are mediated through the p66 subunit mutation, although an involvement of the mutation at the p51 subunit currently cannot be ruled out and should be addressed by biochemical experiments.

In terms of NNRTI resistance, our molecular modeling analysis is consistent with a hypothesis that the mutation is likely to affect the flexibility and mobility of the p66 thumb subdomain. Extensive crystallographic work with HIV-1 RT in several forms, including an unliganded form, in complex with DNA substrates or NNRTIs has revealed that during the course of DNA polymerization, the p66 thumb subdomain undergoes major conformational motions that are critical for efficient catalysis. Alignment of multiple structures of HIV RT suggests that the p66 thumb moves as a rigid body with its base hinged to the palm subdomain exactly near residue 348 (Fig. 3). Residue 348 is proximal to, and likely to affect, the relative interactions between residues of the p66 connection (T351) and p66 thumb subdomains (V317, I270, P272, W239, and eventually Y318). The proximity of residue 348 to this hinge region leads us to believe that changes imparted by the N348I mutation alter the mobility and flexibility of the thumb subdomain. Subtle changes in the interactions between V317 and N348 may also reposition W239 and its neighboring Y318 in the NNRTI-binding pocket. Interestingly, the Y318F mutation affects NNRTI resistance in a similar way as N348I: it decreases susceptibility to NVP and DLV but not to EFV (19, 33). Biochemical binding experiments of RTs with NNRTIs would directly evaluate this hypothesis.

The effect of the N348I mutation on NRTI resistance cannot be rationalized by direct interactions of the mutated residue with the NRTI binding site. It is tempting to speculate that minor changes in the p66 thumb subdomain hinge motions also have minor effects on the positioning of the nucleic acid, which in turn affects the ability to discriminate between NRTI and the normal substrate by an as yet undefined mechanism. However, direct biochemical experimental evidence will be needed to determine the precise molecular details of the specific mechanisms of NRTI resistance.

It has been proposed previously that an imbalance between reverse transcription and RNA degradation plays an important role in NRTI resistance (25). Pathak and colleagues proposed that connection subdomain mutations may result in a slower RNase H reaction, and this in turn may provide an increased time period available for AZT excision, especially with TAMs (28–30). In the case of N348I, Yap et al. recently reported that N348I decreases RNase H enzymatic activity (42). At present, available evidence is consistent with a model in which these connection subdomain mutations alter the affinity of the RT for template/primer, enhance nucleoside excision, and reduce template switching.

Several studies, including recent work by Delviks-Frankenberry et al. and Brehm et al. (4, 11), highlighted the necessity to expand sequencing analysis to include the connection and RNase H subdomains. This contention is further supported by results in this work and by others (16, 28, 42, 43) showing that mutations at the connection subdomain influence susceptibility to some antiretroviral drugs. Hence, there is a growing interest in obtaining genotypic information from expanded areas of RT that would be useful for a more complete analysis of HIV drug resistance. Interestingly, already two out of four commercially available genotypic and phenotypic assay kits are designed to include in their analysis at least part of the connection subdomain (Antivirogram by Virco up to RT residue 400 and ViroSeq by Abbott/Celera Diagnostics up to RT residue 335).

The present study identifies N348I as a MDR mutation in HIV-1 RT. This knowledge provides information that may be useful in designing more efficient therapeutic strategies that can improve clinical outcome and help prevent the emergence of MDR variants, especially in salvage therapy. This work further highlights the functional role of the HIV-1 RT connection subdomain in drug resistance. Future studies that focus on the structural and biochemical properties of connection subdomain RT mutants should reveal the molecular details of NRTI and NNRTI drug resistance caused by connection subdomain residues.

ACKNOWLEDGMENTS

This work was supported by a grant for the promotion of AIDS Research from the Ministry of Health, Labor and Welfare (to A.H., E.K., Y.S., M.M., H.G., M.T., and S.O.), a grant for Research for Health Sciences Focusing on Drug Innovation from The Japan Health Sciences Foundation (E.K and M.M), a grant from the Organization of Pharmaceutical Safety and Research (A.H., H.G., and S.O.) and a grant from the ministry of Education, Culture, Sports, Science, and Technology (E.K).

We thank Yukiko Takahashi and Fujie Negishi for sample preparation and the AIDS Clinical Center coordinator nurses for their dedicated assistance.

REFERENCES

1. **Antinori, A., M. Zaccarelli, A. Cingolani, F. Forbici, M. G. Rizzo, M. P. Trotta, S. Di Giambenedetto, P. Narciso, A. Ammassari, E. Girardi, A. De Luca, and C. F. Perno.** 2002. Cross-resistance among nonnucleoside reverse transcriptase inhibitors limits recycling efavirenz after nevirapine failure. AIDS Res. Hum. Retrovir. **18:**835–838.
2. **Baldanti, F., S. Paolucci, G. Maga, N. Labo, U. Hubscher, A. Y. Skoblov, L. Victorova, S. Spadari, L. Minoli, and G. Gerna.** 2003. Nevirapine-selected mutations Y181I/C of HIV-1 reverse transcriptase confer cross-resistance to stavudine. AIDS **17:**1568–1570.
3. **Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes.** 2002. Nucleoside analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. J. Virol. **76:**9143–9151.
4. **Brehm, J. H., D. Koontz, J. D. Meteer, V. Pathak, N. Sluis-Cremer, and J. W. Mellors.** 2007. Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine. J. Virol. **81:**7852–7859.
5. **Brenner, B., D. Turner, M. Oliveira, D. Moisi, M. Detorio, M. Carobene, R. G. Marlink, J. Schapiro, M. Roger, and M. A. Wainberg.** 2003. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. AIDS **17:**F1–5.
6. **Brown, A. J., H. M. Precious, J. M. Whitcomb, J. K. Wong, M. Quigg, W. Huang, E. S. Daar, R. T. D'Aquila, P. H. Keiser, E. Connick, N. S. Hellmann, C. J. Petropoulos, D. D. Richman, and S. J. Little.** 2000. Reduced susceptibility of human immunodeficiency virus type 1 (HIV-1) from patients with primary HIV infection to nonnucleoside reverse transcriptase inhibitors is associated with variation at novel amino acid sites. J. Virol. **74:**10269–10273.
7. **Caride, E., R. Brindeiro, K. Hertogs, B. Larder, P. Dehertogh, E. Machado, C. A. de Sa, W. A. Eyer-Silva, F. S. Sion, L. F. Passioni, J. A. Menezes, A. R. Calazans, and A. Tanuri.** 2000. Drug-resistant reverse transcriptase genotyping and phenotyping of B and non-B subtypes (F and A) of human immunodeficiency virus type I found in Brazilian patients failing HAART. Virology **275:**107–115.
8. **Cherrington, J. M., A. S. Mulato, M. D. Fuller, and M. S. Chen.** 1996. Novel mutation (K70E) in human immunodeficiency virus type 1 reverse transcriptase confers decreased susceptibility to 9-[2-(phosphonomethoxy)ethyl]adenine in vitro. Antimicrob. Agents Chemother. **40:**2212–2216.
9. **Das, K., S. G. Sarafianos, A. D. Clark, Jr., P. L. Boyer, S. H. Hughes, and E. Arnold.** 2007. Crystal structures of clinically relevant Lys103Asn/Tyr181Cys double mutant HIV-1 reverse transcriptase in complexes with ATP and non-nucleoside inhibitor HBY 097. J. Mol. Biol. **365:**77–89.
10. **de Jong, J. J., J. Goudsmit, V. V. Lukashov, M. E. Hillebrand, E. Baan, R. Huismans, S. A. Danner, J. H. ten Veen, F. de Wolf, and S. Jurriaans.** 1999.

Insertion of two amino acids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs. AIDS **13**:75–80.

11. **Delviks-Frankenberry, K. A., G. N. Nikolenko, R. Barr, and V. K. Pathak.** 2007. Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance. J. Virol. **81**:6837–6845.
12. **Esnouf, R., J. Ren, C. Ross, Y. Jones, D. Stammers, and D. Stuart.** 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Nat. Struct. Biol. **2**:303–308.
13. **Gatanaga, H., S. Oka, S. Ida, T. Wakabayashi, T. Shioda, and A. Iwamoto.** 1999. Active HIV-1 redistribution and replication in the brain with HIV encephalitis. Arch. Virol. **144**:29–43.
14. **Hachiya, A., S. Aizawa-Matsuoka, M. Tanaka, Y. Takahashi, S. Ida, H. Gatanaga, Y. Hirabayashi, A. Kojima, M. Tatsumi, and S. Oka.** 2001. Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (MAGIC-5). Antimicrob. Agents Chemother. **45**:495–501.
15. **Hachiya, A., H. Gatanaga, E. Kodama, M. Ikeuchi, M. Matsuoka, S. Harada, H. Mitsuya, S. Kimura, and S. Oka.** 2004. Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naive patients. Virology **327**:215–224.
16. **Hachiya, A., E. Kodama, S. G. Sarafianos, M. M. Schuckman, M. Matsuoka, M. Takiguchi, G. Gatanaga, and S. Oka.** 2007. A novel mutation, N348I in HIV-1 reverse transcriptase induced by NRTI treatment, confers nevirapine resistance, abstr. 593. Abstr. 14th Conf. Retrovir. Opportunistic Infect., Los Angels, CA.
17. **Hammer, S. M., M. S. Saag, M. Schechter, J. S. Montaner, R. T. Schooley, D. M. Jacobsen, M. A. Thompson, C. C. Carpenter, M. A. Fischl, B. G. Gazzard, J. M. Gatell, M. S. Hirsch, D. A. Katzenstein, D. D. Richman, S. Vella, P. G. Yeni, and P. A. Volberding.** 2006. Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. JAMA **296**:827–843.
18. **Hammond, J. L., U. M. Parikh, D. L. Koontz, S. Schlueter-Wirtz, C. K. Chu, H. Z. Bazmi, R. F. Schinazi, and J. W. Mellors.** 2005. In vitro selection and analysis of human immunodeficiency virus type 1 resistant to derivatives of beta-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine. Antimicrob. Agents Chemother. **49**:3930–3932.
19. **Harrigan, P. R., M. Salim, D. K. Stammers, B. Wynhoven, Z. L. Brumme, P. McKenna, B. Larder, and S. D. Kemp.** 2002. A mutation in the 3′ region of the human immunodeficiency virus type 1 reverse transcriptase (Y318F) associated with nonnucleoside reverse transcriptase inhibitor resistance. J. Virol. **76**:6836–6840.
20. **Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison.** 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science **282**:1669–1675.
21. **Kavlick, M. F., K. Wyvill, R. Yarchoan, and H. Mitsuya.** 1998. Emergence of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 variants, viral sequence variation, and disease progression in patients receiving antiretroviral chemotherapy. J. Infect. Dis. **177**:1506–1513.
22. **Kemp, S. D., C. Shi, S. Bloor, P. R. Harrigan, J. W. Mellors, and B. A. Larder.** 1998. A novel polymorphism at codon 333 of human immunodeficiency virus type 1 reverse transcriptase can facilitate dual resistance to zidovudine and L-2′,3′-dideoxy-3′-thiacytidine. J. Virol. **72**:5093–5098.
23. **Kodama, E. I., S. Kohgo, K. Kitano, H. Machida, H. Gatanaga, S. Shigeta, M. Matsuoka, H. Ohrui, and H. Mitsuya.** 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. **45**:1539–1546.
24. **Larder, B. A., S. D. Kemp, and P. R. Harrigan.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269**:696–699.
25. **Lusso, P., F. Cocchi, C. Balotta, P. D. Markham, A. Louie, P. Farci, R. Pal, R. C. Gallo, and M. S. Reitz, Jr.** 1995. Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4$^+$ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. J. Virol. **69**:3712–3720.
26. **Mann, D. L., S. J. O'Brien, D. A. Gilbert, Y. Reid, M. Popovic, E. Read-Connole, R. C. Gallo, and A. F. Gazdar.** 1989. Origin of the HIV-susceptible human CD4$^+$ cell line H9. AIDS Res. Hum. Retrovir. **5**:253–255.
27. **Nameki, D., E. Kodama, M. Ikeuchi, N. Mabuchi, A. Otaka, H. Tamamura, M. Ohno, N. Fujii, and M. Matsuoka.** 2005. Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. J. Virol. **79**:764–770.
28. **Nikolenko, G. N., K. A. Delviks-Frankenberry, S. Palmer, F. Maldarelli, M. J. Fivash, Jr., J. M. Coffin, and V. K. Pathak.** 2007. Mutations in the connection domain of HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resistance. Proc. Natl. Acad. Sci. USA **104**:317–322.
29. **Nikolenko, G. N., S. Palmer, F. Maldarelli, J. W. Mellors, J. M. Coffin, and V. K. Pathak.** 2005. Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. Proc. Natl. Acad. Sci. USA **102**:2093–2098.
30. **Nikolenko, G. N., E. S. Svarovskaia, K. A. Delviks, and V. K. Pathak.** 2004. Antiretroviral drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase increase template-switching frequency. J. Virol. **78**:8761–8770.
31. **Paolucci, S., F. Baldanti, G. Maga, R. Cancio, M. Zazzi, M. Zavattoni, A. Chiesa, S. Spadari, and G. Gerna.** 2004. Gln145Met/Leu changes in human immunodeficiency virus type 1 reverse transcriptase confer resistance to nucleoside and nonnucleoside analogs and impair virus replication. Antimicrob. Agents Chemother. **48**:4611–4617.
32. **Paolucci, S., F. Baldanti, M. Tinelli, G. Maga, and G. Gerna.** 2003. Detection of a new HIV-1 reverse transcriptase mutation (Q145M) conferring resistance to nucleoside and non-nucleoside inhibitors in a patient failing highly active antiretroviral therapy. AIDS **17**:924–927.
33. **Pelemans, H., R. M. Esnouf, H. Jonckheere, E. De Clercq, and J. Balzarini.** 1998. Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. J. Biol. Chem. **273**:34234–34239.
34. **Ren, J., L. E. Bird, P. P. Chamberlain, G. B. Stewart-Jones, D. I. Stuart, and D. K. Stammers.** 2002. Structure of HIV-2 reverse transcriptase at 2.35-Å resolution and the mechanism of resistance to non-nucleoside inhibitors. Proc. Natl. Acad. Sci. USA **99**:14410–14415.
35. **Rhee, S. Y., W. J. Fessel, A. R. Zolopa, L. Hurley, T. Liu, J. Taylor, D. P. Nguyen, S. Slome, D. Klein, M. Horberg, J. Flamm, S. Follansbee, J. M. Schapiro, and R. W. Shafer.** 2005. HIV-1 Protease and reverse-transcriptase mutations: correlations with antiretroviral therapy in subtype B isolates and implications for drug-resistance surveillance. J. Infect. Dis. **192**:456–465.
36. **Sarafianos, S. G., K. Das, C. Tantillo, A. D. Clark, Jr., J. Ding, J. M. Whitcomb, P. L. Boyer, S. H. Hughes, and E. Arnold.** 2001. Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA: DNA. EMBO J. **20**:1449–1461.
37. **Shafer, R. W., S. Y. Rhee, D. Pillay, V. Miller, P. Sandstrom, J. M. Schapiro, D. R. Kuritzkes, and D. Bennett.** 2007. HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance. AIDS **21**:215–223.
38. **Shirasaka, T., M. F. Kavlick, T. Ueno, W. Y. Gao, E. Kojima, M. L. Alcaide, S. Chokekijchai, B. M. Roy, E. Arnold, and R. Yarchoan.** 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA **92**:2398–2402.
39. **Sluis-Cremer, N., C. W. Sheen, S. Zelina, P. S. Torres, U. M. Parikh, and J. W. Mellors.** 2007. Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. **51**:48–53.
40. **Tuske, S., S. G. Sarafianos, A. D. Clark, Jr., J. Ding, L. K. Naeger, K. L. White, M. D. Miller, C. S. Gibbs, P. L. Boyer, P. Clark, G. Wang, B. L. Gaffney, R. A. Jones, D. M. Jerina, S. H. Hughes, and E. Arnold.** 2004. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. Nat. Struct. Mol. Biol. **11**:469–474.
41. **Winters, M. A., K. L. Coolley, Y. A. Girard, D. J. Levee, H. Hamdan, R. W. Shafer, D. A. Katzenstein, and T. C. Merigan.** 1998. A 6-base pair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. J. Clin. Investig. **102**: 1769–1775.
42. **Yap, S. H., C. W. Sheen, J. Fahey, M. Zanin, D. Tyssen, V. D. Lima, B. Wynhoven, M. Kuiper, N. Sluis-Cremer, P. R. Harrigan, and G. Tachedjian.** 2007. N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. PLoS Med. **4**:e335.
43. **Yap, S. H., B. Wynhoven, M. Kuiper, C. W. Sheen, N. Sluis-Cremer, R. Harrigan, and G. Tachedjian.** 2007. A mutation in the connection subdomain of the HIV-1 reverse transcriptase (N348I) is selected commonly in vivo and confers decreased susceptibility to zidovudine and nevirapine, abstr. 594. Abstr. 14th Conf. Retrovir. Opportunistic Infect., Los Angels, CA.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 10 (2008) 689–698

