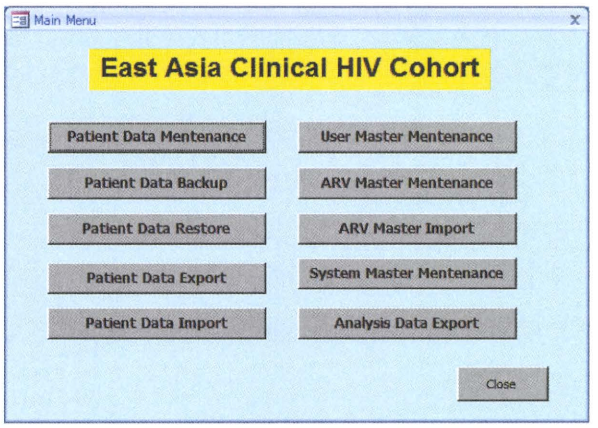
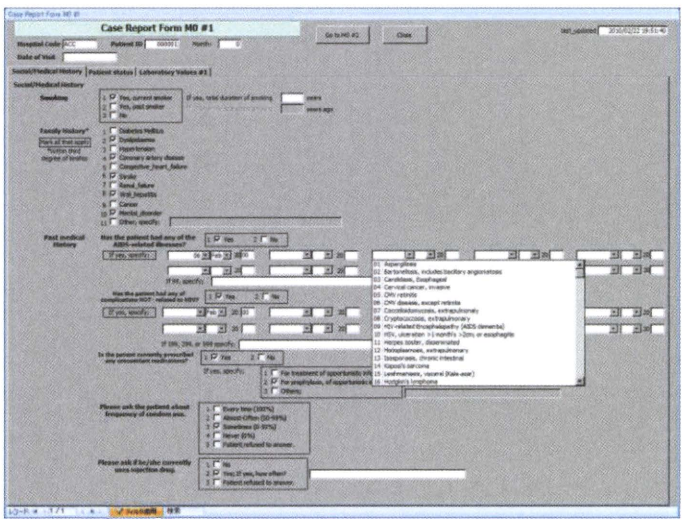
(a) 起動画面



(b) 入力画面 1



(c) 入力画面 2

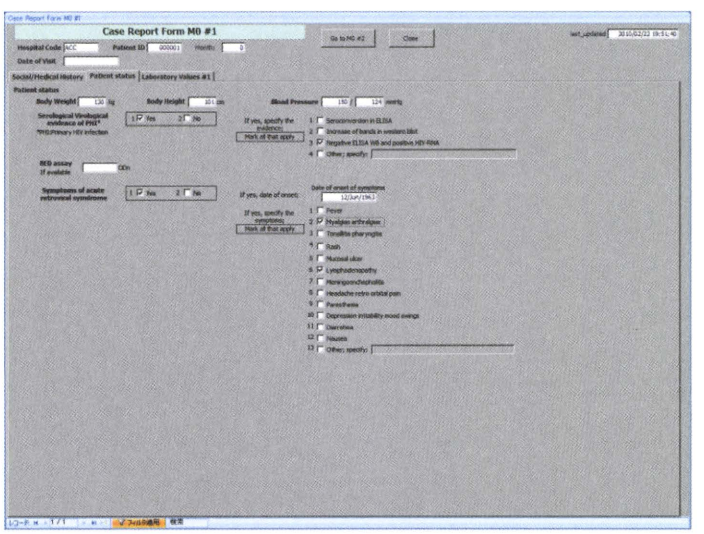


図 1 完成したデータベース

た。しかし、急性期には一時的な CD4 数減少がみられることが少なくないため、実際に治療適応を判断するには一定の観察期間を経なければならない。今回の結果をもって、HIV 感染症の進行が早まっているという結論を出すことはできない。引き続き観察を続けたい。

コホートの多施設化については、データベースのフォーマットが完成したことで、積極的な研究への参加を呼びかけやすくなった。来年度は、倫理性と透明性の高い運用規則を作成し、研究体制の基盤を固めたい。運用規則など研究体制が整ったところで、慢性感染者へ対象を拡大し、最終的に 2000 名以上の患者データ登録を最終目標とする。

E. 結論

多施設共同 HIV 感染者コホート研究において、予定した症例数の登録とデータベースの開発が完了した。今後は、早期 HIV 感染者の予後を解析するとともに、コホートの拡大と運営基盤の盤石化を図る予定である。

健康危機情報

該当なし

F. 研究発表

論文発表

1) Nakamura H, Teruya K, Takano M, Tsukada K, Tanuma J, Yazaki H, Honda H, Honda M, Gatanaga H, Kikuchi Y, Oka S. Clinical symptoms and courses of primary HIV-1 infection in recent years in Japan. Intern Med. 50 (2) :95-101, 2011

学会発表

国際学会

なし

国内学会

なし

G. 知的所有権の出願・取得状況（予定を含む）

なし

参考文献

1) Kawashima Y, Pfafferott K, Frater J et al. Adaptation of HIV-1 to human leukocyte antigen class I. Nature 2009; 458: 641-645.

2) Tanuma J, Fujiwara M, Teruya K, et al. HLA-A*2402-restricted HIV-1 specific T lymphocytes and escape mutation after ART with structured

treatment interruptions. Microbes Infect 2008; 10: 689-698.

3) Nakamura H, Teruya K, Takano M, et al. Clinical symptoms and courses of primary HIV-1 infection in recent years in Japan. Intern Med. 2011; 50: 95-10

4) Panel on Antiretroviral Guidelines for Adults and Adolescents of Department of Health and Human Services., 2010. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services., http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf.

研究協力者一覧

小池　隆夫　（北海道大学病院　第二内科）
遠藤　知之　（北海道大学病院　第二内科）
西尾　充史　（北海道大学病院　第二内科）
藤本　勝也　（北海道大学病院　第二内科）
今村　雅寛　（北海道大学病院　造血細胞治療センター（血液内科I））
田中　淳司　（北海道大学病院　造血細胞治療センター（血液内科I））
橋野　　聡　（北海道大学病院　造血細胞治療センター（第三内科））
近藤　　健　（北海道大学病院　造血細胞治療センター（第三内科））
菊池　　嘉　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
照屋　勝治　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
潟永　博之　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
本田美和子　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
木内　　英　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
矢崎　博久　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
本田　元人　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
渡邊　恒二　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
青木　孝弘　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
西島　　健　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
水島　大輔　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
濱田　洋平　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
橋本　亜希　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
高野　　操　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
小形　幹子　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
石坂美千代　（（独）国立国際医療研究センター　エイズ治療・研究開発センター）
内藤　俊夫　（順天堂大学医学部附属順天堂医院　総合診療科）
齊田　瑞恵　（順天堂大学医学部附属順天堂医院　総合診療科）
鈴木　麻衣　（順天堂大学医学部附属順天堂医院　総合診療科）
吉田　正樹　（東京慈恵会医科大学附属病院　感染制御部）
藤井　　毅　（東京大学医科学研究所附属病院　感染免疫内科）
岩本　愛吉　（東京大学医科学研究所附属病院　感染免疫内科）
鯉渕　智彦　（東京大学医科学研究所附属病院　感染免疫内科）
三浦　聡之　（東京大学医科学研究所附属病院　感染症分野）
中村　仁美　（東京大学医科学研究所附属病院　感染症分野）
松本　和史　（東京大学医科学研究所附属病院　看護部）
宮崎菜穂子　（東京大学医科学研究所附属病院　薬剤部）
角野久美子　（東京大学医科学研究所附属病院　医療安全管理部）
藤原　紀子　（東京大学医科学研究所附属病院　医療安全管理部）
山本　政弘　（（独）国立病院機構九州医療センター　免疫感染症科）
南　　留美　（（独）国立病院機構九州医療センター　免疫感染症科）
横幕　能行　（（独）国立病院機構名古屋医療センター　感染症科）

