for the completeness and accuracy of the presented data.

Statistical Analysis

The tested hypothesis was that more patients in the RAL+DRV/r arm will experience >10% improvement in eGFR from the baseline than patients in the LPV/r+TVD arm after switching from LPV/r+TVD to RAL+DRV/r. Sample size calculation was based on the assumption that 50% of the patients of the RAL+DRV/r arm and 10% of the patients of the LPV/r + TVD arm will experience >10% improvement in eGFR from the baseline to week 48. With a 2-sided alpha level of 0.05 and 80% power, the estimated population sample required in this study was 50 patients (25 per single arm). To account for dropouts, we planned to enroll 27 patients per one arm. The study was not fully powered for secondary analysis. Per protocol population while on the initial randomized regimen was used for the analysis of the primary endpoint.

The primary endpoint was the proportion of patients with >10% improvement in eGFR at 48 weeks from the baseline calculated with the CG equation [35]. The baseline eGFR was estimated from the average of serum creatinine measured at baseline and at screening for enrollment. eGFR at week 48 was estimated from the average of serum creatinine at weeks 36 and 48. The proportion of such patients was compared between the two arms by the Fisher exact test. The following three equations for eGFR were also used for sensitivity analysis: 1) A 3-variable equation for the Japanese set by the Japanese Society of Nephrology (JSN equation): $[194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287} \times (0.739 \text{ for female patients})]$ [37], 2) the Modification of Diet in Renal Disease (MDRD) equation adjusted with coefficient for the Japanese $[0.808 \times 175 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ for female patients})]$ [37], and 3) Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation adjusted for the Japanese $[0.813 \times 141 \times \min(\text{serum creatinine}/\kappa, 1)^{\alpha} \times \max(\text{serum creatinine}/\kappa, 1)^{-1.209} \times (0.993)^{\text{age}} \times (1.018 \text{ for females})]$ (where κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, *min* represents the minimum of serum creatinine/ κ or 1, and *max* is the maximum of serum creatinine/ κ or 1) [38]. Furthermore, the percent improvement in eGFR from baseline to week 48, calculated with all four equations described above, was compared between the two arms by the Student's t-test. Because the percent improvement in eGFR may depend on the baseline value, a correlation between the percent improvement in eGFR and the baseline value was tested, and the results showed very weak correlation ($0.001 < r < 0.2$) for all four equations for eGFR. Accordingly, the comparison of the percent improvement was conducted by the t-test as described above.

The secondary renal endpoint was changes in per protocol renal tubular markers from the baseline to week 48, and the results were compared by the Mann-Whitney test. The secondary efficacy endpoint was the proportions of patients with HIV-1 RNA <50 copies/mL at weeks 24 and 48. Data of both per protocol population and the intent-to-treat (ITT) population, comprising all randomized treatment-exposed subjects were used for the assessment of efficacy. With regard to analysis on the viral efficacy in this study, per protocol analyses were more important than ITT analyses, because some patients enrolled in the RAL+DRV/r arm were expected to develop adverse events due to switching to the new medications and subsequent discontinuation of the allocated regimen, whereas new adverse events were not likely in patients of the LPV/r+TVD arm solely by continuing the same regimen as before. Baseline parameters were compared between the two arms by the Student's t-test for continuous variables and by either the χ^2 test or Fisher's exact test for categorical variables. Statistical significance was defined as two-sided *p* values <0.05. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 21.0 (SPSS, Chicago, IL).

Results

Patient disposition and baseline characteristics

Between February and December of 2011, 59 patients from 11 centers were enrolled in the study and randomized. Of these, 29 and 30 patients were allocated to the RAL+DRV/r and the LPV/r+TVD arm, respectively (Figure 1). One patient in the RAL+DRV/r arm withdrew consent before starting the allocated regimen, thus was excluded from the analysis. The baseline demographics and characteristics of the participating patients are listed in Table 1. Most patients were men who have sex with men, with well-maintained CD4 count. Patients of the LPV/r+TVD arm were younger (*p*=0.040) and had lower CD4 count (*p*=0.029) than those of the RAL+DRV/r arm. All other major variables were similar between the two arms.

Primary endpoint

At week 48, six patients (25%) out of 24 in the RAL+DRV/r arm and 3 patients (11%) out of 28 in the LPV/r+TVD arm, experienced >10% improvement in eGFR from baseline, and the difference was not statistically significant (*p*=0.272, 95% CI -0.067 to 0.354). Sensitivity analysis with three other equations for eGFR (JSN, CKD-EPI, and MDRD) showed the same results; no difference in the proportion of patients with improvement of >10% in eGFR was noted between the two arms (JSN equation: 4/24 in RAL+DRV/r, 3/29 in LPV/r+TVD, *p*=0.688, 95% CI -0.126 to 0.267) (CKD-EPI equation: 2/24 in RAL+DRV/r, 2/29 in LPV/r+TVD, *p*=1.000, 95% CI -0.148 to 0.197) (MDRD equation: 5/24 in RAL+DRV/r, 3/29 in LPV/r+TVD, *p*=0.444, 95% CI -0.093 to 0.313) (Table 2).

Additional analysis showed that the percent improvement in eGFR from the baseline to week 48 calculated using all four equations was not significantly different between the two arms [CG equation: difference in mean % improvement (DRV/r+RAL versus LPV/r+TDF/FTC) -8.7%, 95% CI -18.2 to 0.8, *p*=0.071]

Table 1. Baseline characteristics of the enrolled patients.

	RAL+DRV/r (n=28)	LPV/r+TVD (n=30)	P value
Sex (male), n (%)	28 (100)	29 (97)	1.000
Age (years) [†]	44 (37-51)	39 (34-45)	0.040
CD4 count (/μl) [†]	549 (384-710)	456 (330-592)	0.029
Route of transmission (homosexual contact), n (%)	27 (96)	24 (80)	0.151
History of AIDS, n (%)	10 (36)	11 (37)	1.000
Body weight (kg) [†]	66 (59-75)	66 (59-72)	0.502
Body mass index (kg/m ²) [†]	22 (21-25)	22.6 (19.9-24.6)	0.440
eGFR by JSN equation (ml/min/1.73 m ²) [†]	87 (76-103)	85 (70-90)	0.356
eGFR by CG equation (ml/min) [†]	119 (88-143)	108 (89-120)	0.456
Serum creatinine (mg/dl) [†]	0.78 (0.70-0.87)	0.76 (0.67-0.83)	0.184
Urinary albumin (mg/g Cre) [†]	8 (6-27)	7 (5-12)	0.075
Urinary β2 microglobulin (μg/g Cre) [†]	452 (178-1566)	424 (204-2275)	0.234
Tubular resorption of phosphate (%) [†]	92 (87-93)	90 (86-94)	0.886
NAG (U/g Cr) [†]	6.2 (3.7-11.6)	5.2 (3.7-8.3)	0.183
Hypertension, n (%)	2 (7)	1 (3)	0.605
Dyslipidemia, n (%)	17 (61)	8 (27)	0.016
Diabetes mellitus, n (%)	0 (0)	1 (3)	1.000
Current smoking, n (%)	13 (46)	13 (43)	1.000
Hepatitis C, n (%)	0 (0)	0 (0)	N/A
Duration of tenofovir use (weeks)	163 (109-224)	124 (85-212)	0.721

Hypertension was defined by current treatment with antihypertensive agents or systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg. Dyslipidemia was defined by current treatment with lipid-lowering agents or high-density lipoprotein cholesterol >140 mg/dl, high-density lipoprotein cholesterol <40 mg/dl, total cholesterol >240 mg/dl, or triglyceride >500 mg/dl. IQR: interquartile range, AIDS: acquired immunodeficiency syndrome, eGFR: estimated glomerular filtration rate, LDL: low-density lipoprotein, JSN: the Japanese Society of Nephrology equation [37], CG: Cockcroft-Gault equation [35]

[†] median (interquartile range)

(JSN equation: -1.1%, -6.9 to 4.8, *p*=0.720) (CKD-EPI equation: -1.6%, 95% CI -4.7 to 1.6, *p*=0.323) (MDRD equation: -1.1%, 95% CI -6.9 to 4.8, *p*=0.722) (Table 2). Thus, this study demonstrated that switching to NRTI-sparing regimen of RAL+DRV/r did not increase the proportion of patients who showed >10% improvement in eGFR, compared to continuation of LPV/r+TVD.

Secondary renal endpoints

Among the four renal tubular markers used in this study, the improvement in urinary β2 microglobulin from baseline to week 48 was significantly larger in the RAL+DRV/r arm (*n*=23) than