www.elsevier.com/locate/micinf

Original article

HLA-A*2402-restricted HIV-1-specific cytotoxic T lymphocytes and escape mutation after ART with structured treatment interruptions

Junko Tanuma [a,*], Mamoru Fujiwara [b], Katsuji Teruya [a], Saori Matsuoka [a], Hikaru Yamanaka [a], Hiroyuki Gatanaga [a], Natsuo Tachikawa [a], Yoshimi Kikuchi [a], Masafumi Takiguchi [b], Shinichi Oka [a]

[a] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan*
[b] *Division of Viral Immunology, Center for AIDS research, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan*

Received 7 January 2008; accepted 17 March 2008
Available online 29 March 2008

Abstract

Although a limited duration of immune activation of structured treatment interruptions (STIs) has been reported, the immune escape mechanism during STIs remains obscure. We therefore investigated the role of three immunodominant cytotoxic T lymphocyte (epitopes) in 12 HLA-A*2402-positive patients participating longitudinally during the clinical study of early antiretroviral treatment (ART) with five series of structured treatment interruptions (STIs). The frequency of HLA-A*2402-restricted CTLs varied widely and a sustained CTL response was rarely noted. However, a Y-to-F substitution at the second position in an immunodominant CTL epitope Nef138-10 (Nef138-2F), which was previously demonstrated as escape mutation, was frequently detected in seven patients primarily and emerged in the remaining five patients thereafter, and the existence of escape mutations was correlated with high pVL levels early in the clinical course. These findings suggest that escape mutation in the immunodominant CTL epitope may be one of the mechanisms to limit HIV-1-specific immune control in STIs.

Keywords: Structured treatment interruptions; Cytotoxic T lymphocyte; HLA-A*2402; Escape variant

1. Introduction

Structured treatment interruption (STI) is considered one of the immune stimulatory interventions for HIV-1 infection, based on the hypothesis that viral rebound during treatment interruption might induce HIV-specific immune responses [1–3]. Since the 1999 case report of the early-treated patient who achieved sustained viral suppression without highly antiretroviral therapy (HAART) after two occasional treatment interruptions [1], the STI strategy has been studied in various clinical settings [4–7]. Because cytotoxic T lymphocytes (CTLs) play a critical role in the control of HIV-1 replication and HIV-specific CD4+ T-cell response is important to maintain effective HIV-1-specific CTLs [8-11], early treatment that can preserve HIV-1-specific-CD4+ T cells is considered to have the greater impact on STI in early infection than in chronic infection [11–13]. However, the majority of previous STI trials revealed the limitation of immune activation with risk of viral resistance [4,14,15] and the mechanisms of viral control failure in STI strategy have remained unclear.

Viral mutation in immunodominant epitopes is one of the obstacles to HIV-1 vaccine development [16–21]. Since HIV-1-specific T-cell responses are restricted by HLA alleles, its escape variant can be transmitted and adopted in populations sharing some dominant HLA alleles [19–21]. In Japan where HLA-A*2402 is the most frequent HLA class I allele with 70% prevalence, HLA-A*2402-restricted CTLs and its immunodominant epitopes have been extensively assessed [22]. Nef138-10, which has been proved previously as an HLA-A*2402-restricted CTL epitope provoking strong cytolytic activity [22], is one of the immunodominant CTL epitopes in HLA-A*2402-positive Japanese patients [21,22].

* Corresponding author. Tel.: +81 3 3202 7181x5642; fax: +81 3 3202 7198.
E-mail address: jtanuma@imcj.hosp.go.jp (J. Tanuma).

1286-4579/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2008.03.007

Our previous study showed that a Y-to-F substitution at the second position in Nef138-10 epitope (Nef138-2F) impairs the ability of the Nef138-10-specific CTLs to suppress HIV-1 replication, indicating that Nef138-2F is an escape mutation from CTLs [23]. Since Nef138-2F is observed in both HLA-A*2402-positive and -negative patients, Nef138-2F variant may be stable and adopted at a population level [21].

In the present study of early antiretroviral treatment with five series of STIs for HLA-A *2402 positive Japanese patients, we investigated the longitudinal magnitudes of HIV-1-specific HLA-A*2402-restricted CTLs by using HLA-epitope tetramer binding assay and sequenced the most immunodominant epitopes Nef138-10 to evaluate whether escape mutation might negatively influence viral control in an STI study.

2. Methods

2.1. Study design and patient population

This trial was designed as a prospective study at the AIDS Clinical Center, International Medical Center of Japan. Between November 2000 and December 2001, patients with early HIV infection, with or without acute retroviral symptoms, were recruited. Early HIV infection was confirmed within 6 months before recruitment by a documented history of seroconversion in enzyme-linked immunosorbent assay (ELISA) or longitudinal increase of bands in Western blot test. Patients with active opportunistic infections or psychological disorders, or those treated with immunomodulatory agents were excluded. Antiretroviral therapy was initiated after obtaining a signed informed consent. The first-choice regimen for this study consisted of stavudine, lamivudine and indinavir boosted with ritonavir, but the patient was allowed to use other antiretroviral drugs when the first regimen could not be tolerated. To avoid emergence of drug resistance to indinavir, ritonavir-boosting was stopped more than 1 week before treatment interruption. The duration of treatment interruption was fixed for 3 weeks. The first treatment was interrupted after more than 3 months of HAART, when CD4+ cell count was $>500/mm^3$ and plasma viral load (pVL) had been <50 copies/ml for at least 1 month. Other interruptions were also carried out when pVL became <50 copies/ml and CD4+ cell count was $>300/mm^3$. Five series of STIs were scheduled during the treatment.

The study protocol was approved by the institutional ethical review boards (IMCJ-H13-10).

2.2. Monitoring and sample collection

Patients were monitored monthly during HAART and at approximately a 4-month interval after treatment discontinuation. Unscheduled visits were permitted according to clinical needs. At each visit, clinical assessment and routine laboratory tests were performed. Blood specimens were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes, separated into peripheral blood mononuclear cells (PBMCs) and plasma, and stored at −80 °C for assessment of HIV-1-specific CTLs and sequence of the dominant epitope region. pVL was quantified by using the Amplicor HIV-1 Monitor test 1.5 (Roche Diagnostics, Indianapolis, IN) with a detection limit of 50 copies/ml. Antiretroviral drug resistance-associated mutations were examined at baseline and after HAART including STIs in all 26 participants. Each mutation was identified according to the revised August 2006 International AIDS Society Resistance-USA Panel [24].

2.3. HLA typing and epitope-HLA-A*2402 tetramer cytometry assays

High-resolution HLA class I typing was performed by a PCR-sequence-specific primer method. If HLA-A*2404 was positive, HIV-1 specific CTLs were investigated by using peptide-HLA-A*2402 tetrameric complex synthesized as described previously [21,22,25]. Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C-terminus of the heavy chain, and then mixed with phycoerythrin (PE)-conjugated avidin (extravidin-PE; Sigma−Aldrich, St. Louis, MO) at a molar ratio of 4:1. Cryopreserved PBMCs ($0.5-1 \times 10^6$ cells) were stained by the tetramer at 37 °C for 30 min. After double washing with washing buffer (10% fetal calf serum in RPMI 1640), the cells were stained by fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 mAb (BD Biosciences, San Jose, CA) at 4 °C for 30 min. The cells were then washed twice and analyzed using a FACS Calibur with Cell Quest software (Becton Dickinson, San Jose, CA). Based on our previous study [22], three immunodominant epitopes of HLA-A*2402 restricted CTLs; Nef138-10, Gag28-9 and Env584-9, were chosen for this assay. Since we found a high frequency of Y-to-F substitution at the second position in Nef138-10 gene (Nef138-2F) which has been suspected as an escape variant in previous studies [21], Nef138-2F-specific CTLs (Nef138-2F-CTLs) were also measured by tetramers using Nef138-2F variant alone and by competitive double staining using two types of tetramers of both wild type and Nef138-2F variant to compare the frequencies of the two types of HIV-1-specific CTLs.

2.4. Sequence analyses of Nef138-10 gene

For evaluation of escape variants from CTLs, we sequenced the region coding Nef138-10, which is the immunodominant HLA-A*2402-restricted epitope, while Nef138-2F has been suspected as escape mutation in this epitope, using the method described here. Total RNA was extracted from plasma with a High Pure viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by RT−PCR with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan) to amplify the HIV-1 Nef DNA segment (2341 bp) as described previously [21]. The PCR products were purified with SUPREC-02 (TaKaRa Shuzo) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 5.1 (Software Development, Tokyo).