堀場　昌英　((独) 国立病院機構 東埼玉病院　呼吸器科)
金田　　暁　((独) 国立病院機構 千葉医療センター　消化器科)
立川　夏夫　(横浜市立市民病院　感染症内科)
吉村　幸治　(横浜市立市民病院　感染症内科)
内海　英貴　(群馬大学医学部附属病院　生体統御内科)
鄭　　真徳　(長野県厚生農業協同組合連合会 佐久総合病院　総合診療科)
高添明日香　(長野県厚生農業協同組合連合会 佐久総合病院　総合診療科)
竹田　徹朗　(獨協医科大学越谷病院　腎臓内科)
影向　　晃　(新潟大学大学院医歯学総合研究科　総合地域医療学講座)
茂呂　　寛　(新潟大学医歯学総合病院　第二内科)
上田　幹夫　(石川県立中央病院　血液免疫内科)
高田　清式　(愛媛大学医学部附属病院　総合臨床センター)
藤井　輝久　(広島大学病院　輸血部)
斎藤　誠司　(広島大学病院　血液内科・エイズ医療対策室)
鍵浦　文子　(広島大学病院　エイズ医療対策室)
日笠　　聡　(兵庫医科大学病院　血液内科)
松下　修三　(熊本大学エイズ学研究センター　松下プロジェクト研究室)
宮川　寿一　(熊本大学医学部附属病院　血液内科)
健山　正男　(琉球大学医学部附属病院　第一内科)
仲村　秀太　(琉球大学医学部附属病院　第一内科)

研究成果の刊行に関する一覧

1. Tsukada K, Teruya K, Tasato D, Gatanaga H, Kikuchi Y, and **Oka S**. Raltegravir-associated perihepatitis and peritonitis: a single case report. ***AIDS*** (correspondence) 24: 160-161, 2010.

2. Gatanaga H, Ode H, Hachiya A, Hayashida T, Sato H, Takiguchi M, and **Oka S**. Impact of HLA-B*51-restricted CTL Pressure on Mutation Patterns of Non-nucleoside Reverse Transcriptase Inhibitor Resistance. ***AIDS*** (Fast Track) 24: F15-22, 2010.

3. Sakai K, Gatanaga H, Takata H, **Oka S**, and Takiguchi M. Comparison of CD4+ T-cell-subset distribution in chronically infected HIV+ patients with various CD4 nadir counts. ***Microb Infect*** 12: 374-381, 2010.

4. Gatanaga H, Ode H, Hachiya A, Hayashida T, Sato H, **Oka S**. Combination of V106I and V179D Polymorphic Mutations in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Resistance to Efavirenz and Nevirapine but not to Etravirine. ***Antimicrob Agents Chemother*** 54: 1596-1602, 2010.

5. Phan TT, Ishizaki A, Phung DC, Bi X, **Oka S**, and Ichimura H. Characterization of HIV type 1 genotypes and drug resistance mutations among drug-naive HIV type 1-infected patients in Northern Vietnam. ***AIDS Res Hum Retroviruses*** 26: 233-235, 2010.

6. Koizumi H, Hashimoto M, Fujiwara M, Chikata T, Borghan MA, Hachiya A, Kawashima Y, Takata H, Ueno T, **Oka S**, and Takiguchi M. Different *in vivo* effects of HIV-1 immunodominant epitope-specific CTLs on selection of escape mutant viruses. ***J Virol*** 84: 5508-5519, 2010.

7. Kawashima Y, Kuse N, Gatanaga H, Naruto T, Fujiwara M, Dohki S, Maenaka K, Goulder P, **Oka S**, and Takiguchi M. Long-term control of HIV-1 by HIV-1 pol-specific CTLs in hemophiliacs carrying slow-progressing allele HLA-B*5101. ***J Virol*** 84: 7151-7160, 2010.

8. Gatanaga H, Oowa M, and **Oka S**. Introduction of TaqMan HIV-1 assay increased unnecessary drug resistance testing. ***AIDS Patient Care*** (Letter to the Editor) 24: 1-2, 2010.

9. Tanimoto T, Nguyen HC, Ishizaki A, Chung PT, Hoang TT, Nguyen VT, Kageyama S, **Oka S**, Pham VT, Ichimura H. Multiple routes of hepatitis C virus transmission among injection drug users in Hai Phong, Northern Vietnam. ***J Med Virol*** 82: 1355-1363, 2010.

10. Takarabe D, Rokukawa Y, Takahashi Y, Goto A, Takaichi M, Okamoto M, Tsujimoto T, Noto H, Kishimoto M, Kaburagi Y, Yasuda K, Yamamoto-Honda R, Tsukada K, Honda M, Teruya K, Kajio H, Kikuchi Y, **Oka S**, Noda M. Autoimmune Diabetes in HIV-Infected Patients on Highly Active Antiretroviral Therapy. ***J Clin Endocrinol Metab*** 95: 4056-4060, 2010.

11. **Tanuma J**, Hachiya A, Ishigaki K, Gatanaga H, Lien TTM, Hien ND, Kin NV, Kaku M , and **Oka S.** Impact of CRF01_AE-specific polymorphic mutations G335D and A371V in the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on susceptibility to nucleoside RT inhibitors. ***Microb Infect*** 12: 1170-1177, 2010.

12. Watanabe K, Honda M, Watanabe T, Tsukada K, Teruya K, Kikuchi Y, **Oka S**, and Gatanaga H. Emergence of raltegravir-resistant HIV-1 in the central nerve system. ***Int J STD AIDS*** (case report) 21: 840-841, 2010.

13. Hattori J, Shiino T, Gatanaga H, Yoshida S, Watanabe D, Minami R, Sadamasu K, Kondo M, Mori H, Ueda M, Tateyama M, Ueda A, Kato S, Ito T, Oie M, Takata N, Hayashida T, Nagashima M, Matsuda M, Ibe S, Ota Y, Sasaki S, Ishigatsubo Y, Tanabe Y, Koga I, Kojima Y, Yamamoto M, Fujita J, Yokomaku Y, Koike T, Shirasaka T, **Oka S**, and Sugiura W. Trends in transmitted drug-resistant HIV-1 and demographic characteristics of newly diagnosed patients: nationwide surveillance from 2003 to 2008 in Japan. ***Antiviral Res*** 88: 72-79, 2010.

14. Watanabe T, and **Oka S**. Serum (1→3) β-D-Glucan as a Noninvasive Adjunct Marker for the Diagnosis and Follow-Up of Pneumocystis jiroveci Pneumonia in Patients with HIV Infection. ***Clin Infect Dis*** (correspondence) 50: 451-452, 2010.

15. Mwimanzi P, Hasan Z, Tokunaga M, Gatanaga H, **Oka S**, and Ueno T. Naturally arising HIV-1 Nef variants conferring escape from cytotoxic T lymphocytes influence viral entry co-receptor expression and susceptibility to superinfection. ***Biochem Biophys Res Commun*** 403: 422-427, 2010.

16. Zhou J, Sirisanthana T, Kiertiburanakul S, Chen YM, Han N, Lim PL, Kumarasamy N, Choi JY, Merati TP, Yunihastuti E, **Oka S**, Kamarulzaman A, Phanuphak P, Lee CK, Li PC, Pujari S, Saphonn V, Law MG. Trends in CD4 counts in HIV-infected patients with HIV viral load monitoring while on combination antiretroviral treatment: results from The TREAT Asia HIV Observational Database. ***BMC Infect Dis*** 10: 361, 2010.