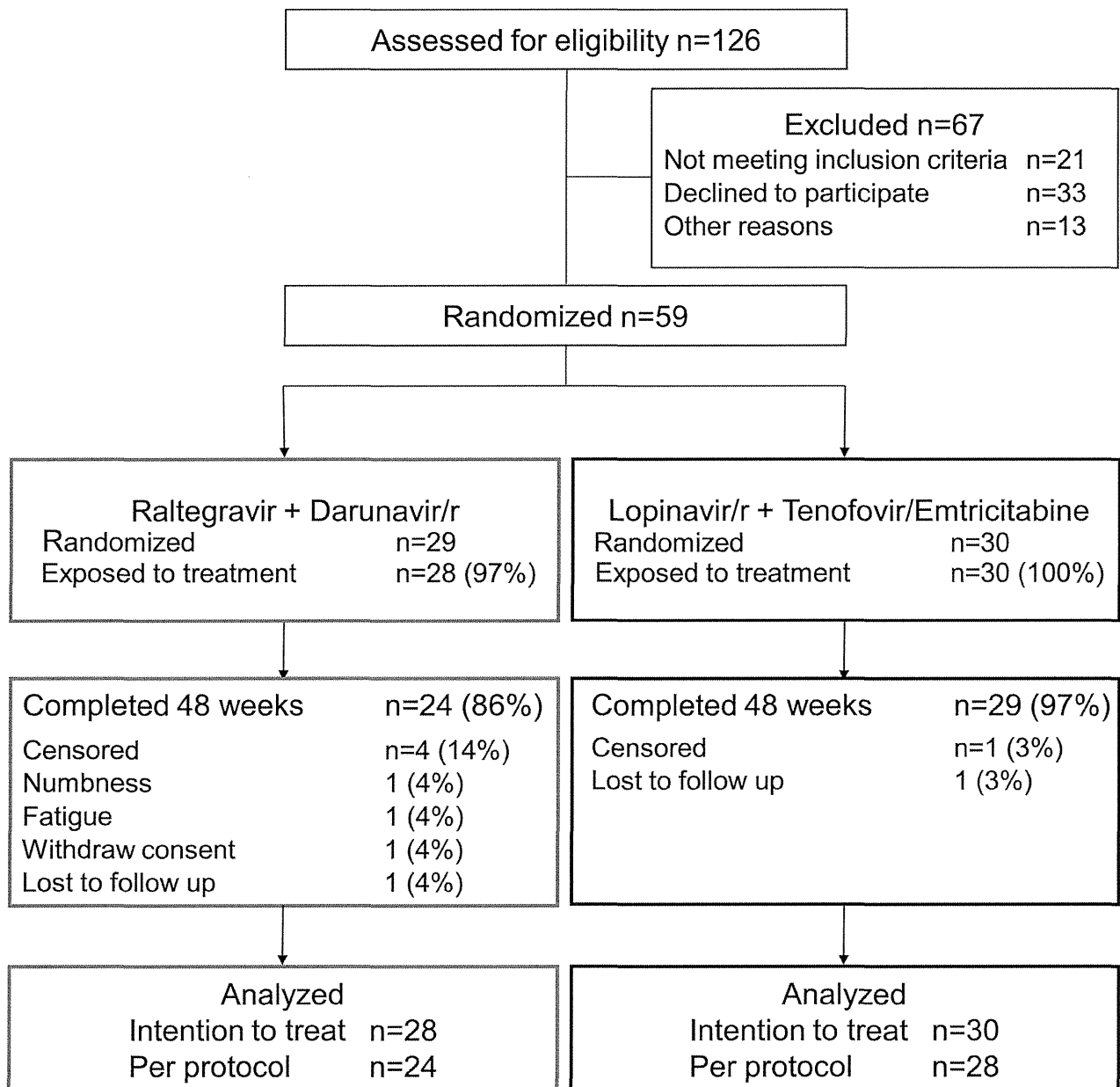


Figure 1. Enrollment, randomization, and disposition of patients. Darunavir/r, ritonavir-boosted darunavir; Lopinavir/r, ritonavir-boosted lopinavir.

doi: 10.1371/journal.pone.0073639.g001

in the LPV/r+TVD arm ($n=28$) (-271 versus -64 $\mu\text{g/g Cr}$, $p=0.026$) (Figure 2A). However, urinary albumin, the percent tubular resorption of phosphate, and NAG showed little change from baseline, and the observed changes were not significantly different between the two arms (Figure 2B, C, D).

Secondary efficacy endpoints

Among the per protocol population, the proportion of patients with HIV RNA <50 copies/mL was 96.2% for the RAL+DRV/r

arm and 96.7% for the LPV/r+TVD arm at week 24, with a difference of -0.5% (95% CI, -10% to 9%), and 100% for the both arms at week 48, with a difference of 0% (95% CI -0.1 to 0.1) (Figure 3A). ITT analysis showed that the proportion was 89.3% and 96.7% for the RAL+DRV/r and LPV/r+TVD arms, respectively, at week 24, with a difference of -7% (95% CI, -21% to 6%), and 85.7% and 96.7%, respectively, at week 48, with a difference of -11% (95% CI, -25% to 4%) (Figure 3B). There was no significant difference in viral efficacy between the

Table 2. Proportion of patients with >10% and mean percent improvement in eGFR at 48 weeks from the baseline calculated by the four equations.

	Cases with >10% increase from baseline	P value (95% CI)	Mean % improvement in eGFR from baseline	Difference in mean % improvement (95% CI) (DRV/r + RAL versus LPV/r + TDF/FTC)	P value
CG equation					
DRV/r + RAL	6/24	0.272 (-0.067 to 0.354)	5.4%	-8.7% (-18.2 to 0.8)	0.071
LPV/r + TDF/FTC	3/28		-3.3%		
JSN equation					
DRV/r + RAL	4/24	0.688 (-0.126 to 0.267)	2.5%	-1.1% (-6.9 to 4.8)	0.720
LPV/r + TDF/FTC	3/29		1.5%		
CKD-EPI equation					
DRV/r + RAL	2/24	1.000 (-0.148 to 0.197)	1.9%	-1.6% (-4.7 to 1.6)	0.323
LPV/r + TDF/FTC	2/29		1.7%		
MDRD equation					
DRV/r + RAL	5/24	0.444 (-0.093 to 0.313)	2.7%	-1.1% (-6.9 to 4.8)	0.722
LPV/r + TDF/FTC	3/29		1.7%		

DRV/r: ritonavir-boosted darunavir, RAL: raltegravir, LPV/r: ritonavir-boosted lopinavir, TDF: tenofovir, FTC: emtricitabine, CG: Cockcroft-Gault equation [35], JSN: the Japanese Society of Nephrology equation [37], CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation adjusted for the Japanese [38], MDRD: the Modification of Diet in Renal Disease equation adjusted with coefficient for the Japanese [37]

two arms at weeks 24 and 48. At week 48, all patients of the RAL+DRV/r arm on the allocated regimen (n=24) had a viral load of <50 copies/mL.

Safety and tolerability

One patient from each arm was lost to follow-up. Three patients of the RAL+DRV/r arm discontinued the allocated regimen by week 48 (one discontinued the regimen at week 4 due to weakness in the lower extremities and one at week 24 because of fatigue, which was later found to be related to acute hepatitis B infection). The other patient withdrew consent at week 24, because it was easier for him to maintain a good medication adherence with once-daily LPV/r+TVD (the regimen the patient used before enrollment). None of the patients of the LPV/r+TVD arm discontinued the allocated regimen by week 48. Thus, at week 48, 24 patients (86%) out of 28 in the RAL+DRV/r arm and 29 (97%) of 30 in the LPV/r+TVD arm, were on the allocated regimens.

The following grade 3 or 4 laboratory data or abnormal symptoms that were at least one grade higher than the baseline were encountered in this study: RAL+DRV/r arm: a rise in ALT (due to acute hepatitis B infection, n=1), and elevated LDL-cholesterol (n=3), LPV/r+TVD arm: elevated LDL-cholesterol (n=1), and hypophosphatemia (n=3). The above side effects did not lead to discontinuation of the study drugs.

Discussion

This randomized trial elucidated the recovery of TDF-associated nephropathy after discontinuation of TDF. The results demonstrated no significant increase in the proportion

of patients who showed >10% improvement in eGFR after switching to NRTI sparing regimen of RAL+DRV/r, compared to continuation of LPV/r+TVD. This finding could be due to any of the following reasons; 1) Relatively preserved baseline renal function of the enrolled patients, with a median eGFR of 86 ml/min/1.73 m² (IQR 75-97, JSN equation), with only one patient with CKD stage 3 due to persistent +1 proteinuria, and no patients with stage 4 or more. Although the number of patients is relatively small, a previous pilot study of 21 patients reported improvement of eGFR (by CG equation) in most patients after switching from PI/r+TVD to PI/r+RAL in patients with proteinuria and suppressed HIV viral load [39]. Thus, improvement of eGFR after discontinuation of TDF might be more significant in patients with severe to moderately impaired renal function. Larger studies are needed to investigate this issue thoroughly. 2) Study patients had been on TDF for a long period of time at enrollment (median: 136 weeks, range 27-370 weeks, 72% were on TDF for more than 2 years), although shorter duration of TDF therapy is likely to be associated with greater eGFR improvement after discontinuation [26]. Furthermore, because TDF-induced renal dysfunction is mainly observed during the first 6 months after commencement of such therapy [18,19,40], it is possible that patients who developed severe renal dysfunction soon after starting TDF might have already discontinued TDF and therefore not included in the study.

Although the present study did not show an increase in eGFR after discontinuation of TDF, it is noteworthy that the value of urinary β 2 microglobulin, a sensitive marker for TDF-induced tubulopathy [41,42], improved significantly in the RAL+DRV/r arm compared to LPV/r+TVD, even in patients with relatively preserved eGFR. It is of importance considering that

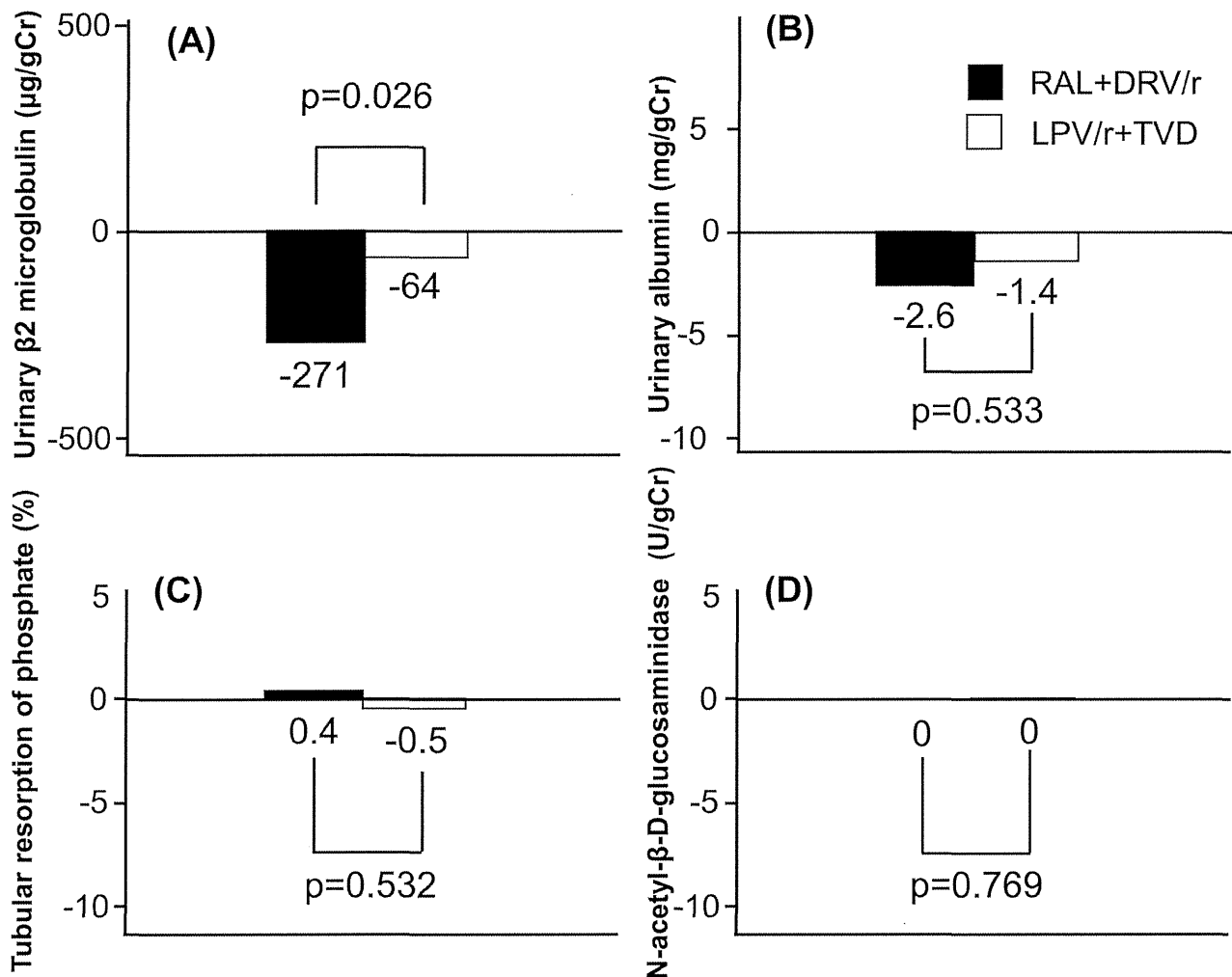


Figure 2. Median changes in markers of renal tubular function between baseline and 48 weeks. (A) Urinary β 2 microglobulin, (B) Urinary albumin, (C) Percent tubular resorption of phosphate, (D) Urinary N-acetyl- β -D-glucosaminidase. RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.

doi: 10.1371/journal.pone.0073639.g002

proximal tubulopathy is associated with bone mineral density abnormality and possible long-term nephrotoxic effect [17,43-45]. Further large and long-term studies are needed to elucidate the long-term impact of TDF-induced tubulopathy on GFR.