2.5. Statistical analysis

Data from patients who completed the treatment protocol including five series of STIs were analyzed. Before analysis, pVL data were log-transformed and undetectable pVL (<50 copies/ml) was considered equivalent to 50 copies/ml. The Mann–Whitney *U*-test was used to compare the pVLs determined every 3 months after treatment cessation to the pVLs of 279 untreated chronic HIV-1 patients in order to assess the durability of viral suppression. The correlation between pVL and percentage of CTLs was assessed by simple regression analysis. Statistical analyses were performed using SPSSII software package for Windows, version 11.0J.

3. Results

3.1. Characteristics of participants

During the enrollment period, 432 new patients were referred to our clinic. Of these, 32 met the criteria of early HIV-1 infection and 6 were excluded due to psychological problems or taking systemic steroid therapy for symptoms associated with acute retroviral syndrome. All 26 recruits were Japanese infected with HIV-1 by sexual intercourse, and 24 were men (92%). The mean age of patients was 35.0 years (range, 21–56 years). The mean pVL at baseline was 5.21 $\log_{10}$ copies/ml (range, 3.28–6.91 $\log_{10}$ copies/ml) and the mean CD4+ cell count at baseline was 413/mm^3 (range, 49–1156/mm^3). Twenty-five patients presented with wide-range clinical symptoms of acute retroviral syndrome. Fifteen out of 26 participants completed the treatment protocol including five series of STI. HAART had to be continued in four patients because CD4+ cell counts had never stabilized above 300/mm^3 despite more than 6 months of treatment. The other seven patients discontinued the treatment protocol after less than five STIs due to adverse events, adherence problems, or no specific problems.

In the protocol-completed 15 patients, 14 were men (92%). The mean age was 34.0 years (range, 21–56 years). At baseline, the median pVL was 5.14 $\log_{10}$ copies/ml (range, 3.28–6.91 $\log_{10}$ copies/ml) and the median CD4+ cell count was 475/mm^3 (range, 245–990/mm^3). The demographic, immunological, and virological factors before initiation of HAART of the protocol-completed group were not statistically different from those of the uncompleted group (Mann–Whitney *U*-test) (data not shown), although baseline CD4+ cell counts of four ART-continued patients: 49, 185, 210, and 351/mm^3 respectively seemed lower than those who completed the treatment protocol. Twelve (80%) patients were positive for HLA-A*2402 and its incidence was similar to those reported previously in Japanese population [21,22]. No specific HLA genotypes that are known to influence the clinical course of HIV infection such as HLA-B*27, HLA-B*57 and HLA-B*35 (except B*3501) [26] were detected in participants. The median length of follow-up after treatment cessation was 961 days (range, 462–1255 days).

No resistance-associated mutations were identified among all the 26 participants at study enrollment except one who had M184V, D30N and L90M mutations despite good virologic responses throughout HAART. There was no increase in resistance-associated mutations during and after five STIs in all participants (data not shown).

3.2. Plasma viral load and CD4+ cell count in protocol-completed 15 patients

Fig. 1 shows serial changes in median pVLs and CD4+ cell counts in protocol-completed 15 patients. Peaks of viral

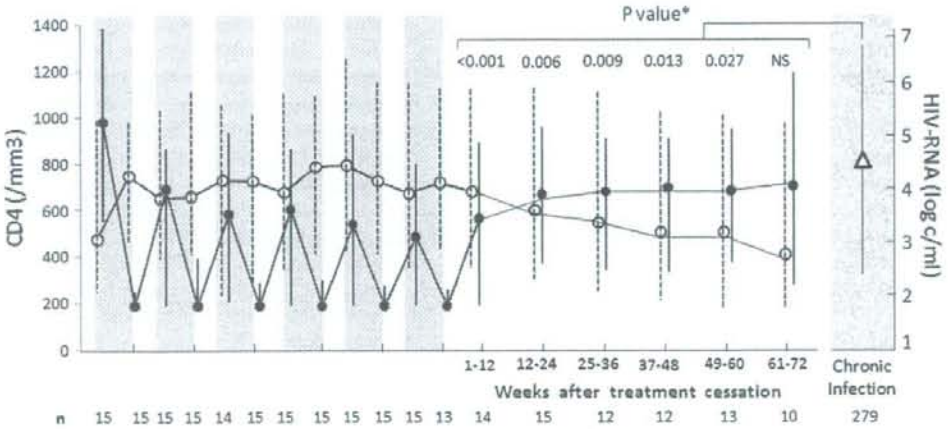

Fig. 1. Serial changes in plasma viral loads and CD4+ cell counts of 15 protocol-completed patients. Plasma viral loads (pVLs) and CD4+ cell counts are expressed as median of 15 protocol-completed patients; at baseline, at the times of treatment interruption, at the peaks of pVL rebound during structured treatment interruption and at every 12 weeks after treatment cessation. Open circles: CD4+ cell counts; solid circles: pVLs; triangle: the median pVL of 279 untreated chronic HIV-1 patients who were referred to our clinic during the study and whose CD4 count was >200/mm^3. Vertical lines provide the ranges with dotted lines in CD4+ cell counts and with light lines in pVLs. Shaded area: time on antiretroviral therapy; unshaded area: time off therapy. Numbers of patients whose data were evaluated at each time point appear at the bottom of the graph. *pVLs of every 12 weeks after treatment cessation were compared to the pVLs of 279 untreated chronic HIV-1 patients by Mann–Whitney *U*-test.

rebounds during treatment interruptions decreased gradually. The pVLs of every 12 weeks after treatment cessation were under 4 $\log_{10}$ copies/ml in most of the patients and they were significantly lower for 60 weeks than the pVLs of 279 untreated chronic HIV-1 patients in our clinic. However, pVLs gradually increased and there was no difference at week 61–72 from pVLs of chronically infected patients. The proportion of patients with a favorable viral control whose median pVL at every 12 weeks after treatment cessation were less than 4.0 $\log_{10}$ copies/ml was 66% in the first 12 weeks but the proportion decreased to 33% in the 61–72 weeks. Along with the increase in pVL, CD4+ cell counts declined after treatment cessation and one patient (KI-134) required restart of HAART because CD4+ cell count decreased below 200/mm^3 at week 52. None of the patients developed episode of opportunistic infections or HIV-related diseases throughout this study.

*3.3. Plasma viral loads and frequency of HLA-A*2402-restricted CTLs*

We investigated induction of 3 HLA-A*2402-restricted immunodominant epitope-specific CTLs in 12 patients with HLA-A*2402 by using the corresponding tetramers. Fig. 2 shows the serial changes in HLA-A*2402-restricted HIV-1-specific CTLs. Overall, the frequency of HLA-A*2402-restricted CTLs varied widely among the patients and a sustained CTL response was rarely noted. We investigated the correlation between pVLs at every 12 weeks after treatment cessation and frequency of HLA-A*2402-restricted CTLs according to the epitope. None of Nef138-10-, Gag28-9- or Env584-9-specific CTLs was statistically correlated to pVLs (Fig. 3A).

3.4. Effect of Nef138-10 escape mutation on suppression of HIV replication

A Y-to-F substitution at the second position of Nef138-10 (Nef138-2F) has been suspected as an escape mutation from HLA-A*2402-restricted Nef138-10-specific CTLs in a previous study [21]. In fact, we recently demonstrated that Nef138-10-specific CTLs fail to suppress replication of Nef138-2F mutant [23]. We therefore performed serial sequence analyses of Nef138-10 epitope and investigated whether this 2F mutation is responsible for the limited duration of viral suppression. As shown in Table 1, we found high frequency of this mutation. Seven out of 12 patients had Nef138-2F variant in viral RNA or proviral DNA in the earliest samples (KI-091, KI-126, KI-134, KI-144, KI-150, KI-154 and KI-163). The Nef138-2F variant was not detected in the earliest samples of the other five patients (KI-092, KI-099, KI-102, KI-158 and KI-161) and these patients were considered to have Nef138-10 wild-type infection except a T-to-C substitution at the fifth position (Nef138-5C) in KI-099 which has also been suspected as one of the escape variants from Nef138-10-specific CTLs in a previous study [21], and an L-to-I substitution at the forth position (Nef138-4I) in KI-161. However, Nef138-2F mutation was detected at the latter stage in all the other five patients.

We speculated that Nef138-10-specific CTLs can control replication of HIV-1 in patients who had been infected with Nef138-WT virus. Therefore we compared pVLs according to the existence of escape mutants Nef138-2F or 138-5C at the earliest sample drawn during early phase of infection before treatment initiation. As shown in Fig. 3B, the pVLs between 13 and 36 weeks were significantly lower in the other four patients who were confirmed as Nef138-WT or Nef138-4I infection than in the remaining eight patients who had Nef138-2F or Nef138-5C variant in the earliest samples, which has been suspected as an escape variant from Nef138-10-specific CTLs in a previous study. These indicate that Nef138-10-specific CTLs control replication of wild-type virus but the presence of either Nef138-2F or Nef138-5C negatively influences viral control.

3.5. Nef138-2F variant specific CTLs

We found Nef138-WT-tetramer and Nef138-2F-tetramer bound to both Nef138-WT-specific CTL clones and Nef138-2F-specific CTL clones. In addition, Nef138-WT-tetramer had stronger affinity to Nef138-WT-specific CTL clones than Nef138-2F-specific CTL clones (Fig. 4A) and vice versa (our unpublished work). Therefore, the double-staining assay using both tetramers simultaneously was performed to differentiate the two types of CTLs.

The frequencies of the two types of CTLs are shown in Table 1. In patients negative for Nef138-2F or Nef138-5C initially, Nef138-WT-CTLs were detected early after the treatment cessation (KI-092, KI-102, KI-158 and KI-161) but declined after evolution of Nef138-2F (KI-092, KI-102, and KI-161). Although only a slight elevation of Nef138-2F-CTLs was noted after emergence of Nef138-2F (KI-092 and KI-161), the magnitude was smaller than that of Nef138-WT-specific CTLs before emergence of Nef138-2F.

In patients having Nef138-2F variant initially and suspected as Nef138-2F variant infection, the frequencies of Nef138-2F-CTLs were relatively smaller than those of Nef138-WT-specific CTLs in Nef138-10 wild-type infection, except KI-144 who had marked increase of Nef138-2F-CTLs in week 37.

Fig. 4B and C illustrate the clinical courses of two representative cases; KI-161 was non-Nef138-2F variant infection and KI-144 was suspected as Nef138-2F variant infection. In KI-161 (Fig. 4B), Nef138-WT-CTL response diminished after the emergence of Nef138-2F mutation. Interestingly, the pVL of this patient seemed to increase along with the fall in Nef138-WT-CTLs (Fig. 2). In KI-144 (Fig. 4C), Nef138-2F-CTLs were induced but there was no suppression of pVLs. These results indicate that either infection or emergence of Nef138-2F variant might limit the CTL induction.

4. Discussion

In this study, we could not demonstrate the lowered set-point pVLs in patients who received HAART with five series of STIs in early HIV-1 infection. Previous studies revealed that a vigorous HIV-1-specific CD4 response is associated