17. Nakamura H, Teruya K, Takano M, Tsukada K, **Tanuma J**, Yazaki H, Honda H, Honda M, Gatanaga H, Kikuchi Y, and **Oka S**. Clinical symptoms and courses of primary HIV-1 infection in recent years in Japan. ***Intern Med*** 50: 95-101, 2011.

18. Watanabe T, Murakoshi H, Gatanaga H, Koyanagi M, **Oka S**, and Takiguchi M. Effective recognition of HIV-1-infected cells by HIV-1 Integrase-specific HLA-B*4002-restricted T cells. ***Microb Infect*** 13: 160-166, 2011.

19. Ishikawa N, Ishigaki K, Ghidinelli MN, Ikeda K, Honda M, Miyamoto H, Kakimoto K, and **Oka S**. Paediatric HIV and elimination of mother-to-child transmission of HIV in the ASEAN region: a call to action. ***AIDS Care*** 23: 413-416, 2011.

20. Honda K, Zheng N, Murakoshi H, Hashimoto M, Sakai K, Borghan MA, Chikata T, Koyanagi M, Tamura Y, Gatanaga H, **Oka S**, and Takiguchi M. Selection of escape mutant by HLA-C-restricted HIV-1 Pol-specific cytotoxic T lymphocytes carrying strong ability to suppress HIV-1 replication. ***Eur J Immunol*** 41:97-106, 2011.

21. Hachiya A, Kodama EN, Schuckmann MM, Kirby KA, Michailidis E, Sakagami Y, **Oka S**, Singh K, and Sarafianos SG. K70Q adds high-level tenofovir resistance to "Q151M complex" HIV reverse transcriptase through the enhanced discrimination mechanism. ***PLoS One*** 6, e16242, 2011.

22. Morooka M, Ito K, Kubota K, Yanagisawa K, Teruya K, Hasuo K, Shida Y, Minamimoto R, Kikuchi Y, and **Oka S**. Usefulness of F-18 FDG PET/CT in a case of Kaposi sarcoma with an unexpected bone lesion. ***Clin Nucl Med*** 36:231-234, 2011.

23. Davaalkham J, Unenchimeng P, Baigalmaa C, Erdenetuya G, Nyamkhuu D, Shiino T, Tsuchiya K, Hayashida T, Gatanaga H, and **Oka S.** Identification of a current hot spot of HIV-1 transmission in Mongolia by molecular epidemiological analysis. ***AIDS Res Hum Retrovisus*** (in press)

24. Honda M, Ishisaka M, Ishizuka N, Kimura S, **Oka S** on behalf of Japanese Anti-HIV-1 QD Study Group. Open-label randomized multicenter selection study of once daily antiretroviral treatment regimen comparing ritonavir boosted atazanavir to efavirenz with fixed dose abacavir and lamivudine. ***Intern Med*** (in press)

Raltegravir-associated perihepatitis and peritonitis: a single case report

Raltegravir, the first approved HIV integrase inhibitor, has demonstrated an excellent safety and tolerability profile in several clinical trials [1] and is currently used widely as one of the key components of salvage regimens. However, the duration of clinical use is relatively short, and unknown adverse effect may occur. Here, we report one case of peritonitis associated with use of raltegravir. Abdominal symptoms appeared within 2 weeks of commencement of treatment, and raltegravir had to be stopped due to worsening of clinical condition.

Case report

The patient was a 49-year-old Japanese hemophiliac coinfected with HIV and hepatitis C virus (HCV). HIV-RNA was undetectable, and $CD4^+$ cell count was above 500 cells/μl for more than 5 years under the combination of abacavir, nevirapine and lopinavir/ritonavir. In January 2009, lopinavir/ritonavir was replaced with raltegravir because of bleeding tendency related to the use of a protease inhibitor. Abacavir and nevirapine were continued, and no other drugs were modified. The patient visited the hospital on day 18 after the use of raltegravir, complaining of a gradually worsening pain in the right upper abdomen and lower chest wall for 3 days. A nonsteroidal anti-inflammatory drug was not effective, and a computed tomography (CT) scan performed 11 days after the onset of the symptom revealed contrast enhancement of the liver surface (Fig. 1a) and fatty stranding of the greater omentum (Fig. 1b), which are findings compatible with perihepatitis and peritonitis. Oral prednisone (60 mg/day for 3 days, then 30 mg/day for 3 days) was prescribed, and all the symptoms resolved immediately. However, abdominal symptoms developed again after withdrawal of prednisone, necessitating its reintroduction on day 31 at 30 mg/day. Attempts to taper prednisone led to worsening of abdominal pain and development of stomatitis, resulting in continuation of treatment at 20 mg/day. Raltegravir was switched to lopinavir/ritonavir 11 weeks after the onset of abdominal pain and, finally, all antiretroviral drugs were terminated 4 days later because of diarrhea and bleeding related to lopinavir/ritonavir. Abdominal symptoms gradually improved, and prednisone could be tapered to 10 mg/day within 2 weeks. A CT scan performed 10 days after cessation of antiretroviral therapy showed an improvement of perihepatic enhancement. C-reactive protein levels increased to 1.42 mg/dl during raltegravir use and fell to normal levels 6 days after discontinuation of raltegravir. Other laboratory data including transaminase levels showed no changes, and $CD4^+$ cell count and HIV-RNA were stable throughout the course.

This is the first reported case of severe peritonitis associated with raltegravir use. Although not described here, we have experienced several other cases with similar abdominal symptoms that disappeared after raltegravir termination. Several case reports have recently described previously unknown adverse effects related to raltegravir, such as rhabdomyolysis [2] and exacerbation of depression [3]. However, to our knowledge, raltegravir-associated peritonitis has not been reported. In the BENCHMRK

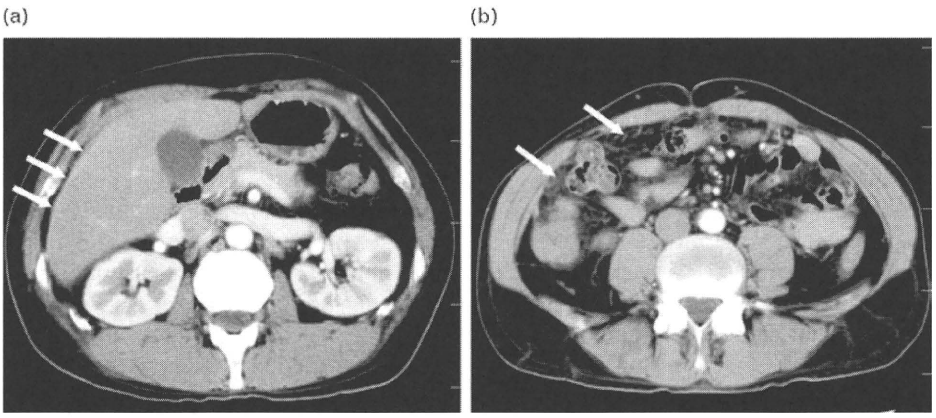


Fig. 1. A computed tomography scan performed 11 days after the onset of the symptoms. A computed tomography scan shows contrast enhancement around the liver surface (a) and fatty stranding of the greater omentum (b).

(Blocking integrase in treatment Experienced patients with a Novel Compound against HIV: MeRcK, MK-0518) study [1], abdominal symptoms, such as diarrhea and nausea, were noted in patients on raltegravir, and some of which might be associated with mild peritonitis.