With regard to the viral efficacy and safety of RAL+DRV/r, all patients in that arm who continued the allocated regimen accomplished viral suppression of <50 copies/ml at week 48 ($n=24$). Only one (3.6%) patient discontinued RAL+DRV/r due to a side effect possibly related to RAL+DRV/r (weakness of the lower extremities), confirming the safety of this combination. To our knowledge, this is the first study to examine the viral efficacy of RAL+DRV/r in patients with suppressed viral load. The KITE study, an industry-sponsored pilot study, examined the viral efficacy of RAL+LPV/r in patients with suppressed viral load [46]. However, LPV/r is placed as an

alternative PI in the American Department of Health and Human Services Guidelines, mainly because of the higher rates of gastrointestinal side effects and hyperlipidemia compared with other PIs (URL: <http://aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>). Because the number of enrolled patients is relatively small and this study does not have sufficient power to elucidate viral efficacy, further studies are needed to confirm the viral efficacy of RAL+DRV/r in patients with suppressed viral load. If the NRTI sparing regimen of RAL+DRV/r is proved to be efficacious in maintaining viral suppression in treatment-experienced patients, switching to this combination for patients with suppressed viral load should become an attractive treatment option for patients who cannot tolerate NRTI toxicity or to prevent further NRTI toxicity.

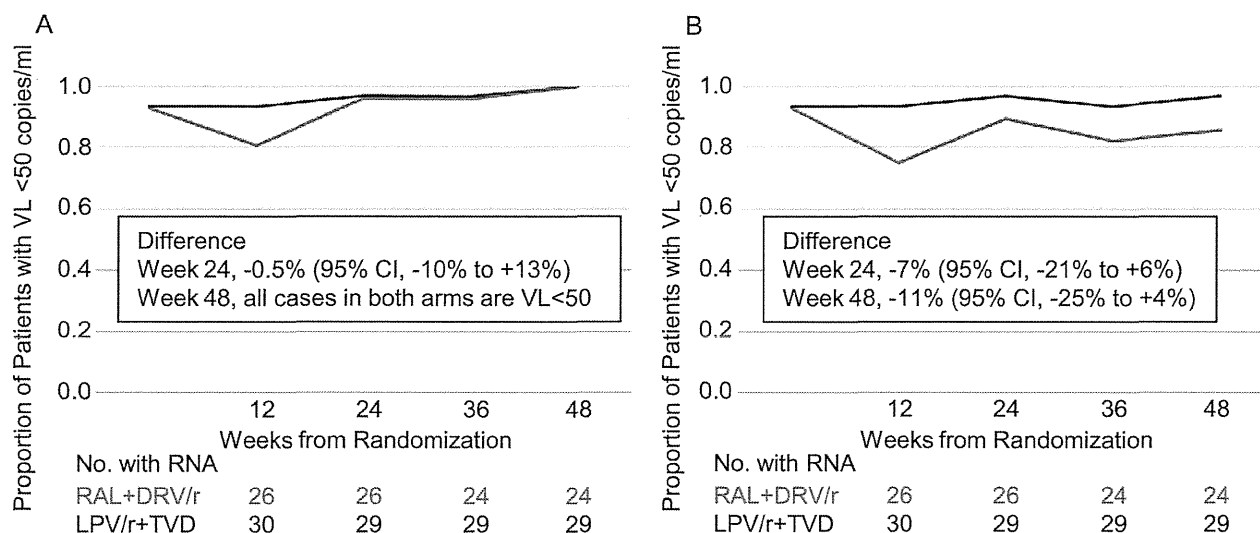


Figure 3. Proportion of patients with HIV RNA <50 copies/ml at 24 and 48 weeks. (A) Per protocol analysis. (B) Intention-to-treat analysis. VL, viral load; RAL, raltegravir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir; TVD, fixed dose of tenofovir/emtricitabine.

doi: 10.1371/journal.pone.0073639.g003

Several limitations must be acknowledged. First, as mentioned above, this trial has sufficient power for the primary endpoint only; other results should be interpreted with caution. Further larger studies are needed to confirm the improvement in urinary β_2 microglobulin after switching ritonavir-boosted PI to NRTI sparing regimen of RAL+DRV/r and the viral efficacy of RAL+DRV/r in patients with suppressed viral load. Second, the enrolled patients had relatively preserved renal function. This was a study-design related issue; patients with severely impaired eGFR, the population in whom TDF nephrotoxicity can be reversible is clinically important, were excluded from the study. Based on the study design and need for randomization, patients of one arm needed to continue treatment with TDF, and it was considered ethically inappropriate to have patients with impaired renal function to continue TDF. Third, all study subjects were Japanese and almost exclusively men (mostly men who have sex with men). Further studies are needed to determine whether the findings of this study are also applicable to females, patients with different routes of transmission, and patients of different racial background.

In conclusion, this trial showed that discontinuation of LPV/r +TVD and switching to NRTI-sparing regimen of RAL+DRV/r did not result in improvement of renal function among patients with relatively preserved eGFR and suppressed HIV viral load. However, urinary β_2 microglobulin, a sensitive marker of TDF-induced tubulopathy, improved after discontinuation of TDF plus ritonavir-boosted PI, suggesting switching TDF to NRTI sparing regimen might be beneficial in the long-term. RAL+DRV/r showed favorable viral efficacy and safety in patients with suppressed viral load, but further larger studies are needed to confirm the viral efficacy of this combination.

Supporting Information

Protocol S1. Trial protocol.
(DOCX)

Checklist S1. CONSORT checklist.
(DOC)

Acknowledgements

We thank the patients for participation in this study.

The SPARE trial team includes the following members:

Michiyo Ishisaka¹, Mikiko Ogata¹, Misao Takano¹, Akiko Nakano¹, Masahiro Imamura², Junji Tanaka², Satoshi Hashino², Takeshi Kondo², Mitsufumi Nishio², Katsuya Fujimoto², Hiroshi Moro³, Aikichi Iwamoto⁴, Tomohiko Koibuchi⁴, Takeshi Fujii⁴, Toshiyuki Miura⁴, Hitomi Nakamura⁴, Nahoko Miyazaki⁴, Kazufumi Matsumoto⁴, Kumiko Sumino⁴, Noriko Fujiwara⁴, Mizue Saita⁵, Akihito Suzuki⁵, Mika Tanei⁵, Rino Sakamoto⁵, Satoshi Kimura⁶, Kunihiko Okada⁷, Asuka Takasoe⁷, Seiji Saito⁸, Sayaka Sugihara⁸, Fumiko Kagiura⁸, Yuichi Murakami⁹, Rumi Minami¹⁰, Soichiro Takahama¹⁰, Junichi Kiyasu¹⁰, Shuzo Matsushita¹¹, Naoki Ishizuka¹, Yoshimi Kikuchi¹, Katsuji Teruya¹, Kunihisa Tsukada¹, Junko Tanuma¹, Hirohisa Yazaki¹, Haruhito Honda¹, Ei Kinai¹, Koji Watanabe¹, Takahiro Aoki¹, Daisuke Mizushima¹, Ikumi Genka¹, Miwako Honda¹, Masayuki Chida¹, Junichi Masuda¹, Mai Nakamura¹, and Fumihide Kanaya¹

¹National Center for Global Health and Medicine, Tokyo, Japan;

²Hokkaido University Hospital, Sapporo, Japan;

³Niigata University Medical and Dental Hospital, Niigata, Japan;

⁴Research Hospital of the Institute of Medical Science, The University of Tokyo, Tokyo, Japan;

⁵Juntendo University School of Medicine, Tokyo, Japan;

⁶Tokyo Teishin Hospital, Tokyo, Japan;

⁷Saku Central Hospital, Nagano, Japan;

⁸Hiroshima University Hospital, Hiroshima, Japan;

⁹Ehime University Hospital, Ehime, Japan;

¹⁰National Hospital Organization Kyushu Medical Center, Fukuoka, Japan;

¹¹Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

Author Contributions

Conceived and designed the experiments: T. Nishijima HG TS TE MH T. Naito TF KT MY TM YT HM SO. Performed the experiments: T. Nishijima HG TE MH MK T. Naito II MT TF KT MY TM YT SO. Analyzed the data: T. Nishijima HG TS HK SO. Wrote the manuscript: T. Nishijima HG TS HK TE MH MK T. Naito II MT TF KT MY TM YT HM SO.