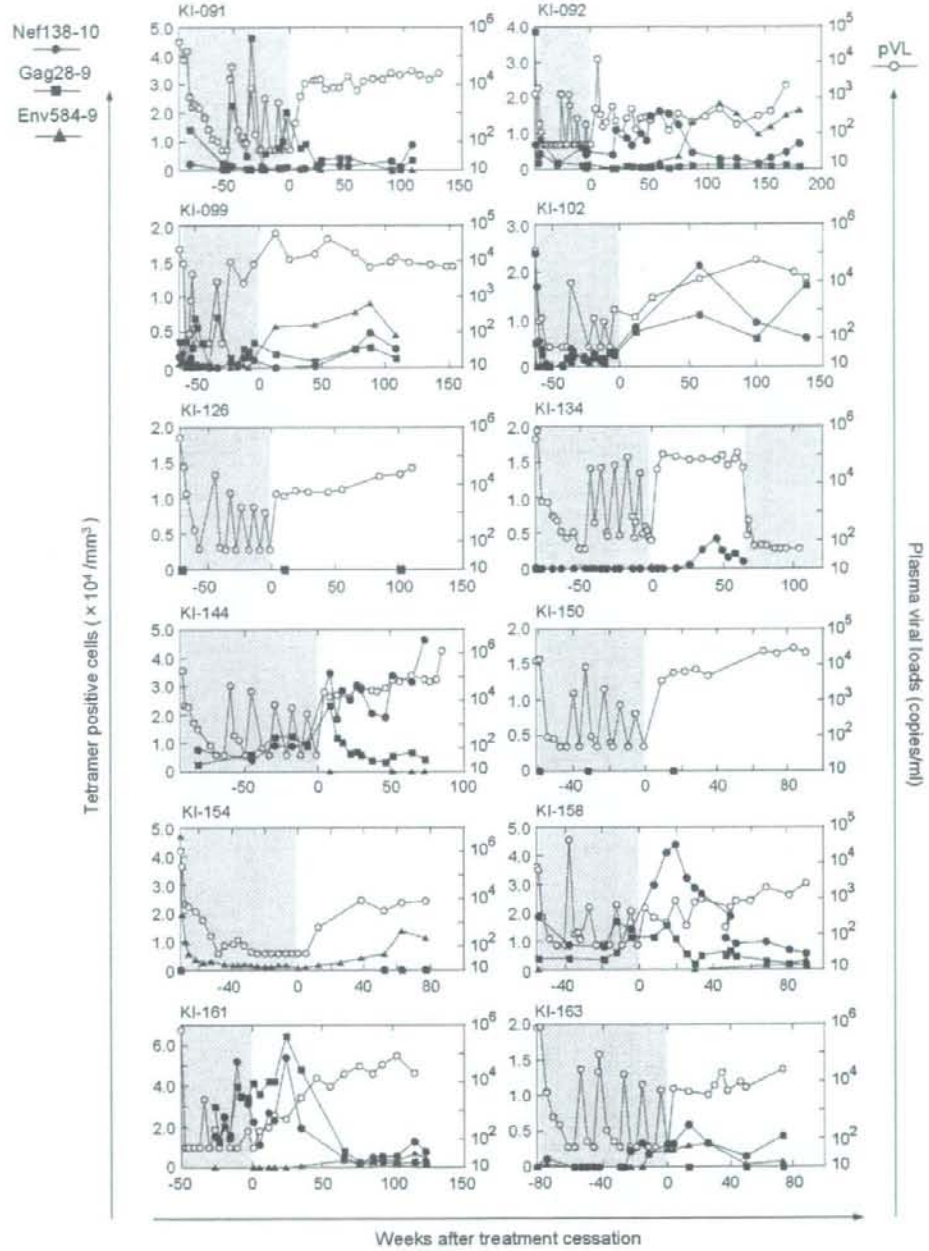

Fig. 2. Frequencies of HLA-A*2402 restricted HIV-1-specific CTLs determined by tetramer binding assay. HLA-A*2402 restricted HIV-1-specific CTLs in PBMCs were determined by using tetrameric complexes of HLA-A*2404 and each of the three types of epitopes. Solid circle: Nef138-10-specific CTL; solid squares: Gag28-9-specific CTL; solid triangles: Env584-9-specific CTL; open circles: plasma viral load. Shaded area: time on antiretroviral therapy; unshaded area: time off therapy.

with a slower disease progression [8–11]; however, despite some reports of boosted immunological responses in acutely treated patients, the evidence of clinical benefits of early treatment has not been established [12,13]. In line with these trials of early initiation of HAART with or without STI, the CTL responses in our study were mostly transient and did not correlate with pVL levels.

We adopted HLA-epitope tetramer analysis for evaluating CTL responses, which provides specific information on HLA class I allele and HLA-restricted epitopes, because CTL

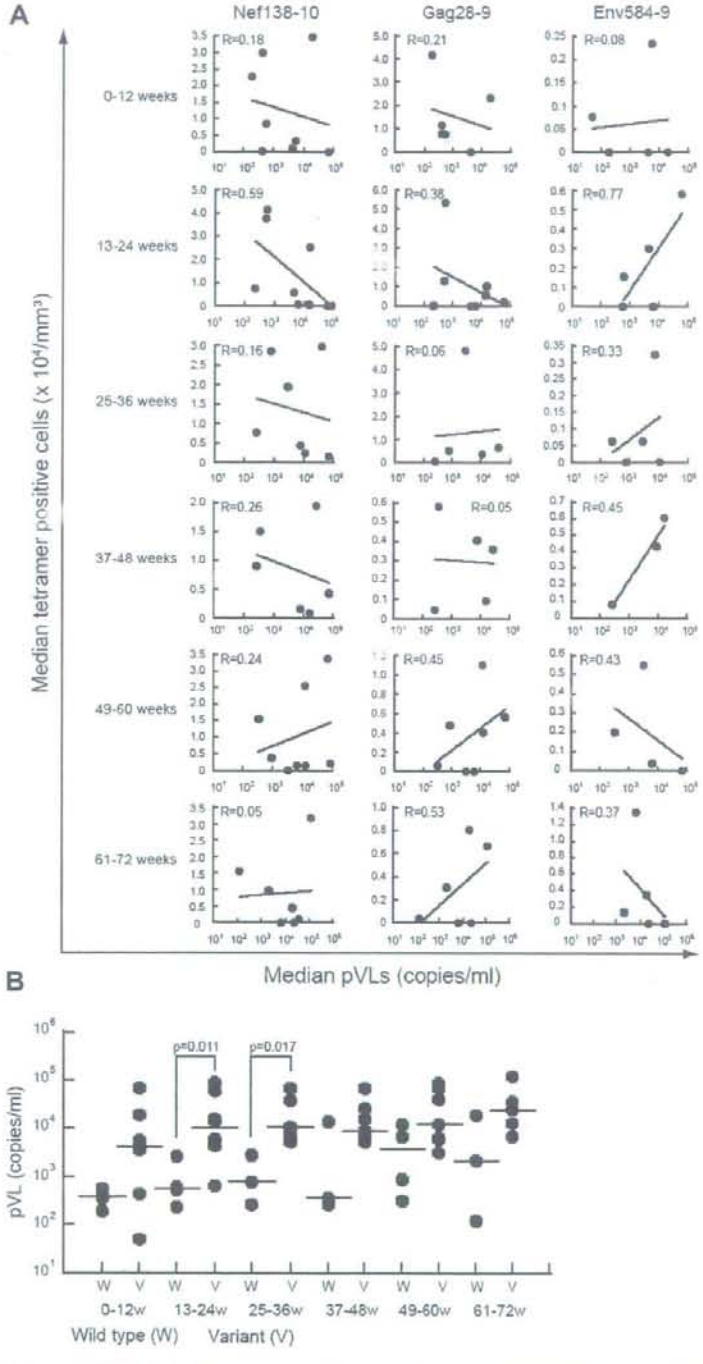

Fig. 3. (A) Plasma viral loads and frequency of HLA-A*2402-restricted HIV-1-specific CTLs. The correlation between the pVL values of every 12 weeks after treatment cessation and frequency of HLA-A*2402-restricted CTLs was assessed by simple regression analysis according to the epitope in 12 HLA*A2402-positive patients. None of Nef138-10-, Gag28-9- or Env584-9-specific CTLs was statistically correlated to pVLs at any time point. R: correlation coefficient. (B) Plasma viral loads and initial type of virus. pVL was compared according to the existence of escape variant in the earliest sample drawn during early phase of infection. Wild type group (W) includes four patients: KI-092, KI-102, KI-158 and KI-161. Variant type group (V) includes eight patients: KI-091, KI-099, KI-126, KI-134, KI-144, KI-150, KI-154 and KI-163, having Nef138-2F or Nef138-5C, which were previously reported as escape variants, in viral RNA or proviral DNA in the earliest samples. The pVLs between 12 and 36 weeks were significantly higher in Variant type group than in Wild-type group. Horizontal lines: median values.

Table 1
Nef138-10 sequence and Nef138-specific CTLs in HLA-A*2402 positive patients

Patient ID	Time (weeks)[a]	Sample	Nef138-10 sequence (RYPLTFGWCF)	Tetramer positive cell (% in CD8+ cells)	
				Wild type	2F
KI-091	−55	Proviral DNA	-F————	NA	NA
	21	RNA	-F————	0	0.46
	89	RNA	-F————	0	0.83
KI-092	39	RNA	————	1.48	0.05
	86	RNA	-F————	0.41	0.13
KI-099	−44	Proviral DNA	——C———	NA	NA
	−4	RNA	-F–C————	0.04	0.06
	44	RNA	-F————	0.02	0.12
KI-102	58	RNA	————	2.11	0.45
	137	RNA	-F————	0.45	0.10
KI-126	−68	Proviral DNA	-F————	NA	NA
	19	RNA	-F————	0.01	0.06
	101	NA	NA	0	0.11
KI-134	9	Proviral DNA	-F————	NA	NA
	49	RNA	-F————	0	0.22
KI-144	−46	Proviral DNA	-F————	NA	NA
	37	RNA	-F————	0.02	2.45
	71	RNA	-F————	NA	NA
KI-150	−43	RNA	-F————	NA	NA
	21	RNA	-F————	0	0.03
	63	NA	NA	0	0.02
KI-154	−70	Proviral DNA	-F————	0.06	0.13
	77	RNA	-F————	0.01	0.35
KI-158	14	Proviral DNA	————	2.91	0.41
KI-161	−26	Proviral DNA	—I————	NA	NA
		RNA	-F-I————		
	24	Proviral DNA	-F-I————	3.94	0.05
		RNA	-F-I————		
	86	Proviral DNA	-F-I————	0.29	0.79
		RNA	-F-I————		
	52	RNA	-F————	0.71	0.66
KI-163	−81	Proviral DNA	-F————	NA	NA
		RNA	-F————		
	26	RNA	-F————	0.09	0.57
	73	NA	NA	0.02	0.59

[a] Time: Time in weeks after treatment cessation. Negative time numbers: before treatment cessation. NA, not available.

responses are different between HLA class I alleles and influenced by viral mutations in epitope regions as described elsewhere [16–22]. HLA-A*2402 is the most frequent HLA class I allele with 70% prevalence in the Japanese population [21,22]. Therefore, the majority of the study participants could be assessed by using HLA-A*2402-epitope tetramer and thus it is most beneficial to evaluate HLA-A*2402 restricted CTL responses for Japanese patients. Moreover, HLA-A*2402-restricted epitopes have been studied extensively [22] and we were able to focus on three immunodominant epitopes. This approach allowed us to find a high frequency of the escape variant Nef138-2F efficiently.

Viral mutation is one of the important mechanisms of immune escape of HIV-1 [16–23,27–29], which occurs at amino acids responsible for HLA binding, T-cell receptor recognition, or in flanking regions that affect antigen presentation. In our study Nef138-2F, which is a mutation in the immunodominant CTL epitope Nef138-10, had emerged in 5 of 12 HLA-A*2402-positive patients. Although the magnitude of Nef138-10-specific CTLs was not significantly correlated with pVLs as previous trials [15], Nef138-2F variant infection was correlated with high pVL levels in early clinical course and seemed to contribute to lower CTL response. Furthermore, we previously demonstrated the strong and weak ability of Nef138-10-specific CTL clones to suppress replication of the wild-type and 2F mutant viruses respectively [23]. In addition, although Nef138-2F-specific CTL clones suppressed the replication of both wild-type and Nef138-2F variant, their ability to suppress the replication of Nef138-2F virus was much weaker than that of Nef138-10-specific CTLs or Nef138-2F-specific CTLs against the wild-type virus replication. Furthermore, the present study demonstrated that 2F mutant appeared at the late phase in patients who had wild-type virus at the early phase. Together with these findings, frequent detection of Nef138-2F in this study strongly supports the idea that Nef138-2F is one of the escape mutations from HLA-A*2402-restricted CTLs and that Nef138-2F virus was selected by CTL pressure.

Nef138-2F mutation could occur not only by positive selection by CTLs but also by Nef138-2F-variant transmission [19–21]. Furutsuki et al. [21] reported frequent detection of