Fortunately, raltegravir-associated peritonitis seemed reversible, at least to some extent. However, the longer use of raltegravir after onset of symptoms may lead to irreversible and lethal sequelae. Cessation of antiretroviral therapy as a result of severe abdominal symptoms is a potential risk for re-emergence of acute retroviral syndrome or the further accumulation of HIV-resistant mutations.

Whether the described side effects are universal or related to Asians, hemophiliacs or those who have underlying liver disease is unknown at present. Careful monitoring of abdominal symptoms and the consideration of an appropriate radiographic examination are warranted after commencement of raltegravir-containing regimens.

Acknowledgements

The authors thank all clinical staff of the AIDS Clinical Center.

All authors contributed to the conception, design and performance of this submission.

This study was supported in part by the Ministry of Health, Labour, and Welfare of Japan.

All authors report no potential conflict of interests.

Kunihisa Tsukada, Katsuji Teruya, Daisuke Tasato, Hiroyuki Gatanaga, Yoshimi Kikuchi and Shinichi Oka, *AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan.*

Correspondence to Kunihisa Tsukada, MD, AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. Tel: +81 3 3202 7181; fax: +81 3 3202 7198; e-mail: ktsukada@imcj.acc.go.jp

Received: 29 September 2009; accepted: 2 October 2009.

References

1. Steigbigel RT, Cooper DA, Kumar PN, Eron JE, Schechter M, Markowitz M, *et al.* **Raltegravir with optimized background therapy for resistant HIV-1 infection.** *N Engl J Med* 2008; **359**:339–354.
2. Zembower TR, Gerzenshtein L, Coleman K, Palella FJ Jr. **Severe rhabdomyolysis associated with raltegravir use.** *AIDS* 2008; **22**:1382–1384.
3. Harris M, Larsen G, Montaner JS. **Exacerbation of depression associated with starting raltegravir: a report of four cases.** *AIDS* 2008; **22**:1890–1892.

DOI:10.1097/QAD.0b013e328333d28d

Impact of human leukocyte antigen-B*51-restricted cytotoxic T-lymphocyte pressure on mutation patterns of nonnucleoside reverse transcriptase inhibitor resistance

Hiroyuki Gatanaga[a,b], Hirotaka Ode[d], Atsuko Hachiya[a,c], Tsunefusa Hayashida[a,b], Hironori Sato[d], Masafumi Takiguchi[c] and Shinichi Oka[a,b]

Objective: The objective of this study is to determine the impact of human leukocyte antigen (HLA)-B*51-restricted cytotoxic T-lymphocyte (CTL) pressure on the development of nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance.

Design: The prevalence of HIV-1 harboring an escape mutation, I135X, in a major epitope of HLA-B*51-restricted CTL located in reverse transcriptase is increasing worldwide. We analyzed the effects of escape mutations on the emerging mutation patterns of NNRTI resistance.

Methods: Monoclonal HIV-1 sequences harboring each of the escape mutations, including I135L (HIV-1_{I135L}), I135V (HIV-1_{I135V}), I135T (HIV-1_{I135T}), and I135R (HIV-1_{I135R}) in reverse transcriptase, and a wild-type monoclonal HIV-1 (HIV-1_{WT}) were cultured in the presence of increasing concentrations of efavirenz. Induced mutations during culture passages of the culture were analyzed.

Results: E138K emerged during the cultural passages of HIV-1_{I135V}, HIV-1_{I135T}, and HIV-1_{I135R}, but not during the passages of HIV-1_{WT}. The combination of I135T, the most frequent escape mutation, and E138K (HIV-$1_{I135T/E138K}$) conferred significant resistance to efavirenz, nevirapine, and etravirine. The HIV-$1_{I135L/E138K}$ and HIV-$1_{I135R/E138K}$ were significantly resistant to nevirapine and etravirine, respectively, though each solo of escape mutations and E138K did not confer significant resistance to NNRTI. Computational analysis indicated that I135T and E138K cooperatively extend the gap between the binding site of reverse transcriptase and NNRTI.

Conclusion: HLA-B*51-restricted CTL can induce novel mutation patterns of NNRTI resistance by selecting escape mutations. The spread of CTL escape variants may alter the mutation patterns of drug resistance.

AIDS 2010, **24**:F15–F22

[a]AIDS Clinical Center, International Medical Center of Japan, Tokyo, [b]Division of Infectious Disease, [c]Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, and [d]Laboratory of Viral Genomics, Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan.

Correspondence to Hiroyuki Gatanaga, MD, AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.

Tel: +81 3 3202 7181; fax: +81 3 5273 6483; e-mail: higatana@imcj.acc.go.jp

Received: 9 November 2009; revised: 28 December 2009; accepted: 19 January 2010.

DOI:10.1097/QAD.0b013e328337b010

Keywords: antiretroviral therapy, cellular immunity, cytotoxic T-lymphocyte escape variant, E138K, major epitope

Introduction

Cytotoxic T lymphocytes (CTLs) are one of the antiretroviral host factors that can modify the clinical course of HIV-1 infection [1]. However, HIV-1 evades these cells by acquiring escape mutations in recognized epitopes, and some of the CTL-escape variants remain stable without reversion even in the absence of such selective pressure [2]. TAFTIPSI (reverse transcriptase 128–135) is a major epitope recognized by human leukocyte antigen (HLA)-B*51-restricted CTL [3], and we recently reported that its escape mutation, I135X, is detected in the majority of HLA-B*51-positive infected individuals and also in a significant proportion of HLA-B*51-negative individuals, and that I135X can exist persistently even in HLA-B*51-negative individuals probably because it does not cause a significant fitness cost [4]. Consequently, I135X can spread as a polymorphic mutation among infected individuals and has in fact accumulated in the HIV-positive populations, especially among the Japanese, in whom HLA-B*51 is highly prevalent. Previous studies reported that I135X was associated with low-level resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) [5–7] and suggested that I135X may be a determinant of evolutional patterns of NNRTI resistance [8,9], though it has also been reported that there is no correlation between the presence of I135X at baseline and efficacy of NNRTI [10]. To determine whether CTL escape mutations alter the development of drug resistance, we focused on I135X and induced NNRTI resistance from I135X-harboring HIV-1s by cultural passages in the presence of increasing concentrations of efavirenz (EFV).

Materials and methods

HIV-1 sequences and human leukocyte antigen types in treatment-naive patients

We recently reported the frequent prevalence of I135X mutations in Japan [4]. To confirm the same and to determine the frequency of each mutation, we used another cohort that included 575 treatment-naive newly diagnosed HIV/AIDS patients recruited from across Japan between January 2003 and December 2004 [11]. Among them, data of HLA typing were available for 97 patients.

Generation of recombinant HIV-1 sequences

The desired mutations were introduced into the *Xma*I-*Nhe*I region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 reverse transcriptase (strain BH10) [12]. The *Xma*I-*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 h after transfection and stored at −80°C until use.

Induction of efavirenz-resistant HIV-1

The infectious HIV-1 clones were propagated in MT-2 cells in the presence of increasing concentrations of EFV [12]. Briefly, MT-2 cells (1×10^5) were exposed to 500 blue cell-forming units (BFUs) in MAGIC-5 cells (CCR5-expressing and CD4-expressing HeLa-LTR-β-D-gal cells) of each monoclonal HIV-1 and cultured in the presence of EFV at an initial concentration of 3 nmol/l. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased by half-log fold. This selection was carried out until the EFV concentration reached 1000 nmol/l. Proviral HIV-1 reverse transcriptase gene in the infected MT-2 cells was amplified and sequenced at several passages.