References

- Gallant JE, DeJesus E, Arribas JR, Pozniak AL, Gazzard B et al. (2006) Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. *N Engl J Med* 354: 251-260. doi: 10.1056/NEJMoa051871. PubMed: 16421366.
- Gallant JE, Staszewski S, Pozniak AL, DeJesus E, Suleiman JM et al. (2004) Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naïve patients: a 3-year randomized trial. *JAMA* 292: 191-201. doi:10.1001/jama.292.2.191. PubMed: 15249568.
- Izzedine H, Hulot JS, Vittecoq D, Gallant JE, Staszewski S et al. (2005) Long-term renal safety of tenofovir disoproxil fumarate in antiretroviral-naïve HIV-1-infected patients. Data from a double-blind randomized active-controlled multicentre study. *Nephrol Dial Transplant* 20: 743-746. doi:10.1093/ndt/gfh658. PubMed: 15741212.
- Nelson MR, Katlama C, Montaner JS, Cooper DA, Gazzard B et al. (2007) The safety of tenofovir disoproxil fumarate for the treatment of HIV infection in adults: the first 4 years. *AIDS* 21: 1273-1281. doi: 10.1097/QAD.0b013e3280b07b33. PubMed: 17545703.
- Arribas JR, Pozniak AL, Gallant JE, DeJesus E, Gazzard B et al. (2008) Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naïve patients: 144-week analysis. *J Acquir Immune Defic Syndr* 47: 74-78. doi: 10.1097/QAI.0b013e3281815acab8. PubMed: 17971715.
- Post FA, Moyle GJ, Stellbrink HJ, Domingo P, Podzamczak D et al. (2010) Randomized comparison of renal effects, efficacy, and safety with once-daily abacavir/lamivudine versus tenofovir/emtricitabine, administered with efavirenz, in antiretroviral-naïve, HIV-1-infected adults: 48-week results from the ASSERT study. *J Acquir Immune Defic Syndr* 55: 49-57. doi:10.1097/QAI.0b013e32811f9c0f7. PubMed: 20431394.
- Kinai E, Hanabusa H (2009) Progressive renal tubular dysfunction associated with long-term use of tenofovir DF. *AIDS Res Hum Retroviruses* 25: 387-394. doi:10.1089/aid.2008.0202. PubMed: 19361280.
- Winston A, Amin J, Mallon P, Marriott D, Carr A et al. (2006) Minor changes in calculated creatinine clearance and anion-gap are associated with tenofovir disoproxil fumarate-containing highly active antiretroviral therapy. *HIV Med* 7: 105-111. doi:10.1111/j.1468-1293.2006.00349.x. PubMed: 16420255.
- Gallant JE, Winston JA, DeJesus E, Pozniak AL, Chen SS et al. (2008) The 3-year renal safety of a tenofovir disoproxil fumarate vs. a thymidine analogue-containing regimen in antiretroviral-naïve patients. *AIDS* 22: 2155-2163. doi:10.1097/QAD.0b013e3283112b8e. PubMed: 18832879.
- Fux CA, Simcock M, Wolbers M, Bucher HC, Hirschel B et al. (2007) Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV Cohort Study. *Antivir Ther* 12: 1165-1173. PubMed: 18240857.
- Nolan D, Mallal S (2004) Complications associated with NRTI therapy: update on clinical features and possible pathogenic mechanisms. *Antivir Ther* 9: 849-863. PubMed: 15651744.
- Lewis W, Day BJ, Copeland WC (2003) Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat Rev Drug Discov* 2: 812-822. doi:10.1038/nrd1201. PubMed: 14526384.
- Kohler JJ, Hosseini SH, Hoying-Brandt A, Green E, Johnson DM et al. (2009) Tenofovir renal toxicity targets mitochondria of renal proximal tubules. *Lab Invest* 89: 513-519. doi:10.1038/labinvest.2009.14. PubMed: 19274046.
- Herlitz LC, Mohan S, Stokes MB, Radhakrishnan J, D'Agati VD et al. (2010) Tenofovir nephrotoxicity: acute tubular necrosis with distinctive clinical, pathological, and mitochondrial abnormalities. *Kidney Int* 78: 1171-1177. doi:10.1038/ki.2010.318. PubMed: 20811330.
- Peyrière H, Reynes J, Rouanet I, Daniel N, de Boever CM et al. (2004) Renal tubular dysfunction associated with tenofovir therapy: report of 7 cases. *J Acquir Immune Defic Syndr* 35: 269-273. doi: 10.1097/00126334-200403010-00007. PubMed: 15076241.
- McComsey GA, Kitch D, Daar ES, Tierney C, Jahed NC et al. (2011) Bone mineral density and fractures in antiretroviral-naïve persons randomized to receive abacavir-lamivudine or tenofovir disoproxil fumarate-emtricitabine along with efavirenz or atazanavir-ritonavir: Aids Clinical Trials Group A5224s, a substudy of ACTG A5202. *J Infect Dis* 203: 1791-1801. doi:10.1093/infdis/jir188. PubMed: 21606537.
- Fux CA, Rauch A, Simcock M, Bucher HC, Hirschel B et al. (2008) Tenofovir use is associated with an increase in serum alkaline phosphatase in the Swiss HIV Cohort Study. *Antivir Ther* 13: 1077-1082. PubMed: 19195333.
- Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T et al. (2012) Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naïve patients with HIV infection. *PLOS ONE* 7: e29977. doi:10.1371/journal.pone.0029977. PubMed: 22242194.
- Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S et al. (2010) Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. *Clin Infect Dis* 51: 496-505. doi:10.1086/655681. PubMed: 20673002.
- Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P et al. (2008) The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. *Clin Pharmacol Ther* 83: 265-272. doi:10.1038/sj.cpt.6100269. PubMed: 17597712.
- Goicoechea M, Liu S, Best B, Sun S, Jain S et al. (2008) Greater tenofovir-associated renal function decline with protease inhibitor-based versus nonnucleoside reverse-transcriptase inhibitor-based therapy. *J Infect Dis* 197: 102-108. doi:10.1086/524061. PubMed: 18171292.
- Tressler R, Godfrey C (2012) NRTI backbone in HIV treatment: will it remain relevant? *Drugs* 72: 2051-2062. doi: 10.2165/11640830-000000000-00000. PubMed: 23083109.
- Moyle G (2000) Clinical manifestations and management of antiretroviral nucleoside analog-related mitochondrial toxicity. *Clin Ther* 22: 911-936; discussion 898. doi:10.1016/S0149-2918(00)80064-8. PubMed: 10972629.
- Scherzer R, Estrella M, Li Y, Choi AI, Deeks SG et al. (2012) Association of tenofovir exposure with kidney disease risk in HIV infection. *AIDS* 26: 867-875. doi:10.1097/QAD.0b013e328351f68f. PubMed: 22313955.
- Bonjoch A, Echeverría P, Perez-Alvarez N, Puig J, Estany C et al. (2012) High rate of reversibility of renal damage in a cohort of HIV-infected patients receiving tenofovir-containing antiretroviral therapy. *Antiviral Res* 96: 65-69. doi:10.1016/j.antiviral.2012.07.009. PubMed: 22892171.
- Wever K, van Agtmael MA, Carr A (2010) Incomplete reversibility of tenofovir-related renal toxicity in HIV-infected men. *J Acquir Immune Defic Syndr* 55: 78-81. doi:10.1097/QAI.0b013e32811d05579. PubMed: 20173649.
- Taiwo B, Swindells S, Berzins B et al. Week 48 results of the Maraviroc Plus Darunavir/ritonavir Study (MIDAS) for treatment-naïve patients infected with R5-tropic HIV-1 [abstract TUPE099]. Presented at: 19th International AIDS Conference; 2012; Washington DC, USA.
- Kozal MJ, Lupo S, DeJesus E, Molina JM, McDonald C et al. (2012) A nucleoside- and ritonavir-sparing regimen containing atazanavir plus

- raltegravir in antiretroviral treatment-naïve HIV-infected patients: SPARTAN study results. *HIV Clin Trials* 13: 119-130. doi:10.1310/hct1303-119. PubMed: 22592092.
29. Clotet B, Bellos N, Molina JM, Cooper D, Goffard JC et al. (2007) 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* 369: 1169-1178. doi:10.1016/S0140-6736(07)60497-8. PubMed: 17416261.
 30. Madruga JV, Berger D, McMurchie M, Suter F, Banhegyi D et al. (2007) 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* 370: 49-58. doi:10.1016/S0140-6736(07)61049-6. PubMed: 17617272.
 31. Taiwo B, Zheng L, Gallien S, Matining RM, Kuritzkes DR et al. (2011) Efficacy of a nucleoside-sparing regimen of darunavir/ritonavir plus raltegravir in treatment-naïve HIV-1-infected patients (ACTG A5262). *AIDS* 25: 2113-2122. doi:10.1097/QAD.0b013e32834bbaa9. PubMed: 21857490.
 32. Dalakas MC, Illa I, Pezeshkpour GH, Laukaitis JP, Cohen B et al. (1990) Mitochondrial myopathy caused by long-term zidovudine therapy. *N Engl J Med* 322: 1098-1105. doi:10.1056/NEJM199004193221602. PubMed: 2320079.
 33. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC et al. (2010) CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. *BMJ* 340: c869. doi:10.1136/bmj.c869. PubMed: 20332511.
 34. Nishijima T, Komatsu H, Gatanaga H, Aoki T, Watanabe K et al. (2011) Impact of small body weight on tenofovir-associated renal dysfunction in HIV-infected patients: a retrospective cohort study of Japanese patients. *PLOS ONE* 6: e22661. doi:10.1371/journal.pone.0022661. PubMed: 21799928.
 35. Cockcroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. *Nephron* 16: 31-41. doi:10.1159/000180580. PubMed: 1244564.
 36. Rodríguez-Nóvoa S, Labarga P, Soriano V, Egan D, Albalater M et al. (2009) Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. *Clin Infect Dis* 48: e108-e116. doi:10.1086/598507. PubMed: 19400747.
 37. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K et al. (2009) Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 53: 982-992. doi:10.1053/j.ajkd.2008.12.034. PubMed: 19339088.
 38. Horio M, Imai E, Yasuda Y, Watanabe T, Matsuo S (2010) Modification of the CKD epidemiology collaboration (CKD-EPI) equation for Japanese: accuracy and use for population estimates. *Am J Kidney Dis* 56: 32-38. doi:10.1053/S0272-6386(10)01197-2. PubMed: 20416999.
 39. Bredeek UF, Guadron R, Yolo R et al. (2012) A Switch From TDF/FTC To Raltegravir In Patients On A Boosted Protease Inhibitor Is Effective In Reducing Proteinuria And Increasing GFR [abstract: H1-1399b]. Presented at: 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy; September 9-12. USA: San Francisco.
 40. Gallant JE, Moore RD (2009) Renal function with use of a tenofovir-containing initial antiretroviral regimen. *AIDS* 23: 1971-1975. doi: 10.1097/QAD.0b013e32832c96e9. PubMed: 19696652.
 41. Gatanaga H, Tachikawa N, Kikuchi Y, Teruya K, Genka I et al. (2006) Urinary beta2-microglobulin as a possible sensitive marker for renal injury caused by tenofovir disoproxil fumarate. *AIDS Res Hum Retroviruses* 22: 744-748. doi:10.1089/aid.2006.22.744. PubMed: 16910829.
 42. Nishijima T, Shimbo T, Komatsu H, Takano M, Tanuma J et al. (2013) Urinary beta-2 microglobulin and alpha-1 microglobulin are useful screening markers for tenofovir-induced kidney tubulopathy in patients with HIV-1 infection: a diagnostic accuracy study. *J Infect Chemother.* PubMed: 23467792.
 43. Dauchy FA, Lawson-Ayayi S, de la Faille R, Bonnet F, Rigotherier C et al. (2011) Increased risk of abnormal proximal renal tubular function with HIV infection and antiretroviral therapy. *Kidney Int* 80: 302-309. doi: 10.1038/ki.2011.124. PubMed: 21544066.
 44. McCormsey GA, Tebas P, Shane E, Yin MT, Overton ET et al. (2010) Bone Disease in HIV Infection: A Practical Review and Recommendations for HIV Care Providers. *Clin Infect Dis* 51: 937-946. doi:10.1086/656412. PubMed: 20839968.
 45. Kudo K, Konta T, Mashima Y, Ichikawa K, Takasaki S et al. (2011) The association between renal tubular damage and rapid renal deterioration in the Japanese population: the Takahata study. *Clin Exp Nephrol* 15: 235-241. doi:10.1007/s10157-010-0392-y. PubMed: 21225309.
 46. Ofotokun I, Sheth AN, Sanford SE, Easley KA, Shenvi N et al. (2012) A switch in therapy to a reverse transcriptase inhibitor sparing combination of lopinavir/ritonavir and raltegravir in virologically suppressed HIV-infected patients: a pilot randomized trial to assess efficacy and safety profile: the KITE study. *AIDS Res Hum Retroviruses* 28: 1196-1206. doi:10.1089/aid.2011.0336. PubMed: 22364141.