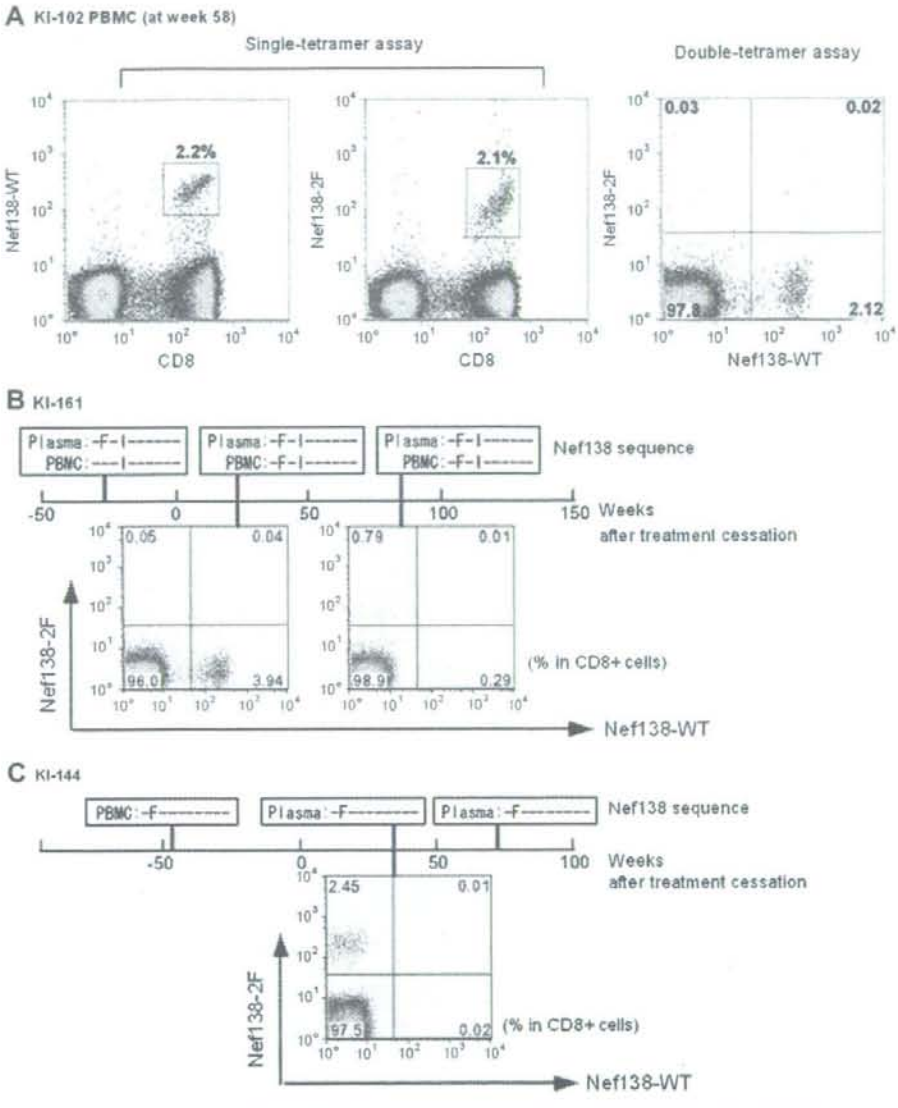

Fig. 4. Nef138-2F variant and CTL specificity. (A) PBMC of KI-102 at week 58, known to coincide with Nef138-10 wild-type infection, were assayed for wild-type Nef138-10-specific CTL (Nef138-WT-CTL) by tetramer-staining with Nef138-WT-tetramer and Nef138-2F-tetramer. The left two charts depict the results of single-tetramer-staining, showing the two tetramers stained for Nef138-WT-CTL equally (2.2% by Nef138-WT-tetramer versus 2.1% by Nef138-2F-tetramer). The right chart depicts the result of double-tetramer-staining with Nef138-WT-tetramer and Nef138-2F-tetramer, showing Nef138-WT-CTL was stained by Nef138-WT-tetramer and was differentiated from Nef138-2F-CTL. (B) Serial changes in Nef138-10 sequence and Nef138-specific-CTLs of KI-161 infected by non-Nef138-2F strain. Top: the Nef138-10 sequence; bottom charts: results of double-staining assay with Nef138-WT-tetramer and Nef138-2F-tetramer. Numbers in each quadrant represent the frequency of tetramer-positive cells among total CD8+ cells. Right lower quadrant: frequency of Nef138-WT-tetramer-positive cells; left upper quadrant: frequency of Nef138-2F-tetramer-positive cells. Note the induction of Nef138-WT-CTL and reduction in their proportion after emergence of Nef138-2F mutation. Nef138-2F-CTLs were induced after emergence of Nef138-2F mutation but their proportion was relatively lower. (C) Serial changes in Nef138-10 sequence and Nef138-specific-CTLs of KI-144 infected by Nef138-2F variant. Note the induction of Nef138-2F-CTL. Nef138-WT-CTLs were never detected throughout the study.

Nef138-2F variant in HLA-A*2402 negative Japanese patients who were infected by sexual intercourse and reversion from Nef138-2F to wild type occurred very slowly over years. These might allow horizontal spread of Nef138-2F variant. Even if the transmission of this variant in Japanese patients is very frequent, our study included the five patients who did not have this variant initially and were considered as wild-type infection, and we provided longitudinal evidence of positive selection of Nef138-2F variant under the pressure of Nef138-WT-CTLs in those.

In conclusion, our study demonstrated that early antiretroviral treatment with five series of STI did not induce a sustained immune response. A high frequency of escape mutation in the immunodominant HLA-A*2402-restricted CTLs was found, which could be one of the causes of limited immune responses by STIs.

Acknowledgments

We thank Manami Satoh and Hirokazu Koizumi (Division of Viral Immunology, Center for AIDS research, Kumamoto University), for assistance in epitope-HLA tetramer assay. No conflicts of interest declared by all authors. This work was supported by grants for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (AIDS-H15-001).

References

[1] J. Lisziewicz, E.S. Rosenberg, J. Lieberman, J. Heiko, L. Lopalco, R. Siliciano, B. Walker, F. Lori, Control of HIV despite the discontinuation of antiretroviral therapy, N. Engl. J. Med. 340 (1999) 1683–1684.

[2] E.S. Rosenberg, M. Altfeld, S.H. Poon, M.N. Phillips, B.M. Wilkies, R.L. Eldridge, G.K. Robbins, R.T. D'Aquila, P.J.R. Goulder, B.D. Walker, Immune control of HIV-1 after early treatment of acute infection, Nature 407 (2000) 523–526.

[3] F. Lori, M.G. Lewis, J. Xu, G. Varga, D.E. Zinn Jr., C. Crabbs, W. Wagner, J. Greenhouse, P. Silvera, J. Yalley-Ogunro, C. Tinelli, J. Lisziewicz, Control of SIV rebound through structured treatment interruptions during early infection, Science 290 (2000) 1591–1593.

[4] J. Lawrence, D.L. Mayers, K.H. Hullsiek, G. Collins, D.I. Abrams, R.B. Reisler, L.R. Crane, B.S. Schmetter, T.J. Dionne, J.M. Saldanha, M.C. Jones, J.D. Baxter, Structured treatment interruption in patients with multidrug-resistant human immunodeficiency virus, N. Engl. J. Med. 349 (2003) 837–846.

[5] A. Lafeuillade, C. Poggi, G. Hittinger, E. Counillon, D. Emilie, Predictors of plasma human immunodeficiency virus type 1 RNA control after discontinuation of highly active antiretroviral therapy initiated at acute infection combined with structured treatment interruptions and immune-based therapies, J. Infect. Dis. 188 (2003) 1426–1432.

[6] J. Plana, F. Garcia, A. Oxenius, G.M. Ortiz, A. Lopez, A. Cruceta, G. Mestre, E. Fumero, C. Fagard, M.A. Sambeat, F. Segura, J.M. Miro, M. Arnedo, L. Lopalcos, T. Pumarola, B. Hirschel, R.E. Phillips, D.F. Nixon, T. Gallant, J.M. Gatell, Relevance of HIV-1-specific CD4+helper T-cell responses during structured treatment interruptions in patients with CD4+T-cell nadir above 400/mm^3, J. Acquir. Immune. Defic. Syndr. 36 (2004) 791–799.

[7] B. Hoen, I. Fournier, C. Lacabaratz, M. Burgard, I. Charreau, M.L. Chaix, J.M. Molina, J.M. Livrozet, A. Venet, F. Raffi, J.P. Aboulker, C. Rouzioux, Structured treatment interruptions in primary HIV-1 infection. The ANRS 100 PRIMSTOP Trial, J. Acquir. Immune. Defic. Syndr. 40 (2005) 307–316.

[8] P. Borrow, H. Lelicki, B.H. Hahn, G.M. Shaw, M.B.A. Oldstone, Virus-specific CD8$^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol. 68 (1994) 6103–6110.

[9] G.S. Ogg, X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D.D. Ho, D.F. Nixon, A.J. McMichael, Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA, Science 279 (1998) 2103–2106.

[10] C. Hess, M. Altfeld, S.Y. Thomas, M.M. Addo, E.S. Rosenberg, T.M. Allen, R. Draenert, R.L. Eldrige, J.V. Luzen, H.J. Stellbrink, B.D. Walker, A.D. Luster, HIV-1 specific CD8+T cells with an effector phenotype and control of viral replication, Lancet 363 (2004) 863–866.

[11] E.S. Rosenberg, J.M. Billingsley, A.M. Caliendo, S.L. Boswell, P.E. Sax, S.A. Kalams, B.D. Walker, Vigorous HIV-1-specific CD4+T cell responses associated with control of viremia, Science 278 (1997) 1447–1450.

[12] D.E. Smith, B.D. Walker, D.A. Cooper, E.S. Rosenberg, J.M. Kaldor, Is antiretroviral treatment of primary HIV infection clinically justified on the basis of current evidence? AIDS 18 (2004) 709–718.

[13] F.M. Hecht, L. Wang, A. Collier, S. Little, M. Markowitz, J. Margolick, J.M. Kilby, E. Daar, B. Conway, S. Holtefor the AIEDRP Network, A multicenter observational study of the potential benefits of initiating combination antiretroviral therapy during acute HIV infection, J. Infect. Dis. 194 (2006) 725–733.

[14] C.L. Tremblay, J.L. Hicks, L. Sutton, F. Giguel, T. Flynn, M. Johnston, P.E. Sax, B.D. Walker, M.S. Hirsh, E.S. Rosenberg, R.T. D'Aquila, Antiretroviral resistance associated with supervised treatment interruptions in treated acute HIV infection, AIDS 17 (2003) 1086–1089.

[15] D.E. Kaufmann, M. Lichterfeld, M. Altfeld, M.M. Addo, M.N. Johnston, P.K. Lee, B.S. Wagner, E.T. Kalife, D. Strick, E.S. Rosenberg, B.D. Walker, Limited durability of viral control following treated acute HIV infection, PLoS Med. 1 (2004) e36.

[16] T.M. Allen, D.H. O'Connor, P. Jing, J.L. Dzuris, B.R. Mothe, T.U. Vogel, E. Dunphy, M.E. Lieble, C. Emerson, N. Wilson, K.J. Kunstman, X. Wang, D.B. Allison, A.L. Hughes, R.C. Desrosiers, J.D. Altman, S.M. Wolinsky, A. Sette, D.I. Watkins, Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia, Nature 407 (2000) 386–390.

[17] D.T. Evans, D.H. O'Connor, P. Jing, J.L. Dzuris, J. Sydney, J. da Silva, T.M. Allen, H. Horton, J.E. Venham, R.A. Rudersdorf, T. Vogel, C.D. Pauze, R.E. Bontrop, R. DeMars, A. Sette, A.L. Hughes, D.I. Watkins, Virus-specific cytotoxic T lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef, Nat. Med. 5 (1999) 1270–1276.

[18] D.H. Barouch, J. Kunstman, M.J. Kuroda, J.E. Schmits, S. Santra, F.W. Peyerl, G.R. Gorgone, D.C. Montefiori, M.G. Lewis, S.M. Wolinsky, N.L. Letvin, Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys, J. Virol. 77 (2003) 7367–7375.

[19] C.B. Moore, M. John, I.R. James, F.T. Christriansen, C.S. Witt, S.A. Mallal, Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level, Science 296 (2002) 1439–1443.

[20] P.J. Goulder, C. Brander, Y. Tang, C.A. Tremblay, R.A. Colbert, M.M. Addo, E.S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S.I. Pelton, S.K. Burchett, K. McIntosh, B.T. Korber, B.D. Walker, Evolution and transmission of stable CTL escape mutations in HIV infection, Nature 412 (2001) 334–338.

[21] T. Furutsuki, N. Hosoya, A. Kawana-Tachikawa, M. Tomizawa, T. Odawara, M. Goto, Y. Kitamura, T. Nakamura, A.D. Kelleher, D.A. Cooper, A. Iwamoto, Frequent transmission of cytotoxic-T-lymphocyte escape mutants of human immunodeficiency virus type 1 in the highly HLA-A-24-positive Japanese population, J. Virol. 78 (2004) 8437–8445.

[22] Y. Ikeda-Moore, H. Tomiyama, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: Strong epitopes are derived from V regions of HIV-1, J. Immunol. 159 (1997) 6242–6252.

[23] M. Fujiwara, J. Tanuma, H. Koizumi, Y. Kawashima, K. Honda, S. Matsuoka-Aizawa, S. Dohki, S. Oka, M. Takiguchi, Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts, J. Virol. 82 (2008) 138–147.

[24] V.A. Johnson, F. Brun-Vezinet, B. Clotet, D.R. Kuritzkes, D. Pillay, J.M. Schapiro, D.D. Richman, Update of the drug resistance mutations in HIV-1: Fall 2006, Top. HIV Med. 14 (2006) 125–130.

[25] J.D. Altman, P.A.H. Moss, P.J.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, M.M. Davis,

Phenotypic analysis of antigen-specific T lymphocytes, Science 274 (1996) 94–96.

[26] M. Carrington, S.J. O'Brien, The influence of HLA genotype on AIDS, Annu. Rev. Med. 54 (2003) 535–551.

[27] H. Tomiyama, H. Akari, A. Adachi, M. Takiguchi, 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 (2002) 7535–7543.

[28] H. Tomiyama, M. Fujiwara, S. Oka, M. Takiguchi, 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 (2005) 36–40.

[29] Y. Yokomaku, H. Miura, H. Tomiyama, A. Kawana-Tachikawa, M. Takiguchi, A. Kojima, Y. Nagai, A. Iwamoto, Z. Matsuda, K. Ariyoshi, Impaired processing and presentation of cytotoxic-T-lymphocyte (CTL) epitopes are major escape mechanisms from CTL immune pressure in human immunodeficiency virus type 1 infection, J. Virol. 78 (2004) 1324–1332.