Drug susceptibility assay

EFV and nevirapine (NVP) were generously provided by Merck Co., Inc. (Rahway, New Jersey, USA) and Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, Connecticut, USA), respectively. Etravirine (ETR) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Recombinant HIV-1 susceptibility to EFV, NVP, and ETR was determined in triplicate using MAGIC-5 cells [12]. The drug susceptibility assay was performed in triplicate and repeated three times. Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1 ($HIV\text{-}1_{WT}$). Drug resistance was considered significant when it was higher than three-fold.

Structural modeling

We constructed structural models of the HIV-1 reverse transcriptase and NNRTI complex by computational analysis. First, we constructed the initial models of wild-type reverse transcriptase with one of the three NNRTIs by homology modeling using Molecular Operating Environment (MOE) 2007.09.02 (http://www.chemcomp.com/). The crystal structures of reverse transcriptase with NNRTI (PDB code: 1IKW [13], 1VRT [14], and 1SV5 [15]) were used for template structures. The ff94 force field and distance-dependent electrostatic energy function were applied in the modeling. Next,

we refined the initial models by energy minimization using sander module of AMBER9 software package through two steps. In the first step, energies for the NNRTI in the complex models were minimized at the gas phase by the conjugated gradient method. In the second step, energies of whole structures were converged up to 0.5 kcal/mol/Å by 50 steps of the steepest descent method and the subsequent conjugated gradient method at implicit water solvent condition. In each minimization, the AMBER ff03 [16,17], the general AMBER force field (gaff) [18], and the generalized Born implicit solvent surface area (GBSA) method (IGB = 2) [19] were applied for potential energy calculations. The charges and atom types of every atom in NNRTI were automatically assigned using the AMBER9 Antechamber module. We also constructed the respective mutant reverse transciptases with the NNRTI by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL version 0.99rc6 (http://www.pymol.org). The structural model of each conformer was refined by a method similar to that used in the wild-type models. Among the refined conformers, we selected those with the lowest energy as each mutant model.

Results

The 135th amino acid in HIV-1 reverse transcriptase and human leukocyte antigen-B*51

We analyzed the relationship between HLA-B*51 and the 135th amino acid of HIV-1 reverse transciptase in 97 infected individuals newly diagnosed in Japan between January 2003 and December 2004 (Table 1). As expected, CTL escape mutations I135X, including I135T, I135L, and I135V, were observed in all but one HLA-B*51-positive patient (94.1%), representing a significantly higher prevalence than in the HLA-B*51-negative patients (Fisher's exact test; $P = 0.01$). However, in the HLA-B*51-negative patients, escape mutations were still observed at a high frequency (62.5%), indicating that I135X variants can transmit from HLA-B*51-positive patients to HLA-B*51negative individuals and can persist even in the absence of HLA-B*51-restricted CTL pressure. Overall, I135X mutations were observed at a high frequency in the treatment-naive patients in Japan, and the most frequent amino acid was I135T (35.1%), which was more frequent than the wild-type I135 (32.0%).

Induction of efavirenz-resistant HIV-1

As described above, I135L, I135V, I135T, and I135R mutations were detected in treatment-naive patients. In order to analyze their effects on the mutation pattern for NNRTI resistance, EFV resistance was induced from monoclonal HIV-1s harboring each of these mutations by culturing them in the presence of increasing concentrations of EFV. These induction experiments were performed independently in triplicate. In one of the three induction experiments on HIV-1_{I135L}, V179D emerged when EFV concentration reached 100 nmol/l, as well as emergence of K103R in the presence of EFV at 1000 nmol/l (Fig. 1a). We previously reported that the combination of K103R and V179D confers significant resistance to NNRTIs [12]. In another experiment, V108I emerged at an EFV concentration of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1b). Both L100I and V108I are listed in the International AIDS Society (IAS)-USA Resistance Table [20] as EFV resistance mutations. In the last experiment on HIV-1_{I135L}, G190A emerged followed by V106A (Fig. 1c). The latter two are also listed in the IAS-USA Table. In one of the three induction experiments on HIV-1_{I135V}, E138K emerged at an EFV of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1d). E138K is a rare mutation and not listed as a resistance mutation in the IAS-USA Table. It was reported that E138K alone did not alter drug susceptibility significantly, though it emerged during resistance induction experiments with ETR and other experimental NNRTIs (Brillant *et al.* 13th International HIV Drug Resistance Workshop, 2004; Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [21–23]. L100I emerged first followed by Y188H in another experiment, and L100I emerged first followed by V108I in the last experiment (Figure 1e and f). In one of the three induction experiments on HIV-1_{I135T}, V108I emerged at an EFV of 100 nmol/l and K101E at an EFV of 1000 nmol/l (Fig. 2a). In another experiment, V106I emerged first followed by V179D (Fig. 2b). The combination of V106I and V179D was confirmed to confer a significant NNRTI resistance by our group (unpublished data). In the last experiment, V108I emerged first followed by E138K and L100I (Fig. 2c). In one of the three induction experiments on HIV-1_{I135R}, L100I emerged at an EFV of 100 nmol/l followed

Table 1. Frequency of amino acids at codon 135 of HIV-1 reverse transcriptase in human leukocyte antigen-B*51-positive and human leukocyte antigen-B*51-negative patients.

135th amino acid	I	L	V	T	R
B*51 (+)/17 (%)	1 (5.9)	3 (17.6)	1 (5.9)	12 (70.6)	0 (0)
B*51 (−)/80 (%)	30 (37.5)	13 (16.3)	11 (13.8)	22 (27.5)	4 (5.0)
Total/97 (%)	31 (32.0)	16 (16.5)	12 (12.4)	34 (35.1)	4 (4.1)

HLA type was determined by standard sequence-based genotyping. HLA, human leukocyte antigen.

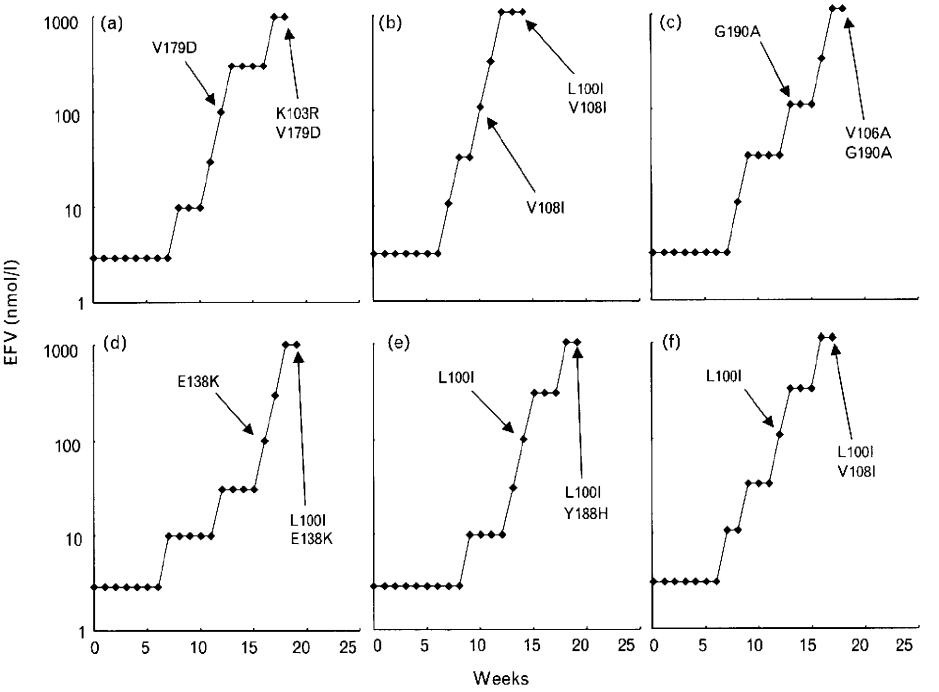


Fig. 1. Induction of efavirenz resistance from HIV-1_{I135L} and HIV-1_{I135V}. HIV-1_{I135L} (a–c) and HIV-1_{I135V} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

by E138K at an EFV of 1000 nmol/l (Fig. 2d). In another experiment, E138K emerged first then G190A and V108I (Fig. 2e). In the last experiment, L100I emerged first followed by K101E (Fig. 2f). In summary, during the induction experiments, all the induced mutations were already known NNRTI-resistance mutations except for E138K, which emerged in one of the three induction experiments on HIV-1_{I135V}, in one of the three induction