Institut Pasteur

Microbes and Infection 15 (2013) 874–886



www.elsevier.com/locate/micinf

Original article

Identification of cross-clade CTL epitopes in HIV-1 clade A/E-infected individuals by using the clade B overlapping peptides

Koji Watanabe ^{a,b,1}, Hayato Murakoshi ^{a,1}, Yoshiko Tamura ^a, Madoka Koyanagi ^a, Takayuki Chikata ^a, Hiroyuki Gatanaga ^{a,b}, Shinichi Oka ^{a,b}, Masafumi Takiguchi ^{a,*}

^a Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto, Japan

^b AIDS Clinical Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo, Japan

Received 12 July 2013; accepted 8 August 2013

Available online 19 August 2013

Abstract

Identification of cross-clade T cell epitopes is one of key factors for the development of a widely applicable AIDS vaccine. We here investigated cross-clade CD8⁺ T cell responses between clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. CD8⁺ T cell responses to 11-mer overlapping peptides derived from Nef, Gag, and Pol clade B consensus sequences were at a similar level to those to the same peptides found in clade B-infected individuals. Fifteen cross-clade CTL epitopes were identified from 13 regions where the frequency of responders was high in the clade A/E-infected individuals. The sequences of 6 epitopes were conserved between the clade B and clade A/E viruses whereas 9 epitopes had different amino acid sequences between the 2 viruses. CD8⁺ T cells specific for the 6 conserved epitopes recognized cells infected with the clade A/E virus, whereas those for 8 diverse epitopes recognized both the clade A/E virus-infected and clade B-infected cells. All of the cross-clade CD8⁺ T cells specific for conserved and diverse epitopes were detected in chronically HIV-1 clade A/E-infected individuals. These results show that in addition to conserved regions polymorphic ones across the clades can be targets for cross-clade CTLs.

© 2013 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.

Keywords: Cross-clade; CTL; HLA; Epitope; HIV

1. Introduction

The HIV-1 genome is characterized by genetic diversity wherein distinct HIV-1 clades are expanding not only in different geographical regions but also even in the same locality [1]. HIV-1 clade B is the most prevalent virus in Japan accounting for more than 80% of the patients in this country. CRF01_AE (clade A/E) is the second most prevalent virus, accounting for 6.1% (Sugiura W, unpublished report). In contrast, the clade A/E virus is mainly prevalent in south-east

Asian countries including Thailand. An RV144 phase III vaccine trial, which was recently performed in Thailand, demonstrated a partial beneficial effect on HIV-1 infection [2]. In this trial, the recombinant canarypox virus-vectored HIV-1 *gag/pollenv* vaccine (ALVAC-HIV) and the recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) were used for priming and boosting, respectively. These vaccines were generated by using genes from both the clade B and A/E viral strains [2] to cover a wider range of potential challenge strains in Thailand, where approximately 80% and 10% of HIV-1-infected individuals are infected with the clade A/E and clade B viruses, respectively [3]. Recent analyses confirmed CD4⁺ cell-mediated and humoral immune responses in vaccines [3–6]. Thus, this clinical trial also highlighted the importance of the cross-clade immune responses to the clade B and A/E viruses. Although T cell functions in the acute and

* Corresponding author. Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan. Tel.: +81 96 373 6529; fax: +81 96 373 6532.

E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

¹ K.W. and H.M. contributed equally to this study.

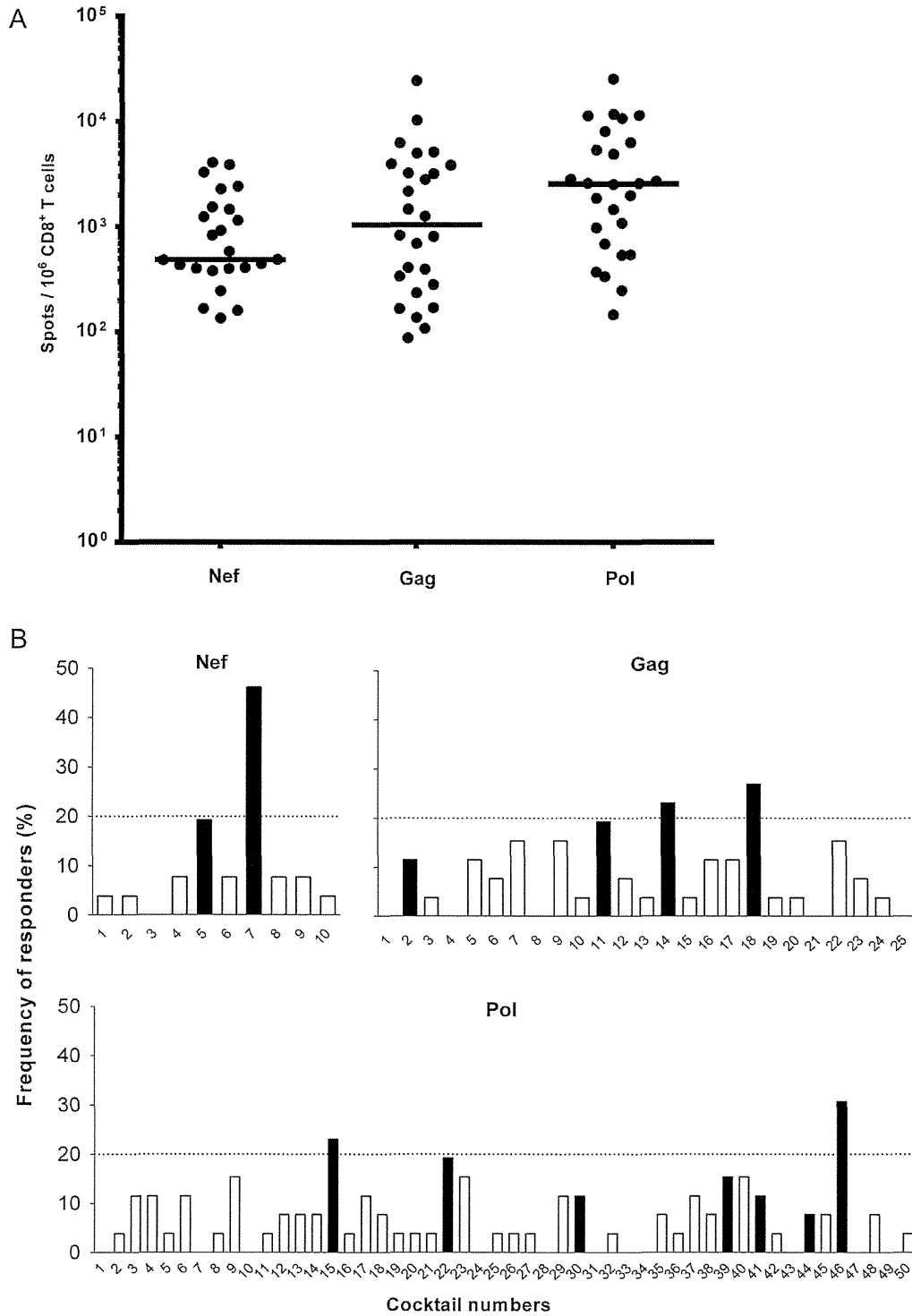
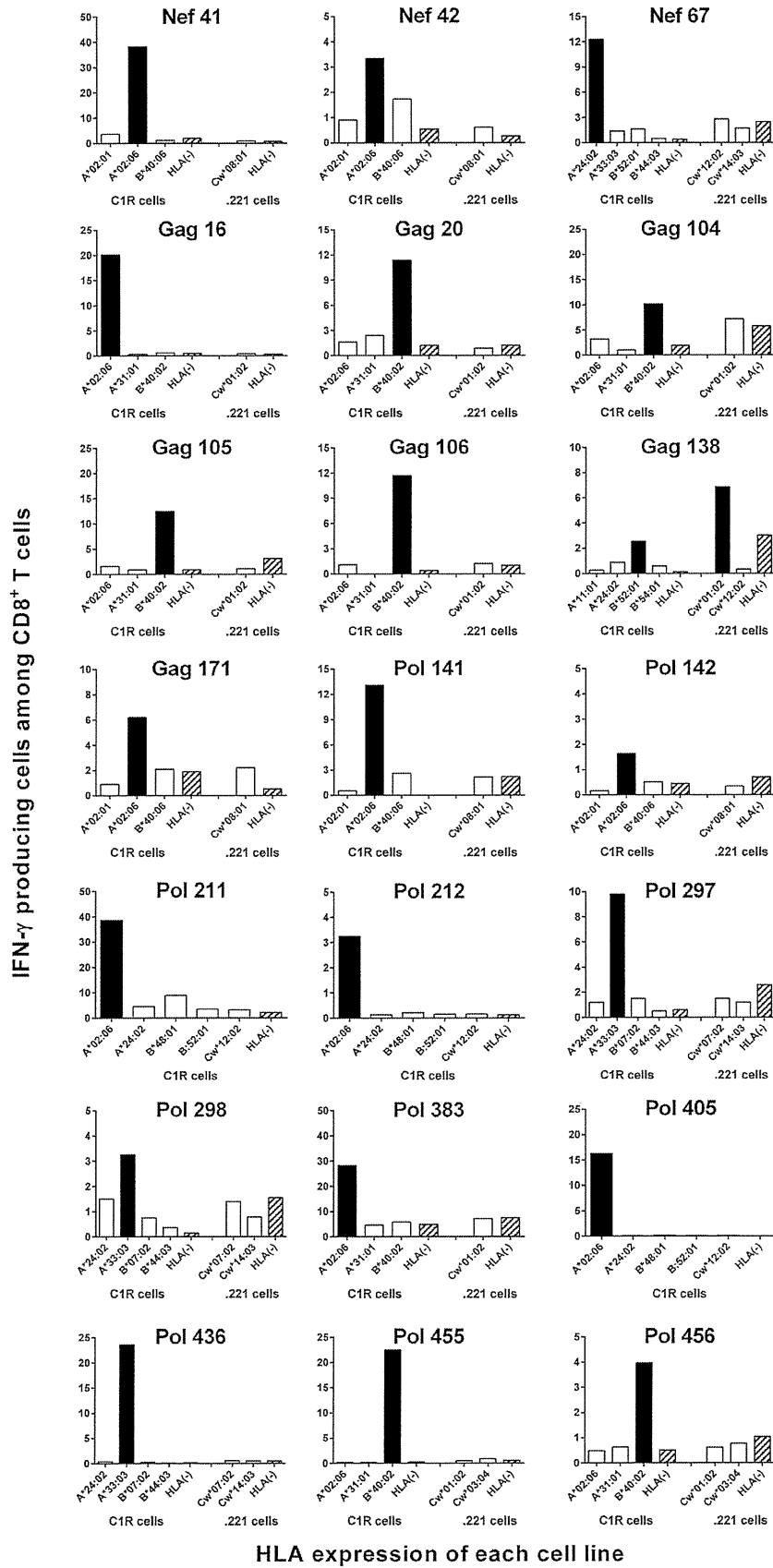