Eur. J. Immunol. 2009. 39: 301–309 DOI 10.1002/eji.200838667

European Journal of Immunology

High frequency and proliferation of $CD4^+FOXP3^+$ Treg in HIV-1-infected patients with low CD4 counts

Xiuqiong Bi[1], Yasuhiro Suzuki[2], Hiroyuki Gatanaga[1] and Shinichi Oka[1]

[1] AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan
[2] Department of Infectious and Respiratory Diseases, Tohoku University, Miyagi, Japan

The frequency of Treg is reported to be higher in patients with chronic HIV type 1 (HIV-1) infection and $CD45RA^+$ Treg exist in normal adults. In this study, we found a lower absolute number (15 cells/μL) but a higher proportion (16.2%) of $FOXP3^+$ cells (Treg) in the $CD4^+$ population in treatment-naïve HIV-1 patients with low CD4 (<200 cells/μL) counts than in those with high CD4 counts (34 cells/μL and 9.3%) or healthy adults (48 cells/μL and 7.5%). In HIV-1 patients, $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets were identified in the Treg population, and the proportion of $CD45RA^-CCR7^-$ Treg was higher (57.9%) in patients with low CD4 than high CD4 counts (38.3%). Treg were in a high proliferation state especially in patients with low CD4 counts. HIV viral load correlated positively with the Treg proliferation rate and the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, the proliferation of Treg correlated positively with the $CD45RA^-CCR7^-$ Treg proportion but negatively with Treg numbers. Successful antiretroviral therapy resulted in a limited increase in Treg numbers, but their frequency was reduced in 1–2 months due to a rapid rebound of $FOXP3^-$ $CD4^+$ cells. Our results suggest that HIV-activating Treg may be a reason for the high frequencies of Treg and $CD45RA^-CCR7^-$ Treg in the peripheral blood of late-stage HIV-1-infected patients.

Key words: Cell proliferation · HIV · Immune regulation · Treg

Supporting Information available online

Introduction

HIV type 1 (HIV-1) infection is characterized by a progressive loss and dysfunction of $CD4^+$ T cells [1, 2]. With regard to reduced T-cell functions, accumulating evidence suggests that the balance between the immune suppression function of natural Treg cells and the effector functions of other types of lymphoid cells influences the magnitude of immune reactions in various types of infections, *e.g.* those caused by *Leishmania major*, *Shistosoma mansonia*, and hepatitis C virus [3–7]. FOXP3 is not only a specific marker but also a critical lineage specification factor for Treg [8–11]. Treg are considered mainly as $CD45RA^-$ cells. However, recent studies have shown that $CD45RA^+$ cells also exist among immune-suppressing $CD25^+CTLA4^+CD4^+$ T cells in adults [12, 13].

The local interaction between Treg and other T cells plays an important role in immune suppression and the local density of Treg determines the course of immune responses to infections [4, 7, 14]. Thus, Treg can be both detrimental and beneficial to the host in response to pathogens [5, 7]. For example, in HIV-infected patients, $CD4^+CD25^+$ Treg have been reported to be proportionally increased, decreased, or highly increased in tonsils, their numbers to correlate with HIV viral load, and to exhibit suppression activity [15–23]. Furthermore, antiretroviral

Correspondence: Dr. Shinichi Oka
e-mail: oka@imcj.hosp.go.jp

therapy (ART) has been reported to have either a negative or no influence on Treg or expression of FOXP3 [18, 23]. In HIV-1-infected individuals, immunodeficiency is often considered when the CD4 cell count falls below 200 cells/μL [1]. However, to our knowledge, there is controversy or little information about the absolute number, frequency, and status of homing markers of Treg in HIV-1-infected patients especially in those with low CD4 counts and late-stage AIDS-related diseases or not on ART [24, 25]. Little is known about the dynamic changes of Treg after ART has been introduced.

It is considered that the CCR7 molecule on T cells is an essential trafficking factor for T cells homing to lymphoid tissues as well as an important marker for defining differentiation stage of T cells with CD45RA molecule [26–28].

The present study was designed to investigate Treg in late-stage HIV-1-infected patients with CD4 count <200 cells/μL and the early impact of ART on Treg. We used the chemokine receptor CCR7 and CD45RA molecules to characterize distinct population of migratory Treg.

Results

High-frequency but low absolute numbers of Treg in HIV-1 patients with low CD4 counts

In this study, we enrolled 95 HIV-1-infected patients and 21 HIV-1-negative Japanese adults as our subjects. Because most AIDS-related diseases occur in HIV-1 patients when their CD4 count decreases to below 200 cells/μL, we classified the patients into two groups, a low CD4 group with a $CD4^+$ T cell count less than 200 cells/μL and a high CD4 group with a $CD4^+$ T cell count not less than 200 cells/μL, for some comparison analysis. Table 1 lists the demographic and clinical characteristics of HIV-1-infected patients and healthy HIV-1-negative controls.

Although FOXP3 expression is considered as the best and most specific marker of Treg, some studies have reported that CD127 and CD25 could distinguish Treg [29, 30]. Accordingly, we first compared the staining of FOXP3 with CD25 and CD127 using PBMC from HIV-1-positive individuals. As shown in Supporting Information Fig. 1A and B, $CD25^+CD127^-$ were a proportion of the CD4 cells. However, gating these cells as Treg seems difficult because of the smear staining of both CD25 and CD127. However, gating FOXP3 in CD4 cells was much easier because of the clear staining of FOXP3. Furthermore, we tested the correlation of the Treg by the two classification markers. Supporting Information Fig. 1C shows a good correlation between the proportion of $FOXP3^+$ and $CD25^+$ $CD127^-$ in CD4 cells in 18 HIV-1 patients. Therefore, in the present study, we considered the $FOXP3^+CD4^+$ cells as Treg, and called $FOXP3^-CD4^+$ cells as conventional $CD4^+$ T cells (Tcon).

In the next step, we investigated the frequency and absolute number of Treg in HIV-1-infected individuals without an ART history and compared them with those of healthy Japanese adults. Figure 1A and B shows FOPX3 expression in $CD4^+$ cells. As shown in Table 2, the proportion of Treg in CD4 cells was $16.2 \pm 2.6\%$ in HIV-1 patients with a low CD4 count and

Table 1. Demographic and clinical characteristics of subjects

	Group[a]		
	A (low CD4)	B (high CD4)	H (healthy)
Characteristics			
Numbers	27	68	21
Age (years, range)	39 (21–65)	38 (21–67)	38 (21–60)
Gender (male:female)	27:0	16:1	3:4
CD4 count (cells/μL, SD)	102 (58)	383 (164)	650 (178)
LogVL (SD)	5 (0.6)	4.2 (0.7)	N/A
AIDS-related diseases[b] (n, %)	23 (85)	11 (16)	N/A
Months of HIV^+ (range)[c]	12.3 (0–97)	21 (0–124)	N/A
Numbers for tests			
Frequency and subsets of Treg[d]	20	39	21
Ki67 staining *versus* FOXP3[e]	11	24	5
CCR7FOXP3 *versus* CD25[f]	3	16	
CD127CD25 *versus* FOXP3[g]	6	12	

[a] Low CD4: <200 cells/μL; high CD4: ≥ 200 cells/μL.
[b] AIDS-related diseases included: candida, herpes simplex virus infection, tuberculosis, pneumocystis jirovici pneumonia, lymphoma (kaposis sarcoma), etc.
[c] Months between the date of the first time of consulting the hospital and the date of blood collected.
[d] Table 2 and Fig. 1.
[e] Figure 2 and Supporting Information Fig. 2.
[f] Figure 1C.
[g] Supporting Information Fig. 1.

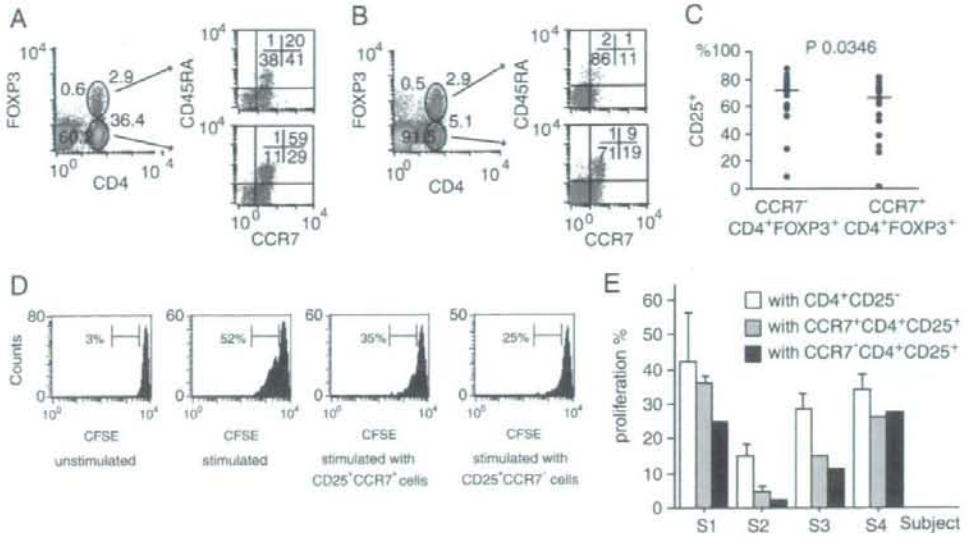

Figure 1. Subsets of Treg in healthy adults and HIV-1-infected patients. (A) Staining of a healthy adult. (B) Staining of an HIV-1-infected patient with low CD4 count. FOXP3 was mainly found in $CD4^+$ T cells both in healthy adults and HIV-1 patients. Treg ($FOXP3^+CD4^+$) cells could be subdivided into $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ subsets, similar to Tcon ($FOXP3^-CD4^+$, conventional CD4 cells). (C) In HIV-1 patients, the proportion of $CD25^+$ among $CCR7^-$ Treg was higher than that among $CCR7^+$ Treg ($p<0.05$, $n = 19$). (D) A representative proliferation of $CD4^+CD25^-$ responder cells cultured with $CCR7^-CD25^+CD4^+$, $CCR7^+CD25^+CD4^+$ cells, or unlabeled $CD25^-CD4^+$ cells stimulated by anti-CD3 mAb with autologous APC (the data are derived from healthy control). (E) $CCR7^+$ and $CCR7^-$ Treg suppression of responder cells in four subjects. S1–S3: healthy subjects, S4: HIV-1-positive patient (the error bars show duplicate or triplicate tests). Horizontal bars represent median values and p value represents comparison result from Wilcoxon-signed rank test.

$9.3 \pm 0.5\%$ in patients with a high CD4 count. The absolute counts of Treg in low CD4 and high CD4 groups were 15 ± 3 and 34 ± 2 cells/μL, respectively. In healthy adults, the mean CD4 count was 650 cells/μL, and the frequency of Treg among $CD4^+$ cells was $7.5 \pm 0.5\%$ with a mean absolute number of 48 ± 4 cells/μL. Therefore, HIV-1 patients with low CD4 counts had a lower absolute count but a significantly higher frequency of Treg than HIV patients with high CD4 and healthy controls.

High proportion of $CD45RA^-CCR7^-$ Treg in HIV-1 patients with low CD4

Considering the distinct homing potentials and effector functions, CD4 T cells could be subdivided into three subsets, namely naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), and effector memory ($CD45RA^-CCR7^-$) cells, based on their surface marker and cytokine secretion [26]. Given that local interaction of Treg and Tcon plays an important role in immune suppression and the local number and/or density of Treg reflects immune suppression, we next investigated whether Treg have the same characteristic phenotype as Tcon. Figure 1A shows that Treg could be divided into three subsets, similar to Tcon, based on CD45RA and CCR7 staining in healthy controls. Interestingly, the proportion of each subset of Treg was different compared with the respective subsets of Tcon (Table 2). In healthy adults, the proportion of $CD45RA^-CCR7^-$ Treg ($39.7 \pm 2\%$) was higher than $CD45RA^-CCR7^-$ Tcon cells ($15.6 \pm 1.2\%$), but the proportion of $CD45RA^+CCR7^+$ Treg ($19.3 \pm 1.6\%$) was lower than $CD45RA^+CCR7^+$ Tcon cells ($45.8 \pm 2.4\%$).

In HIV-1-infected patients, the staining patterns of intracellular FOXP3 and surface CD4, CD45RA, and CCR7 were similar to those in healthy controls (Fig. 1A and B). Figure 1B shows a high proportion of $CD45RA^-CCR7^-$ Treg in a representative patient with a low CD4 count. As shown in Table 2, the proportion of $CD45RA^-CCR7^-$ Treg in the low CD4 group ($57.9 \pm 4.2\%$) was significantly higher than in the high CD4 ($38.3 \pm 1.8\%$) or control groups ($39.7 \pm 2\%$). In contrast, the proportion of $CD45RA^-CCR7^+$ Treg in patients with low CD4 counts was significantly lower than in those with high CD4 counts and the control groups. In all subject groups, the proportions of $CD45RA^-$ cells in Treg were higher than in Tcon. Moreover, we found that in HIV-1-infected patients, the proportion of $CD25^+$ in $CCR7^-$ Treg ($64 \pm 19\%$) was higher than in $CCR7^+$ Treg ($58.8 \pm 21\%$, Fig. 1C).