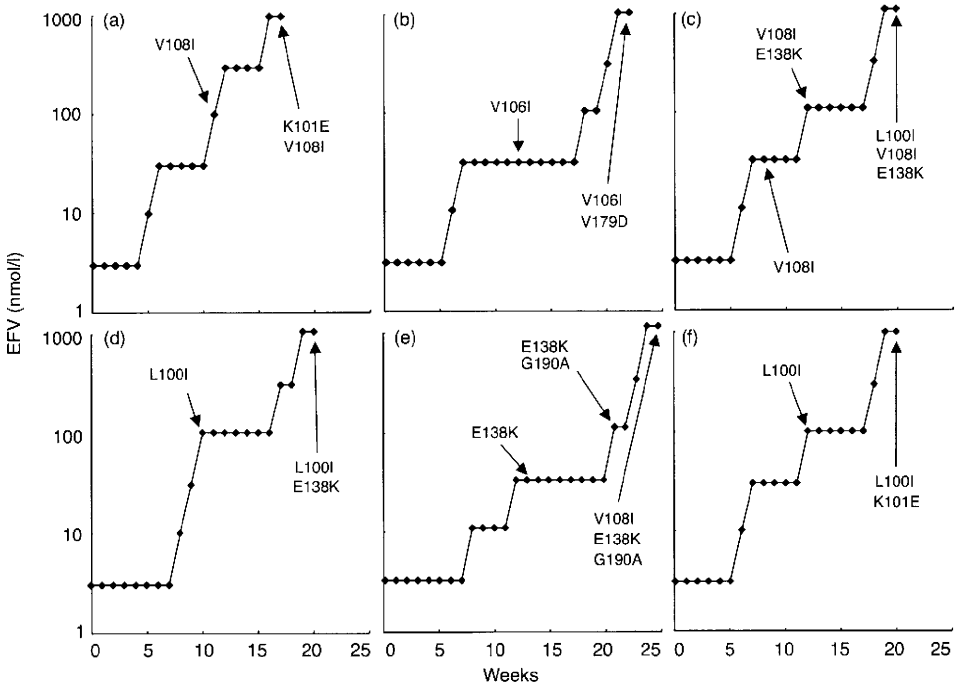


Fig. 2. Induction of efavirenz resistance from HIV-1_{I135T} and HIV-1_{I135R}. HIV-1_{I135T} (a–c) and HIV-1_{I135R} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

experiments on HIV-1_{I135T}, and in two of the three induction experiments on HIV-1_{I135R}. We also performed EFV-resistance induction experiments on HIV-1_{WT} in triplicate using the same procedure. All the induced mutations were already known NNRTI-resistance mutations, whereas E138K did not emerge in any of the three induction experiments on HIV-1_{WT} (data not shown).

Nonnucleoside reverse transcriptase inhibitor resistance conferred by E138K combined with I135X

During the induction experiments on HIV-1s harboring I135X, the emergence of E138K, which is usually a rare mutation, was often observed. To analyze the effects of E138K alone and its combination with I135X on NNRTI susceptibility, a panel of recombinant HIV-1 clones was constructed and their IC_{50} values for EFV, NVP, and ETR were determined. As expected, I135X alone did not confer significant NNRTI resistance (Table 2). The combination of I135T and E138K (I135T/E138K) conferred significant resistance to EFV, NVP, and ETR, though E138K alone did not change NNRTI susceptibility as reported previously (Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [22,23]. I135L/E138K and I135R/E138K conferred significant resistance to NVP and ETR, respectively. In summary, E138K conferred significant resistance when combined with some of the I135X mutations, especially I135T, which is the most prevalent in treatment-naive individuals in Japan (Table 1).

Structural modeling of reverse transcriptase harboring I135T and E138K

The in-vitro drug susceptibility assay described above showed that I135T/E138K conferred the most efficient resistance to EFV and significant resistance to NVP and ETR. To analyze the molecular mechanisms by which E138K combined with I135T alter NNRTI susceptibility, we conducted a structural analysis that included computational methods. A total of 12 structural models of reverse transcriptase-NNRTI complexes were constructed with four reverse transcriptases (wild-type, I135T, E138K, and I135T/E138K) and three NNRTIs (EFV, NVP, and ETR). We first calculated the binding energies between reverse transcriptase and NNRTI. Differences in the binding energies between mutant and wild-type complexes ($\Delta\Delta$Gb) were calculated using the models. The $\Delta\Delta$Gb value correlated positively with the logarithm of fold resistance value obtained by our in-vitro drug susceptibility assay described above: a greater reduction in the binding energy correlated with a greater resistance ($r = 0.77$, $P < 0.02$) [24], suggesting that our modeling appropriately reflects the actual binding mode between the reverse transcriptase molecule and NNRTI. In the 12 models tested, the $\Delta\Delta$Gb value of the I135T/E138K RT-NNRTI complex was persistently larger than wild-type and single mutation reverse transcriptases, indicating that I135T/E138K caused a larger loss of interactions between reverse transcriptase and NNRTI than the single mutations. We then examined the structural changes in the loss of interactions by I135T/E138K. In the wild-type reverse transcriptase, the E138 positioned relatively closely to the EFV, which could contribute to the generation of van der Waals and electrostatic interactions between reverse transcriptase and NNRTI (Fig. 3a). The I135T single substitution caused no significant changes in the steric position of the E138 side chain (Fig. 3b). E138K substitution caused significant changes in the steric position of the E138 side chain (Fig. 3c), whereas the calculated van der Waals energy was similar to that of wild-type reverse transcriptase. I135T/E138K also caused significant changes in the steric position of the K138 side chain, but the orientation of the side chain was different from that of the E138K single mutant reverse transcriptase, possibly due to the interactions between T135 and K138 (Fig. 3d). The K138 conformation in the $RT_{I135T/E138K}$ generated a steric gap between K138 and EFV, and significantly reduced van der Waals energy. In addition, the conformational change necessitated increased electrostatic energy of the reverse transcriptase–EFV complex. These data suggest that an appropriate steric position of the 138th residue is critical for the generation

Table 2. Nonnucleoside reverse transcriptase inhibitor susceptibility of recombinant HIV-1 sequences.