Fig. 1. CD8⁺ T cell responses of clade A/E-infected individuals to HIV-1 clade B-derived overlapping peptides. CD8⁺ T cell responses to peptide cocktails containing clade B consensus overlapping 11-mer peptides were analyzed by performing the ELISPOT assay using CD8⁺ T cells from 26 clade A/E-infected individuals. A. Total magnitude of CD8⁺ T cell responses to the clade B consensus overlapping peptides spanning Nef, Gag and Pol regions. B. Frequency of the responders to each cocktail. Positive response is defined as more than 200 spots. The cocktails for which the frequency of responders was more than 20% or less than 20% are shown as solid bars. In the latter case, at least 1 patient showed a high response (>750 spots).



chronic phases of an HIV-1 infection were well studied in Caucasians infected with the clade B virus and in Africans infected with the clade C virus [7–12], there are only a limited number of studies about the cross-clade reactivity of CTLs [13–17]. However, even in such studies a comprehensive analysis of cross-clade reactivity of the CTLs was not performed.

In the context of HIV vaccine development, it is very important to choose vaccine immunogens capable of eliciting CTLs that can control the variable mutant viruses and exhibit cross-reactivity across the different clade viruses [18,19]. The conserved parts of HIV-1 are good candidates as vaccine antigens [11,12,16,20,21], since they include epitopes conserved among viruses not only in the same clade but also among those clades. Indeed, CTL vaccines containing conserved epitopes have been shown to elicit CTL responses to HIV-1 [22–25]. Although the safety of these consensus CTL vaccines was confirmed in humans [26–32], such vaccines were poorly immunogenic in previous phase I and II trials [27,28,30,31]. Thus more studies on cross-clade effective epitopes will be needed for the development of more potent vaccines.

In the present study, we analyzed cross-clade CD8⁺ T cells between HIV-1 clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. For this analysis, we sought to identify cross-clade CTL epitopes between the clade B and A/E viruses in the Japanese individuals by using 11-mer overlapping peptides derived from the clade B consensus sequence spanning Nef, Gag, and Pol regions. Thereafter, we analyzed cross-clade CD8⁺ T cell recognition for epitope peptides between clade A/E and B as well as CTL recognition for cells infected with the clade B or the A/E virus. This is the first comprehensive study to identify cross-clade CD8⁺ T cells by using overlapping HIV-1 peptides.

2. Materials and methods

2.1. Patient samples

This study was approved by the Ethical Committee of in National Center for Global Health and Medicine and Kumamoto University. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. HIV-1 subtypes were determined by the sequence results on Pol and Gag, and confirmed by Env sequencing. All samples were collected from the cohort in AIDS Clinical Center, National Center for Global Health and Medicine.

2.2. Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (Qiagen). cDNA was synthesized from the RNA by use of the SuperScript III First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen). Nef, Gag, and Pol regions were amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were purified by using ExoSAP-IT (GE). All DNA sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3500 Genetic Analyzer.

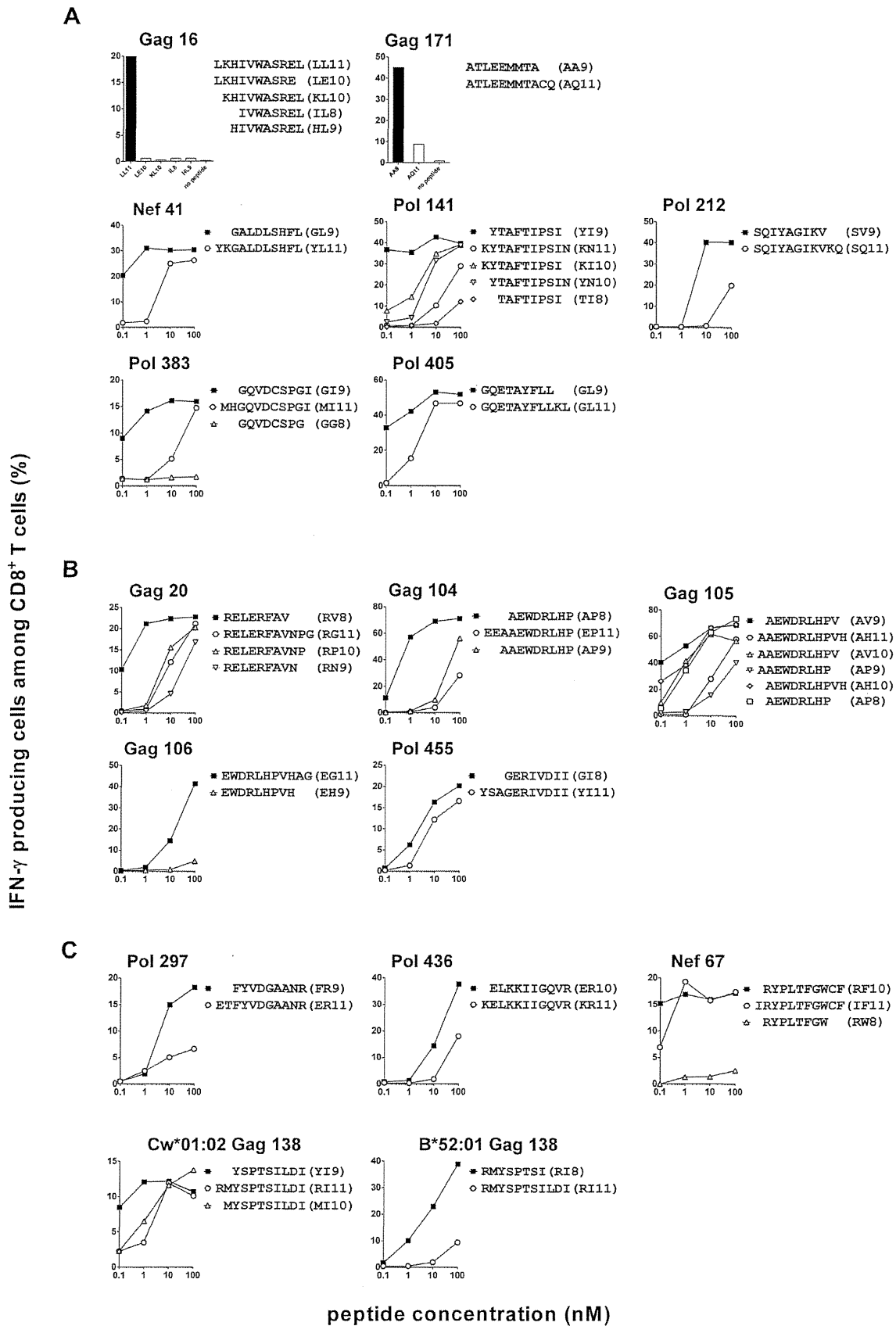
2.3. Synthetic peptides

We previously designed overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences. Each 11-mer peptide was overlapped by 9 amino acids [33]. These 11-mer peptides and truncated peptides were synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.4. ELISPOT assay

CD8⁺ T cells were sorted from cryopreserved PBMCs from 26 chronically HIV-1 clade A/E-infected Japanese individuals by using CD8 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The sorted cells were plated in 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN- γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The appropriate amount of peptide cocktails including 10 overlapping 11-mer peptides were added in a volume of 50 μ L, and then PBMCs were added at 1×10^5 cells/well in a volume of 100 μ L. The plates were incubated for 16 h at 37 °C in 5% CO₂ and then washed with PBS before the addition of biotinylated anti-IFN- γ Mab (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min and then washed with PBS, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min. reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo,

Fig. 2. Identification of HLA restriction of the responses to each 11-mer peptide. Peptide-specific CD8⁺ bulk T cells were induced from PBMCs of the following 6 responders by stimulating the cells with each single peptide. KI-648 for Nef 41, Nef 42, Gag 171, Pol 141, and Pol 142 peptides, KI-632 for Nef 67, Pol 297, Pol 298, and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 211, Pol 212, and Pol 405 peptides, and KI-837 for Pol 455 and Pol 456 peptides. Induced CD8⁺ bulk T cells were stimulated with the corresponding peptide-pulsed C1R cells or 0.221 cells expressing each HLA-class I allele molecule. IFN- γ production by CD8⁺ T cells was detected by performing the intracellular cytokine staining (ICS) assay.



Japan). The CD8⁺ T cells without peptide stimulation were used as a negative control. The number of spots for each peptide-specific T cell response was calculated by subtracting the number of negative-control spots (the number of spots in wells without peptides). Spots giving a mean of more than + 2 SD of the negative-control spots were defined as positive responses. In order to find cross-clade CD8⁺ T cells in our cohort, we performed the ELISPOT assay by the same method with 11-mer single peptides that were the components of the cocktail peptides where 1) the frequency of responders was more than 20% or 2) the frequency was less than 20% but in which case at least 1 patient showed a high spot count (>750 spots).

2.5. Cells

721.221-CD4 cells expressing HLA-A*02:06, -A*33:03 or Cw*01:02 were generated by transfecting both human CD4 gene and one of these HLA-class I genes into 721.221 cells. These cells were maintained in RPMI medium containing 10% fetal calf serum (FCS) and 0.15 mg/mL hygromycin B. C1R cells expressing HLA-A*02:06 and those expressing HLA-A*33:03 were generated by transfecting C1R cells with HLA-A*02:06 and -A*33:03, respectively; and they were maintained in RPMI medium containing 10% FCS and 0.15 mg/mL hygromycin. C1R and 721.221 cells expressing other HLAs used in this study were previously generated and maintained in RPMI medium with 10% FCS and 0.15 mg/mL hygromycin B or 0.2 mg/mL neomycin [33–36].

2.6. Induction of peptide-specific CTLs from PBMCs

PBMCs from HIV-1-infected individuals who showed the responses to the cocktail peptides in the ELISPOT assay were stimulated with 11-mer single peptide or optimal peptide derived from consensus clade B HIV-1 (100 nM) and then cultured in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml interleukin-2) for 2 weeks. These bulk cultured cells were used for intracellular IFN- γ staining assays.

2.7. HIV clones

The replication-competent molecular clones of p93JP-NH1 [37] and pNL-432 [38] reported previously were used in this study. Viral stocks were generated from plasmid DNA as described elsewhere [15,39].