$CD45RA^+$ Treg have been reported to show suppressive function [12]. Based on the finding of a high proportion of $CCR7^-$ Treg in patients with a low CD4 count (Table 2), and considering that $CCR7^+$ cells tend to home to lymphoid tissues whereas $CCR7^-$ cells tend to move to peripheral tissues, we next investigated whether there is any difference in the suppressive activity between $CCR7^+$ and $CCR7^-$ Treg. The results showed

Table 2. Comparison of Treg and Tcon in healthy persons and HIV-1-infected patients[a)]

	Healthy (H)	HIV-1(+)ART(−)		*p* value		
		CD4<200 (A)	CD4≥200 (B)	A *versus* B	A *versus* H	B *versus* H
Number of subjects	21	20	39			
Lymphocytes (cells/μL)	1718 (381)	1028 (447)	1661 (579)	<0.0001	<0.0001	NS
CD4 (cells/μL)	650 (178)	108 (58)	395 (195)	<0.0001	<0.0001	<0.0001
CD4 (%)	38.4 (8.6)	11.4 (7.6)	20.5 (8.5)	0.0001	<0.0001	<0.0001
Treg (cells/μL)	48 (16)	15 (11)	34 (14)	<0.0001	<0.0001	0.0008
Treg (%)	7.5 (2.4)	16.2 (11.8)	9.3 (3.4)	0.0137	0.0004	0.0464
Treg (%)						
$CCR7^+$	57	40.1	59.6	0.0001	0.0029	NS
$CD45RA^+CCR7^+$	19.3	13.4	21.1	0.0109	0.0504	NS
$CD45RA^-CCR7^-$	39.7	57.9	38.3	0.0001	0.0006	NS
$CD45RA^-CCR7^+$	37.7	26.7	38.5	0.0005	0.0057	NS
$CD45RA^-$	77.4	84.6	76.8	0.0131	0.0419	NS
Tcon (%)						
$CCR7^+$	81.3	55.8	74.8	0.0178	0.0035	NS
$CD45RA^+CCR7^+$	45.8	31.9	41.1	NS	0.0217	NS
$CD45RA^-CCR7^-$	15.6	36.8	22.1	0.0283	0.0035	0.04
$CD45RA^-CCR7^+$	35.5	23.9	33.7	0.0048	0.0045	NS
$CD45RA^-$	51.1	60.7	55.8	NS	NS	NS
p Value (Treg *versus* Tcon)						
$CCR7^+$	<0.0001	0.0187	<0.0001			
$CD45RA^+CCR7^+$	<0.0001	0.0001	<0.0001			
$CD45RA^-CCR7^-$	<0.0001	0.0004	<0.0001			
$CD45RA^-CCR7^+$	NS	NS	0.005			
$CD45RA^-$	<0.0001	<0.0001	<0.0001			

[a)] Data are means (SD). NS: not significant. CD4<200, CD4≥200: 200 cells/μL. Mann–Whitney *U*-test was used for comparison between groups (A *versus* B, A *versus* H, B *versus* H). Wilcoxon-signed rank test was used for comparison in group (Treg *versus* Tcon).

that both $CCR7^+$ and $CCR7^-$ $CD25^+CD4^+$ cells suppressed the proliferation of responder cells (Fig. 1D). The suppressive activity was observed in three healthy controls and one HIV-1 patient (Fig. 1E), although no difference was found in the suppression function between the $CCR7^+$ and $CCR7^-$ Treg.

The above results demonstrated the existence of $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ Treg subsets, similar to Tcon. The proportion of $CCR7^+$ Treg was lower than $CCR7^+$ Tcon cells in both healthy controls and HIV-1 patients. However, the proportion of $CD45RA^-CCR7^-$ Treg was higher than $CD45RA^-CCR7^-$ Tcon, particularly in patients with low CD4 count.

High proliferation of Treg correlates with HIV-1 viral load

Immune cells are activated in HIV-infected patients and such activation is linked to CD4 cell depletion [31]. To determine the mechanism of the high frequency of Treg and $CD45RA^-CCR7^-$ Treg in advanced HIV patients, we stained CD4 cells for the proliferation markers Ki67 in 24 patients (including 11 patients with low CD4 counts and 13 patients with high CD4 counts) and five healthy controls. Figure 2A shows that there was no difference between gating the Ki67 in Treg and Tcon in a healthy control and an HIV-1-infected person. As shown in Fig. 2, the proportions of Ki67-stained cells among Treg in low CD4, high CD4, and control groups (41.7, 24.5, and 22.3%, respectively) were higher than those in Tcon cells (18.1, 11.8, and 7.4%, respectively) (Fig. 2B). The expression of Ki67 in both Treg and Tcon cells was higher in patients with low CD4 counts than in those with high CD4 counts and healthy controls. Furthermore, in the 24 HIV-1-infected patients assessed for Ki67, HIV-1 viral load showed a positive correlation with the frequency of Ki67 in Treg and the proportion of $CD45RA^-CCR7^-$ in Treg. However, the CD4 count showed a negative correlation with the frequency of Ki67 in Treg (Fig. 2C). Moreover, the frequency of Ki67 in Treg correlated negatively with the Treg count and the proportion of $CD45RA^+CCR7^+$ in Treg, but positively with the proportion of $CD45RA^-CCR7^-$ in Treg (Fig. 2D). The same correlation was also observed in Tcon cells (Supporting Information Fig. 2).

ART reduces the frequency of Treg

In HIV-1-infected patients, ART can effectively reduce the HIV viral load and improve CD4 counts. In highly active ART-treated patients, a depleted or normalized Treg was observed in

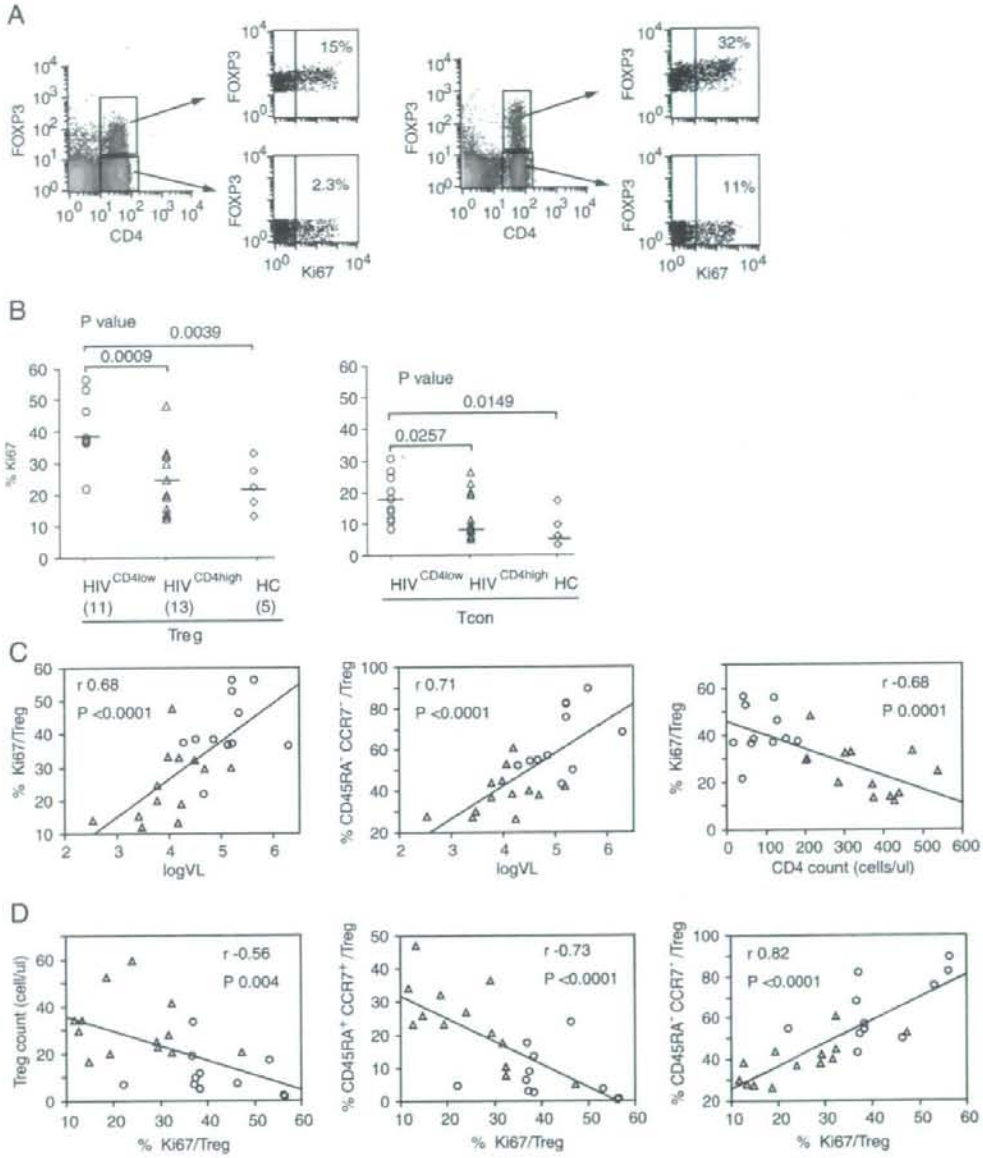

Figure 2. Ki67 staining and high proliferation rate of Treg is associated with viral load. (A) Gating of Ki67 in FOXP3$^+$ and FOXP3$^-$ CD4$^+$ cells in a healthy control (left panel) and an HIV-1-infected person (right panel). (B) Proportion of Ki67-positive Treg (left panel) is higher than that of Ki67-positive Tcon cells (right panel) in healthy controls (HC), HIV-1-infected patients with low CD4 count (HIV^{CD4low}) and HIV-1-infected patients with high CD4 count (HIV^{CD4high}) (numbers in parentheses represent the number of subjects tested). The percentages of Ki67-positive Treg and Tcon cells in the low CD4 group are higher than those in the high CD4 group and healthy control, respectively. (C) HIV-1 viral load shows a positive correlation with the percentage of Ki67 in Treg (left panel) and the proportion of CD45RA$^-$CCR7$^-$ Treg (middle panel). The CD4 count shows a negative correlation with the percentage of Ki67 in Treg (right panel). (D) The percentage of Ki67 in Treg shows correlation negatively with Treg count (left panel) and the proportion of CD45RA$^+$CCR7$^+$ Treg (middle panel) but positively with the proportion of CD45RA$^-$CCR7$^-$ Treg (right panel). Horizontal bars represent median values and p values represent results from Wilcoxon-signed rank test. Simple regression was used for correlation analysis.

PBMC and mucosal tissue [23, 32]. To investigate the impact of ART on Treg, we checked the dynamic change in Treg, their proliferation state, and subsets in nine patients until 9 months after commencement of ART (Fig. 3). The plasma viral load decreased sharply soon after commencement of ART (Fig. 3A). Associated with the decrease in viral load was a rise in the $CD4^+$ count especially in the first 2 months of ART. The CD4 count increased more than 100 cells/μL average in the first month (Fig. 3B). The absolute count of Treg increased in the first month but decreased to some extent thereafter (Fig. 3C); the frequency of Treg decreased rapidly to normal levels within 1–2 months of commencement of ART in all patients (Fig. 3D). On the other hand, the change in the proportion of Ki67 among Tcon and Treg showed a complex pattern. The proportion of Ki67 among Tcon cells increased in the first month of treatment and then decreased and fluctuated on a small scale thereafter (Fig. 3E). However, in the first 1–2 months of ART, the proportion of Ki67 among Treg decreased but maintained high levels until 9 months of ART (Fig. 3F). There was no significant change in each subset in both Treg and Tcon (Fig. 3G and H). However, the $CD45RA^-CCR7^-$ subset still accounted for a high proportion, especially in Treg (Fig. 3G and H, the right panels). The detailed change of each item in each patient is shown in Supporting Information Fig. 3. These results suggest that after initiation of ART, the slow change in the absolute number of Treg and the rapid rebound of Tcon counts resulted in a rapid normalization of the frequency of Treg in HIV-1 patients.

Discussion

Regulation of the immune response is important in maintaining self-tolerance. However, in individuals with immunodeficiency, such as patients with HIV infection, severe immune suppression may contribute to progression of AIDS. Previous studies reported activation of the immune system in HIV-1-infected patients and indicated that human $CD4^+CD25^{high}FOXP3^+$ Treg cells are derived through rapid turnover of memory populations *in vivo* [31, 33, 34].