	Mean IC_{50} (μmol/l) ± SD (fold resistance*)		
HIV-1	EFV	NVP	ETR
Wild-type	0.002 ± 0.0007	0.05 ± 0.01	0.0012 ± 0
I135L	0.003 ± 0.0005 (1.5)	0.07 ± 0.01 (1.4)	0.0012 ± 0.0002 (1)
I135V	0.0024 ± 0.0003 (1.2)	0.04 ± 0.01 (0.8)	0.0011 ± 0.0001 (0.9)
I135T	0.002 ± 0.001 (1)	0.06 ± 0.01 (1.2)	0.0016 ± 0.0002 (1.3)
I135R	0.003 ± 0.001 (1.5)	0.03 ± 0.01 (0.6)	0.0012 ± 0.0002 (1)
E138K	0.004 ± 0.0004 (2)	0.08 ± 0.01 (1.6)	0.0026 ± 0.0001 (2.2)
I135L/E138K	0.003 ± 0.001 (1.5)	0.23 ± 0.02 (4.6)	0.0033 ± 0.0006 (2.8)
I135V/E138K	0.006 ± 0.001 (3)	0.04 ± 0.01 (0.8)	0.0033 ± 0.0006 (2.8)
I135T/E138K	0.014 ± 0.002 (7)	0.19 ± 0.06 (3.8)	0.005 ± 0.0002 (4.2)
I135R/E138K	0.005 ± 0.002 (2.5)	0.14 ± 0.04 (2.8)	0.0047 ± 0.0003 (3.9)

The drug susceptibility assay was performed in triplicate and repeated three times. EFV, efavirenz; ETR, etravirine; NVP, nevirapine.
*Fold resistance was calculated by comparing viral IC_{50} with that of wild-type HIV-1.

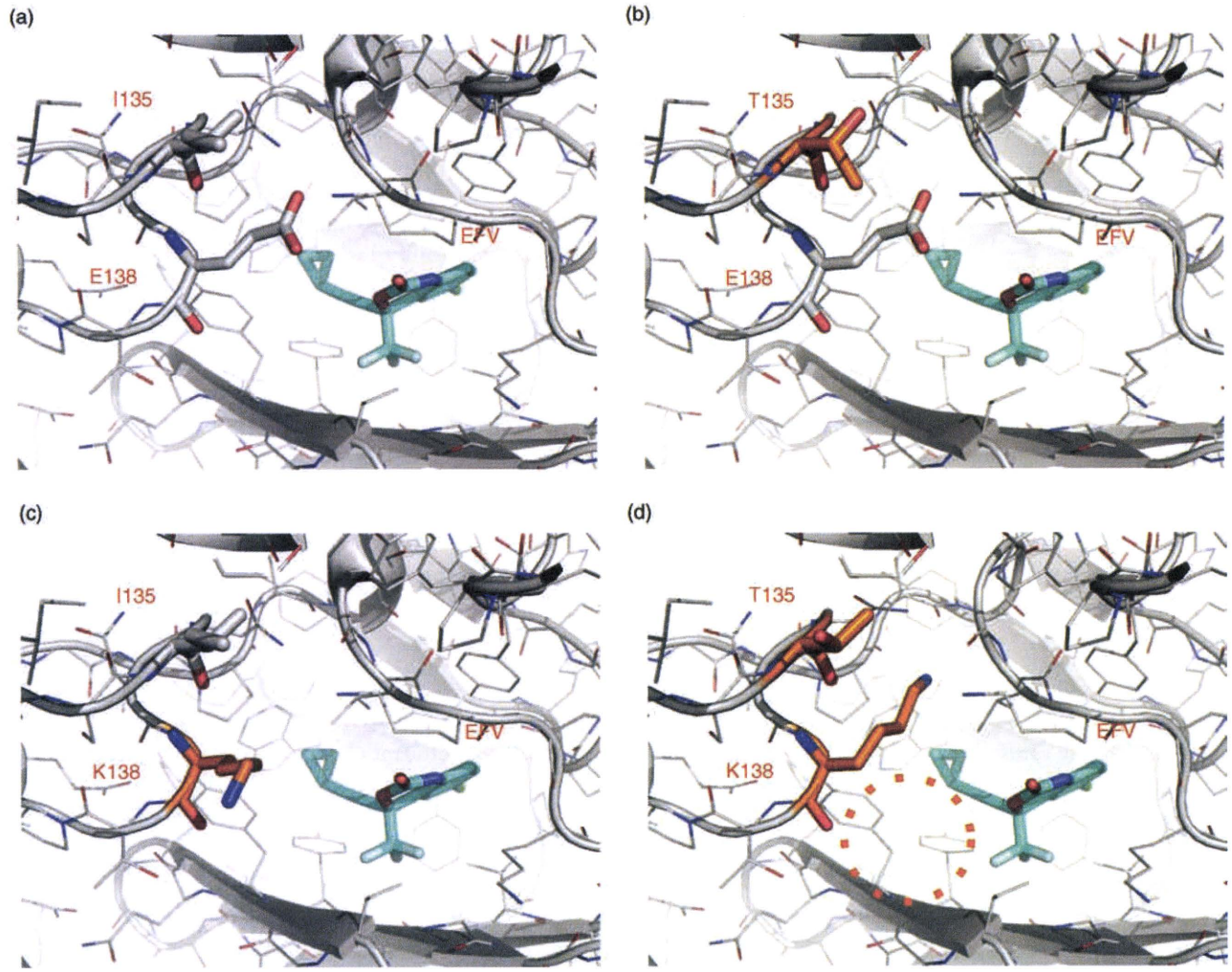


Fig. 3. Structural models of HIV-1 reverse transcriptase–efavirenz complexes. The binding clefts of four complex models are shown. (a) $RT_{wild\text{-}type}$, (b) RT_{I135T}, (c) RT_{E138K} and (d) $RT_{I135T/E138K}$. Sticks indicate the amino acids at positions 135 and 138 of RT, and the atoms of EFV. The mutated residues (I135T and E138K) and the EFV atoms are highlighted with orange and cyan sticks, respectively. The dotted circle in panel D indicated the enlarged gap by I135T/E138K mutations. EFV, efavirenz; RT, reverse transcriptase.

of an optimal EFV binding pocket, and that I135T/E138K, but not the single mutations, effectively break the binding pocket for EFV.

Discussion

As the HIV-1 pandemic progresses, viral genetic diversity is increasing and becoming geographically heterogeneous [25,26]. We recently indicated that HIV-1 adapts to CTL by acquiring escape mutations in the CTL epitopes, and that such escape variants are increasing in the populations at an alarming high rate of corresponding HLA alleles [4]. When escape mutations occur in drug target proteins, they may alter the mutation patterns of drug resistance even if they do not confer drug resistance themselves. In this study, we focused on I135X in reverse transcriptase, which are escape mutations of HLA-B*51-restricted CTL, because I135X are the prevailing mutations and accumulating in Japan, where the frequency of HLA-B*51 is high (~20%). Cultural passages of HIV-1 sequences harboring I135X in the presence of increasing concentrations of EFV induced the emergence of E138K, which is not listed as a resistance mutation in the IAS-USA Table. The analysis of recombinant HIV-1 sequences showed that the combination of E138K and some of the I135X, especially I135T, which is most frequent, conferred significant resistance to NNRTI, though solo E138K did not alter drug susceptibility significantly. However, E138K did not always emerge in triplicate experiments of EFV-resistance induction from HIV-1 sequences harboring I135X, whereas the already known NNRTI-resistance mutations emerged. Importantly, variable mutation patterns emerged under the same conditions of resistance induction experiments,

indicating that the drug selective pressure is one of the driving forces making the genetic diversity of HIV-1 at population levels as CTL pressure does (HLA-B*51-restricted CTL pressure selects not only I135T but also other I135Xs).

In clinical data, Richard *et al.* [27] examined HIV-1 reverse transcriptase sequences in treated Ugandans. In their longitudinal cohort, the HIV-1 infecting one patient (JLT05) acquired I135T/E138K during EFV-containing treatment without any other NNRTI resistance-associated mutations (GenBank: AY556834). Marconi *et al.* [28] performed genotypic resistance testing in patients who experienced virologic failure during their first antiretroviral therapy, and the HIV-1 in one patient (SW065) was found to have I135T/E138K after the failure of EFV-containing treatment (EU308076). In tipranavir clinical trials, the HIV-1s of seven cases who experienced NNRTI treatment failure harbored I135T/E138K (DQ880123, DQ880358, DQ879290, DQ880378, DQ877823, DQ878145, and DQ878874) [29]. These data indicate that I135T/E138K confers significant NNRTI resistance *in vivo* also, suggesting that HLA-B*51-restricted pressure may alter the mutation patterns of NNRTI resistance by inducing escape mutations.