2.8. HIV-1 infection of .221-CD4 cells expressing HLA molecules or not

.221-CD4 cells expressing HLA molecules or not were exposed to each virus for several days. These infected cells were used as stimulator cells for performing an intracellular cytokine staining assay (ICS) when approximately 30–60% of the cells had been infected, which infection was confirmed by intracellular staining for HIV-1 p24 antigen (KC-57-FITC; Beckman Coulter).

2.9. Intracellular cytokine staining assay (ICS)

After .221 cells or C1R cells had been incubated for 60 min with each peptide (0.01–100 nM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed or HIV-1-infected .221-CD4 cells (1×10^5 cells per well) and bulk cultured cells (2×10^4 cells per well) were added to wells of a 96-well round-bottomed plate, and then the cells were incubated for 2 h at 37 °C. Brefeldin A (10 μ g/ml) was then added, after which the cells were incubated for a further 4 h. After having been stained with APC-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark), the cells were fixed with 4% paraformaldehyde and then made permeable with the permeabilizing buffer (0.1% saponin and 5% FCS in PBS). Thereafter the cells were stained with FITC-labeled anti-IFN- γ mAb (BD Bioscience, CA). The percentage of IFN- γ ⁺CD8⁺ cells was analyzed by flow cytometry.

3. Results

3.1. CD8⁺ T cell responses to HIV-1 clade B-derived overlapping peptides by HIV-1 clade A/E-infected individuals

To clarify cross-clade responses of CD8⁺ T cells between the clade B and A/E, we analyzed cross-clade responses of CD8⁺ T cells from 26 clade A/E-infected Japanese individuals to 11-mer overlapping peptides derived from the consensus sequence of HIV-1 clade B Nef, Gag, and Pol regions. We measured the responses of CD8⁺ T cells to cocktails including ten 11-mer overlapping peptides by performing the ELISPOT assay. The median of total magnitudes of the CD8⁺ T cell responses to Nef, Gag, and Pol cocktails were 483, 1037, and 2538, respectively (Fig. 1A). There were no significant differences in total magnitude of the CD8⁺ T cell responses between the clade A/E-infected and 401 clade B-infected Japanese individuals (the median of total magnitude against Nef, Gag and Pol in the clade B-infected individuals were 529,

Fig. 3. Identification of optimal epitopes Truncated peptides were designed based on HLA binding motif, and CD8⁺ bulk T cells were induced from PBMCs of the following 6 responders. KI-648 for Nef 41, Gag 171, and Pol 141 peptides, KI-632 for Nef 67, Pol 297 and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 212 and Pol 405 peptides, and KI-837 for Pol 455 peptide. IFN- γ production by CD8⁺ bulk T cells was measured by performing the ICS assay using the target C1R or .221 cells expressing HLA molecules prepulsed with truncated peptide or 11-mer peptide at a concentration of 100 nM. When the same level of response was seen at 100 nM, the ICS assay was performed again at concentrations from 0.1 to 100 nM. A. responses of HLA-A*02:06-restricted CD8⁺ bulk T cells B. responses of HLA-B*40:02-restricted CD8⁺ bulk T cells C. responses of HLA-A*33:03, A*24:02, Cw*01:02 or B*52:01-restricted CD8⁺ bulk T cells.

1774, and 2300, respectively; H. Murakoshi et al. unpublished observation), although the identities of amino acid sequence in Nef, Gag, and Pol between clade the A/E and the clade B were 80.1, 84.3 and 92.3%, respectively. These results strongly suggest that cross-clade CD8⁺ T cells were frequently elicited in the clade A/E-infected individuals.

3.2. Identification of cross-clade CD8⁺ T cells elicited in HIV-1 clade A/E-infected individuals

To identify cross-clade CD8⁺ T cells in the clade A/E-infected individuals, we focused on the CD8⁺ T cell responses found to be strong or at a high frequency in these individuals (see Materials and methods). We selected the CD8⁺ T cell responses to 13 cocktails including 2 Nef, 4 Gag, and 7 Pol cocktails (solid bars in Fig. 1B). First, to clarify which 11-mer peptides were recognized by the specific CD8⁺ T cells, we selected the clade A/E-infected responders (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964) and measured the CD8⁺ T cell responses to ten 11-mer peptides in each cocktail by using the ELISPOT assay. We found positive responses to three 11-mer Nef peptides (Nef cocktail 5: Nef 41 and 42, Nef cocktail 7: Nef 67), to ten 11-mer Gag peptides (Gag cocktail 2: Gag 11, 16, 19, and 20, Gag cocktail 11: Gag 101, 104, 105, and 106, Gag cocktail 14: Gag 138, Gag cocktail 18: Gag 171), and to eleven 11-mer Pol peptides (Pol cocktail 15: Pol 141 and 142, Pol cocktail 22: Pol 211 and 212, Pol cocktail 30: Pol 297 and 298, Pol cocktail 39: Pol 383, Pol cocktail 41: Pol 405, Pol cocktail 44: Pol 436, Pol cocktail 46: Pol 455 and 456) (data not shown). We next sought to determine HLA restriction molecules in these responses. PBMCs from these responders were stimulated with the 11-mer peptides and then cultured for 14 days. In order to determine the HLA restriction molecules, responses of the cultured cells against the corresponding peptides were analyzed by performing the intracellular cytokine staining (ICS) assay using HLA class I gene-transfected C1R cells or 721.221 cells as stimulators. We found 10 HLA-A*02:06-restricted responses, 6 HLA-B*40:02-restricted responses, 3 A*33:03-restricted responses, 1 HLA-A*24:02-restricted response, 1 HLA-B*52:01-restricted response, and 1 Cw*01:02-restricted response (Fig. 2).

We first analyzed the responses to the 10 HLA-A*02:06-restricted responses. Concerning the responses to overlapping peptides at 3 locations (Nef 41/42, Pol 141/142, and Pol 211/212), we speculated that they would be the same epitope-specific CD8⁺ T cell responses since the responses to these overlapping peptides were restricted by HLA-A*02:06. Therefore we focused on analyzing the response to Nef 41, Pol 141 or Pol 211, which showed higher responses than those to the other overlapping peptides (data not shown). We generated truncated peptides that were speculated based on HLA-A*02:06 binding motif (Ala, Thr or Gln at position 2) [40–43] and then analyzed these CD8⁺ T cell responses to Nef 41, Pol 141, and Pol 212 by using them. As shown in Fig. 3A, we identified 3 optimal epitopes: Nef GL9 (GALDLSHFL), Pol Y19 (YTAFTIPSI), and Pol SV9

(SQIYAGIKV). CD8⁺ T cell responses to Nef GL9 and Pol SV9 were detected among the responses to other overlapping peptides, Nef 42 and Pol 211, respectively (data not shown), indicating that the responses to Nef GL9 and Pol SV9 reflected those to Nef 42 and Pol 212, respectively. In contrast, Pol 142 did not contain Pol Y19. We analyzed the CD8⁺ bulk T cells induced by Pol 142 by using the truncated peptides and identified Pol T18 (TAFTIPSI) as an optimal epitope. However, the response to Pol T18 in CD8⁺ bulk T cells induced by Pol 142 (4.24% IFN- γ secretion at 100 nM peptide concentration) was much lower than that to Pol T18 in CD8⁺ bulk T cells induced by Pol 141 (12.07% at 100 nM; Fig. 3A), suggesting that Pol T18 may have been a very weak epitope. Similarly, we analyzed the other 4 HLA-A*02:06-restricted responses (Gag16, Gag171, Pol383, and Pol405) by using truncated peptides and identified 4 optimal epitopes; Gag LL11 (LKHIVWASREL), Gag AA9 (ATLEEMMTA), Pol GI9 (GQVDCSPGI), and Pol GL9 (GQETAYFLL; Fig. 3A).

By using the same method identified the HLA-A*02:06-restricted epitopes, we attempted to identify other epitopes. We generated truncated peptides based on HLA binding motif [7,33,40,44–50] and then the responses to these truncated peptides were analyzed using the ICS assay. We finally identified 5 HLA-B*40:02-restricted epitopes (Gag RV8, Gag AP8, Gag AV9, Gag EG11, and Pol GI8; Fig. 3B), 2 HLA-A*33:03-restricted epitopes (Pol FR9 and Pol ER10; Fig. 3C), 1 HLA-A*24:02-restricted epitope (Nef RF10; Fig. 3C), 1 HLA-B*52:01-restricted epitope (Gag RI8; Fig. 3C) and 1 HLA-Cw*01:02-restricted epitope (Gag Y19; Fig. 3C).

Eleven of the above 17 peptides were reported as epitopes in previous studies [7,33,40–43,45,47–50], whereas the other 6 peptides (HLA-A*02:06-restricted Gag LL11, Pol SV9, and Pol GI9, as well as HLA-B*40:02-restricted Gag RV8, Gag AP8, and Gag EG11) had not been previously reported to be epitopes. Therefore, we examined whether the CD8⁺ bulk T cells specific for these 6 epitopes could recognize HIV-1 clade B virus-infected cells. We measured the IFN- γ production from the CD8⁺ bulk T cells for target cells infected with HIV-1 clade B clone, NL4-3. These CD8⁺ bulk T cells effectively produced IFN- γ (data not shown), indicating that these 6 peptides had been naturally processed and presented in cells infected with HIV-1.

3.3. Cross-recognition between the clade B and A/E

We sequenced each epitope region in 26 HIV-1 clade A/E-infected Japanese individuals and then compared these sequences to those from the clade A/E and B viruses reported in the database of the Los Alamos National Library. The results showed that the consensus amino acid sequences of these epitopes in our cohort were the same as those in the database of Los Alamos National Library. The clade B consensus sequences of 6 epitopes (Gag AA9, Pol Y19, Pol SV9, Pol GI9, Pol GL9, and Pol ER10) were identical to the clade A/E consensus ones, whereas other 11 epitopes showed different consensus sequences between the clade B and A/E viruses (Table 1).

Table 1
Frequency of amino-acid sequence for each epitope region of clade A/E viruses from Los Alamos National Library database and our cohort patients.