In the present study, we found that untreated HIV-1-infected patients with low CD4 counts have a high frequency of Treg and $CD45RA^-CCR7^-$ Treg. Cell proliferation was higher in Treg than Tcon cells, especially in HIV-1 patients with low CD4 counts. In these patients, both Tcon and Treg showed a high proliferation state, particularly about 40% Treg were Ki67-positive. Ndhlovu *et al.* [22] reported that $FOXP3^+CD127^{lo}$ $CD4^+$ T cells in PBMC showed a strong negative correlation with T-cell activation during the early chronic stage of HIV infection. In our study, we also found a negative correlation between the proliferating frequency of Treg and Treg absolute count. However, we found that the proliferation of Treg correlated positively with the proportion of $CD45RA^-CCR7^-$ Treg. Furthermore, HIV viral load showed a positive correlation with both Treg proliferation and the proportion of $CD45RA^-CCR7^-$ Treg. These results suggest that HIV infection may activate Treg and result in an increased proportion of $CD45RA^-CCR7^-$ among Treg. On the other hand, Epple *et al.* [32] reported that the frequency and absolute counts of mucosal Treg were highly increased in untreated HIV patients. This finding may be considered another reason for our results because $CCR7^+$ lymphocytes tend to home to lymph nodes and lymphoid tissues. Therefore, we consider that in HIV-infected patients, HIV could simultaneously activate the differentiation of Treg as well as stimulate $CCR7^+$ Treg homing to lymph nodes and lymphoid tissues. These two effects of HIV on Treg result in the high frequency of Treg and a high proportion of $CD45RA^-CCR7^-$ Treg in peripheral blood in patients with low CD4 counts.

ART has been a great success in controlling HIV replication and aiding the recovery of CD4 T cells. However, data about its impact on Treg, especially in detail, are rare. In the current study, we observed that with the rapid decrease in viral load was a robust rebound of Tcon 1–2 months after ART initiation; however, the number of Treg increased in some patients but was almost unchanged in others. The unbalanced change in Tcon and Treg resulted in the frequency of Treg decreasing precipitously to normal levels in the first 1–2 months of therapy. Although the viral load decreased to a very low level in a short period after ART introduction, the proliferative state of Tcon and Treg did not decrease significantly. On the contrary, both Tcon and Treg maintained a high proliferation level, especially Treg. Moreover, the three subsets, *i.e.* $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, and $CD45RA^-CCR7^-$ in Tcon and Treg did not show a robust change till 9 months. The results suggest that the recovery of phenotypes needs a much longer period, even if they can recover after ART.

Chase *et al.* [23] observed Treg depletion in highly active ART-treated HIV-1 patients but not in elite suppressors. Here, we did not observe depletion of Treg counts after ART introduction, but we indeed noticed a rapid normalization of the Treg frequency. As we know, to do the suppression assay *in vitro*, an appropriate ratio of Treg to responder cells is needed for observing significant suppression. Considering the suppressive function of both $CCR7^+$ and $CCR7^-$ Treg, we think that the high frequency of Treg, but not the low absolute number of Treg, provides a much better suppressive marker in treatment-naïve HIV-1 patients with low CD4 counts. On the other hand, ART may induce some improvement of the immune suppression because it could reduce the frequency of Treg.

In summary, our results of high frequencies of Treg and $CD45RA^-CCR7^-$ Treg, which tend to migrate to non-lymphoid tissues, in untreated HIV-1 patients with low CD4 counts, emphasize the potential role of Treg in immune deficiency in late-stage HIV-1 infection. Furthermore, anti-HIV treatment could result in a rapid rebound of conventional T cells but not a robust improvement of Treg within 9 months after ART initiation. The different response of Treg and Tcon to ART leads to a rapid decrease in the frequency of Treg. Recently, immune reconstitution syndrome (IRS) is becoming an important problem in HIV treatment. Most IRS occurs in 1–3 months after commencement

Eur. J. Immunol. 2009. 39: 301–309

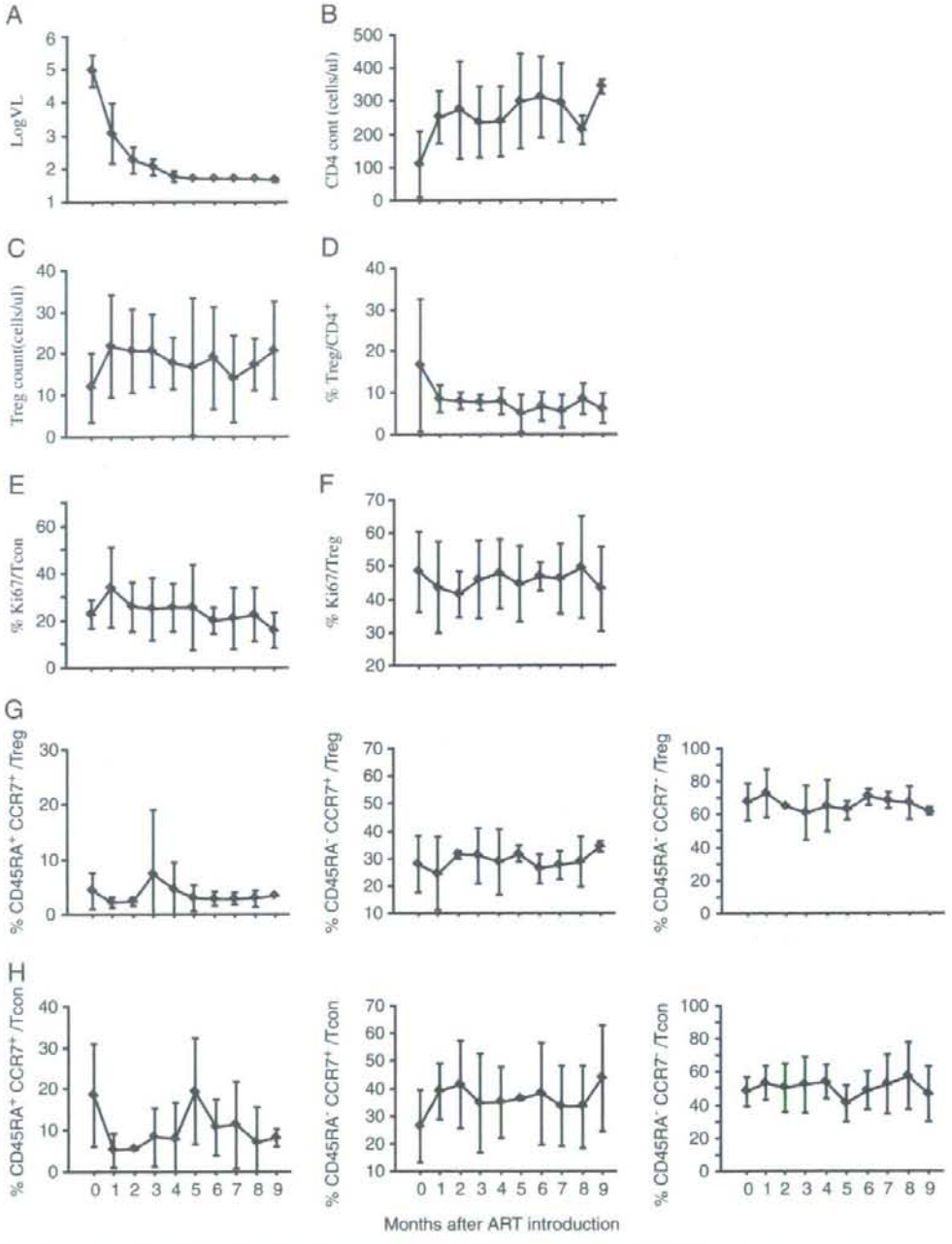

Figure 3. Serial changes in Treg and Tcon cells after commencement of ART. Commencement of ART resulted in rapid reduction in HIV viral load (A) and increase in $CD4^+$ cell count (B). Treatment caused increase in the absolute number of Treg in the first month, then fluctuated slightly thereafter (C), but resulted in a sharp decrease in their percentages in 1 month (D). The proportion of Ki67-positive Tcon increased in the first month but decreased in some extent thereafter (E), while the proportion of Ki67-positive Treg showed some change but still retained a high level at 9 months of commencement of ART (F). At 9 months after ART started, the recovery of the proportion of $CD45RA^+CCR7^+$ Treg (G, left panel) and Tcon (H, left panel) seems very slow, while the proportion of $CD45RA^-CCR7^+$ Treg (G, middle panel) and Tcon (H, middle panel) increased in some extent. However, the proportion of $CD45RA^-CCR7^-$ Treg (G, right panel) and $CD45RA^-CCR7^-$ Tcon (H, right panel) showed a small-scale change, but $CD45RA^-CCR7^-$ Treg maintained a high proportion till 9 months. (A–F) was from nine patients, while (G–H) was from six of them. Vertical bars represent mean ± 1SD.

of ART. Thus, we suppose that the unbalanced improvement of conventional CD4 cells and Treg after commencement of ART might be a factor for IRS. However, this issue needs more investigation.

Materials and methods

Subjects

The subjects were 95 HIV-1-infected patients who have not received any ART and gave written consent before enrollment in this study at the AIDS Clinical Center, International Medical Center of Japan, Tokyo. Nine patients who started ART were followed up for investigation of the impact of ART on Treg. Twenty-one HIV-1-negative adults were recruited as healthy controls. The demographic and clinical characteristics of the subjects are listed in Table 1. HIV-1 viral load was quantified by AMPLICOR HIV-1 MONITOR Test (Roche Diagnostics).

Cell preparation

PBMC were prepared from blood samples collected into EDTA-containing tubes by Ficoll-paque gradient centrifugation. Ki67 staining and evaluation of the ART-treated patients were carried out using cryopreserved PBMC.

For suppression assay, $CD4^+$ cells were isolated from freshly prepared PBMC by using $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to instructions provided by the manufacturer. $CD4^+$ cells were separated by anti-CD25 mAb (PE) and anti-PE Multisort Kit (Miltenyi) into $CD25^-$ and $CD25^+$ cells. After microbeads release, $CD25^+$ cells were sorted into $CCR7^+$ and $CCR7^-$ cells by using anti-CCR7 mAb (FITC, mouse IgG2a, R&D Systems, Minneapolis, MN) and Rat Anti-Mouse IgG2a+b Microbeads (Miltenyi). The $CD4^+$ $CD25^-$ cells were labeled by 2 μM 5-6-CFSE as responder cells in the suppression assay. Unlabeled $CD4^+CD25^-$ cells were used as non-Treg for cell number control. PBMC that were depleted of $CD3^+$ cells by CD3 MicroBeads (Miltenyi) and irradiated with 3000 rad were used as APC.

Cell staining and flow cytometry

Freshly isolated PBMC were surface stained and also stained intracellularly for FOXP3 (PE/APC labeled, clone PCH101, eBioscience, San Diego, CA) and other markers. The stained cells were analyzed on Becton Dickinson FACSCalibur with CellQuest software (BD Bioscience, San Jose, CA). The monoclonal antibodies used in these staining procedures included anti-CCR7-FITC, anti-CD4-perCP, anti-CD25-PE, anti-CD45RA-APC/perCP, anti-Ki67-PE (BD PharMingen, San Diego, CA), and anti-CD127-FITC (eBioscience).

In vitro suppression assay

In a 96-well, round-bottom plate coated with anti-CD3 mAb (0.25–0.5 μg/mL), 5×10^4 CFSE-labeled $CD4^+CD25^-$ cells were seeded and followed by adding autologous APC (2.5×10^4). For testing Treg suppression, the same number of $CD4^+CD25^+$ $CCR7^+$ or $CCR7^-$ cells was added as regulatory cells. In control wells, the same number of unlabeled non-Treg $CD4^+CD25^-$ cells was added in order to adjust cell numbers in each well. After 3–4 days culture in an incubator at 37°C under 5% CO_2, the cells were harvested and analyzed on FACSCalibur. Live cells were gated and the dilution of CFSE was measured as proliferation of responder cells.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups or stratified groups were examined for statistical significance using Mann–Whitney *U*-test and Wilcoxon-signed rank test. Simple linear regression was used for correlation analysis. All analyses were conducted using the StatView software (version 5.0). A p value of <0.05 was considered statistically significant.

Acknowledgements: We are grateful to Dr. Yoshimi Kikuchi, Tsunefusa Hayashida, Dr. Kiyoto Tsuchiya, and the entire staff of the AIDS Clinical Center, International Medical Center of Japan for their help and cooperation. We thank Dr. F.G. Issa, Word-Medex Pty Ltd., Sydney, Australia for English editing. This work was supported by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan, and by the Japanese Foundation of AIDS Prevention (X.Bi).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Douek, D. C., Picker, L. J. and Koup, R. A.,** T cell dynamics in HIV-1 infection. *Annu. Rev. Immunol.* 2003. **21**: 265–304.

2 **Gandhi, R. T. and Walker, B. D.,** Immunologic control of HIV-1. *Annu. Rev. Med.* 2002. **53**: 149–172.

3 **Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M.,** Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 1995. **155**: 1151–1164.

4 **Mills, K. H.,** Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* 2004. **4**: 841–855.

5 **Rouse, B. T., Sarangi, P. P. and Suvas, S.,** Regulatory T cells in virus infections. *Immunol. Rev.* 2006. **212**: 272–286.