Epitope	HXB2 region	Sequence	Frequency of amino acid sequence of each clade virus		
			Clade B viruses for Los Alamos database	Clade A/E viruses for Los Alamos database	Clade A/E viruses for our cohort patients
Nef GL9	Nef(83–91)	GALDLSHFL ^a	529/1494	0/76	1/26
		—F—F—	12/1494	47/76	16/26
		Others	953/1494	29/76	9/26
Nef RF10	Nef(134–143)	RYPLTFGWCF ^a	800/1494	0/76	2/25
		—C—	94/1494	56/76	10/25
		others	600/1494	20/76	13/25
Gag LL11	p17(31–41)	LKHIVWASREL ^a	1182/1644	18/315	2/26
		M—L—	0/1644	147/315	14/26
		others	462/1644	150/315	10/26
Gag RV8	p17(39–46)	RELERFAV ^a	1299/1644	20/315	4/26
		—L—	111/1644	217/315	17/26
		others	234/1644	78/315	5/26
Gag AP8	p24(78–85)	AEWDRRLHP ^a	1263/1644	53/315	1/26
		—V—	128/1644	185/315	10/26
		others	253/1644	77/315	15/26
Gag AV9	p24(78–86)	AEWDRRLHPV ^a	1135/1644	47/315	1/26
		—V—	121/1644	178/315	8/26
		others	388/1644	90/315	17/26
Gag EG11	p24(79–89)	EWDRRLHPVHAG ^a	921/1644	48/315	1/26
		—V—	107/1644	149/315	5/26
		others	616/1644	118/315	20/26
Gag RI8	p24(143–150)	RMYSPTSI ^a	1033/1644	30/315	2/26
		—V—	351/1644	212/315	15/26
		others	260/1644	73/315	9/26
Gag YI9	p24(145–153)	YSPTSILDI ^a	1032/1644	30/315	2/26
		—V—	345/1644	212/315	15/26
		others	267/1644	73/315	9/26
Gag AA9	p24(209–217)	ATLEEMMTA ^a	1468/1644	285/315	25/26
		others	176/1644	30/315	1/26
		others	429/1003	15/59	10/26
Pol YI9	RT(127–135)	YTAFTIPSI ^a	574/1003	44/59	16/26
		others	429/1003	15/59	10/26
		others	565/1003	21/59	8/26
Pol SV9	RT(268–276)	SQIYAGIKV ^a	438/1003	38/59	18/26
		others	565/1003	21/59	8/26
		others	471/1003	45/59	19/26
Pol FR9	RT(440–448)	FYVDGAANR ^a	838/1003	9/59	9/26
		—S—	471/1003	45/59	15/26
		others	118/1003	5/59	2/26
Pol GI9	Integrase(50–60)	GQVDCSPGI ^a	915/1003	55/59	26/26
		others	88/1003	4/59	0/26
		others	484/1003	7/59	7/26
Pol GL9	Integrase(94–102)	GQETAYFLL ^a	519/1003	52/59	19/26
		others	484/1003	7/59	7/26
		others	233/1003	19/59	5/26
Pol ER10	Integrase(157–166)	ELKKIIGQVR ^a	770/1003	40/59	21/26
		others	233/1003	19/59	5/26
		others	378/1003	0/57	0/26
Pol GI8	Integrase(197–204)	GERIVDII ^a	535/1003	0/57	0/26
		—I—	378/1003	56/59	23/26
		others	90/1003	3/59	3/26

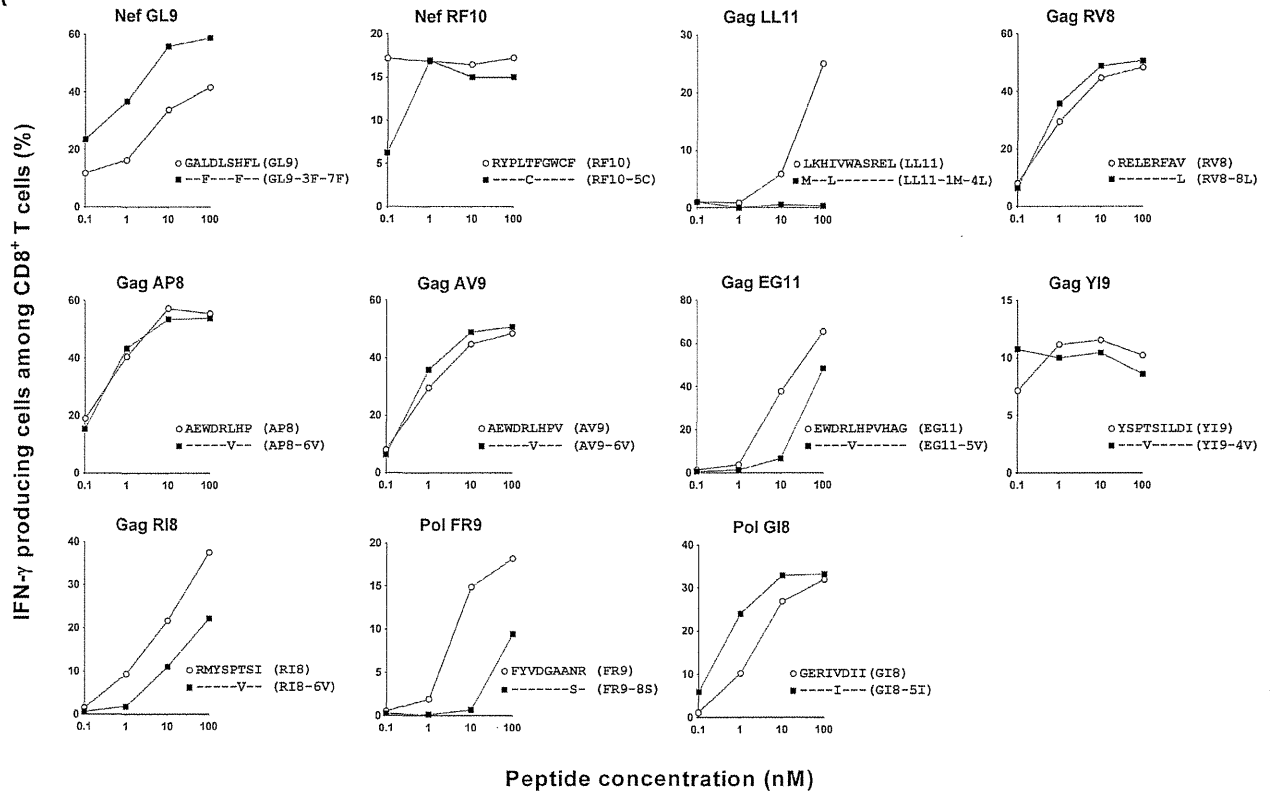
^a Amino acid sequence in clade B consensus used in this study.

We investigated the cross-recognition of these 11 epitope peptides by CD8⁺ T cells that had been induced by stimulating PBMCs from clade A/E virus-infected individuals with clade B-derived epitope peptides. The CD8⁺ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, or Gag YI9 peptides recognized both clades B and A/E peptides evenly (Fig. 4A). The CD8⁺ bulk T cells induced by Gag EG11, Gag RI8, or Pol FR9 more strongly recognized the clade B-derived peptide than the clade A/E-derived one, whereas those induced by Nef GL9 or Pol GI8 more strongly recognized the clade A/E-derived peptide than the clade B-derived one (Fig. 4A). Interestingly, the CD8⁺ bulk T cells induced by Gag 16 LL11

failed to recognize the clade A/E-derived Gag LL11-1M-4L peptide (Fig. 4A).

Next, we investigated whether CD8⁺ T cells recognizing these 10 clade A/E peptides could recognize clade A/E-infected cells. We measured IFN- γ production from the CD8⁺ bulk T cells for target cells infected with HIV-1 clade A/E clone or for those infected with HIV-1 clade B clone. The CD8⁺ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, Gag EG11, Gag YI9, Gag RI8, or Pol GI8 recognized not only the clade B virus-infected cells but also the clade A/E virus-infected ones (Fig. 4B), indicating that these cross-clade epitopes had been naturally processed

A



B

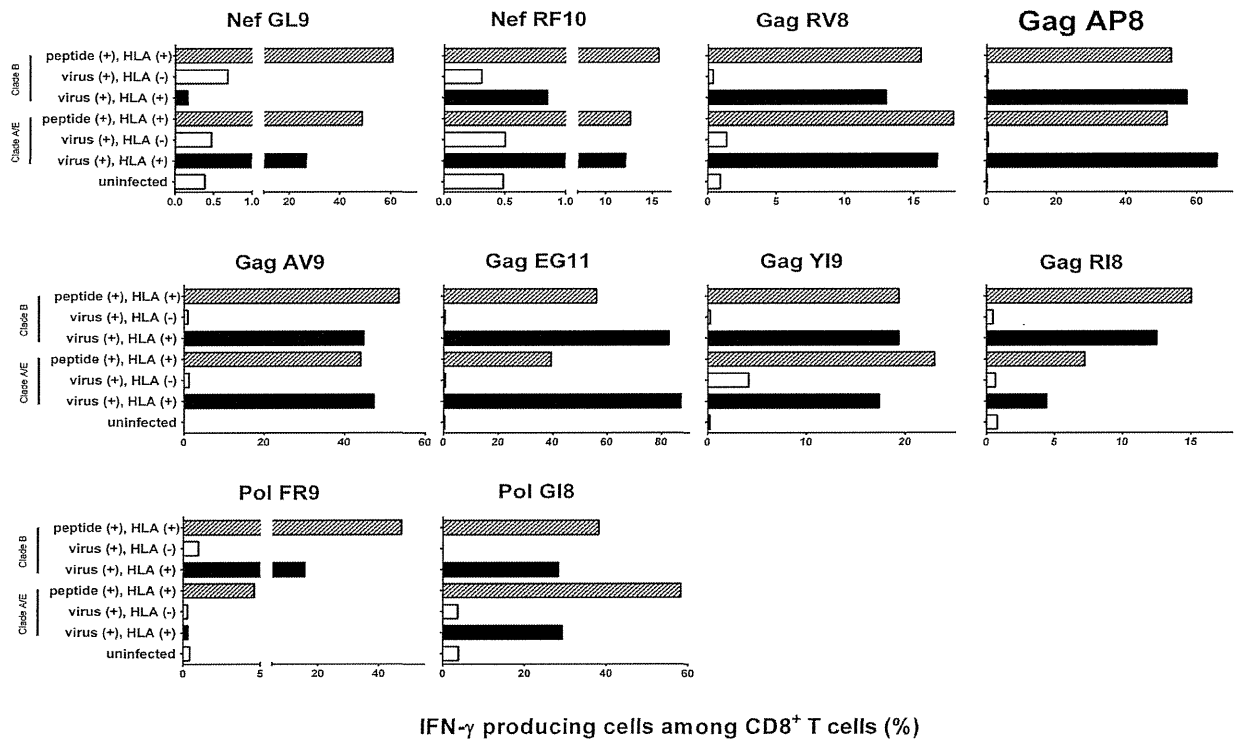


Fig. 4. Cross-recognition by CD8⁺ T cells from HIV-1 clade A/E-infected individuals IFN- γ production by CD8⁺ bulk T cells in response to stimulator cells with optimal epitope peptides and to cells infected with clade B or clade A/E viruses was measured by use of the ICS assay. Nef GL9 specific bulk T cells were induced from PBMCs of KI-648, Nef RF10 and Pol FR9 specific bulk T cells were induced from PBMCs of KI-632, Gag LL11, Gag RV8, Gag AP8, Gag AV9 and Gag EG11 specific bulk T cells were induced from PBMCs of KI-388, Gag RI8 and Gag YI9 specific bulk T cells were induced from KI-724, and Pol GI8 specific bulk