Evidences for the interactions between CTL and drug resistance mutations are accumulating [30–34]. Considering that HIV-1 adapts to particular human HLA alleles and evolves among infected individuals, drug mutation patterns may be affected and altered in currently prevailing viruses. Analysis of drug resistance mutations and development of new antiretroviral agents against laboratory HIV-1 strains derived from isolates obtained decades ago may not always be a suitable strategy. The use of recently obtained clinical isolates may be critical and indispensable in some studies.

Acknowledgements

This work was supported in part by a Grant-in Aid for AIDS research from the Ministry of Health, Labor, and Welfare (H20-AIDS-002), and the Global Center of Excellence Program (Global Education and Research Center Aiming at the Control of AIDS) from the Ministry of Education, Science, Sports and Culture of Japan.

H.G. designed and executed the study, analyzed the data and wrote the manuscript. H.O. and H.S. performed computational analysis and wrote the manuscript. A.H. and T.H. executed the study and collected data. M.T. provided the hypothesis and participated in discussion and review. S.O. participated in discussion and review and supervised the study.

There are no conflicts of interest.

References

1. Carrington M, O'Brien SJ. **The influence of HLA genotype on AIDS.** *Annu Rev Med* 2003; **54**:535–551.
2. Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, Addo MM, *et al.* **Evolution and transmission of stable CTL escape mutations in HIV infection.** *Nature* 2001; **412**:334–338.
3. Tomiyama H, Sakaguchi T, Miwa K, Oka S, Iwamoto A, Kaneko Y, *et al.* **Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101.** *Hum Immunol* 1999; **60**:177–186.
4. Kawashima Y, Pfafferott K, Frater J, Matthews P, Payne R, Addo M, *et al.* **Adaptation of HIV-1 to human leukocyte antigen class I.** *Nature* 2009; **458**:641–645.
5. Leigh Brown AJ, Precious HM, Whitcomb JM, Wong JK, Quigg M, Huang W, *et al.* **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* 2000; **74**:10269–10273.
6. Gao Y, Paxinos E, Galovich J, Troyer R, Baird H, Abreha M, *et al.* **Characterization of a subtype D human immunodeficiency virus type 1 isolate that was obtained from an untreated individual and that is highly resistant to nonnucleoside reverse transcriptase inhibitors.** *J Virol* 2004; **78**:5390–5401.
7. Shafer RW, Schapiro JM. **HIV-1 drug resistance mutations: an updated framework for the second decade of HAART.** *AIDS Rev* 2008; **10**:67–84.
8. Ceccherini-Silberstein F, Svicher V, Sing T, Artese A, Santoro MM, Forbici F, *et al.* **Characterization and structural analysis of novel mutations in human immunodeficiency virus type 1 reverse transcriptase involved in the regulation of resistance to nonnucleoside inhibitors.** *J Virol* 2007; **81**:11507–11519.
9. Tossonian HK, Raffa JD, Grebely J, Viljoen M, Mead A, Khara M, *et al.* **Clinical implications of mutations at reverse transcriptase codon 135 on response to NNRTI-based therapy.** *Open Virol J* 2007; **1**:8–13.
10. Harrigan PR, Hertogos K, Verbiest W, Larder B, Yip B, Brumme ZL, *et al.* **Modest decreases in NNRTI susceptibility do not influence virological outcome in patients receiving initial NNRTI-containing triple therapy.** *Antiviral Ther* 2003; **8**:395–402.
11. Gatanaga H, Ibe S, Matsuda M, Yoshida S, Asagi T, Kondo M, *et al.* **Drug-resistant HIV-1 prevalence in patients newly diagnosed with HIV/AIDS in Japan.** *Antiviral Res* 2007; **75**:75–82.
12. Gatanaga H, Hachiya A, Kimura S, Oka S. **Mutations other than 103N in human immunodeficiency virus type 1 reverse transcriptase (RT) emerge from K103R polymorphism under nonnucleoside RT inhibitor pressure.** *Virology* 2006; **344**:354–362.
13. Lindberg J, Sigurdsson S, Lowgren S, Andersson HO, Sahlberg C, Noreen R, *et al.* **Structural basis for the inhibitory efficacy of efavirenz (DMP-266), MSC194 and PNU142721 towards the HIV-1 RT K103N mutant.** *Eur J Biochem* 2002; **269**:1670–1677.
14. Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, *et al.* **High resolution structures of HIV-1 RT from four RT-inhibitor complexes.** *Nat Struct Biol* 1995; **2**:293–302.
15. Das K, Clark AD Jr, Lewi PJ, Heeres J, De Jonge MR, Koymans LM, *et al.* **Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related nonnucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants.** *J Med Chem* 2004; **47**:2550–2560.
16. Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, *et al.* **A point-charged force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations.** *J Comput Chem* 2003; **24**:1999–2012.
17. Lee MC, Duan Y. **Distinguish protein decoys by using a scoring function based on a new AMBER force field, short molecular dynamics simulations, and the generalized born solvent model.** *Proteins* 2004; **55**:620–634.
18. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. **Development and testing of a general amber force field.** *J Comput Chem* 2004; 25:1157–1174.
19. Onufriev A, Bashford D, Case DA. **Exploring protein native states and large-scale conformational changes with a modified generalized born model.** *Proteins* 2004; **55**:383–394.
20. Johnson VA, Brun-Vezinet F, Clotet B, Gunthard HF, Kuritzkes DR, Pillay D, *et al.* **Update of the drug resistance mutations in HIV-1.** *Top HIV Med* 2008; **16**:138–145.

21. Balzarini J, Karlsson A, Perez-Perez MJ, Vrang L, Walbers J, Zhang H, *et al.* **HIV-1-specific reverse transcriptase inhibitors show differential activity against HIV-1 mutant strains containing different amino acid substitutions in the reverse transcriptase.** *Virology* 1993; **192**:246–253.
22. Balzarini J, Karlsson A, Sardana VV, Emini EA, Camarasa MJ, De Clercq E. **Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K)RT HIV-1 mutants or select for highly resistant (Y181C->C181I)RT HIV-1 mutants.** *Proc Natl Acad Sci U S A* 1004; **91**:6599–6603.
23. Pelemans H, Aertsen A, Van Laethem K, Vandamme AM, De Clercq E, Perez-Perez MJ, *et al.* **Site-directed mutagenesis of human immunodeficiency virus type 1 reverse transcriptase at amino acid position 138.** *Virology* 2001; **280**:97–106.
24. Shenderovich MD, Kagan RM, Heseltine PN, Ramnarayan K. **Structure-based phenotyping predicts HIV-1 protease inhibitor resistance.** *Protein Sci* 2003; **12**:1706–1718.
25. Stephens HA. **HIV-1 diversity versus HLA class I polymorphism.** *Trends Immunol* 2005; **26**:41–47.
26. Gifford RJ, de Oliveira T, Rambaut A, Pybus OG, Dunn D, Vandamme AM, *et al.* **Phylogenetic surveillance of viral genetic diversity and the evolving molecular epidemiology of human immunodeficiency virus type 1.** *J Virol* 2007; **81**:13050–13056.
27. Richard N, Juntilla M, Abraha A, Demers K, Paxinos E, Galovich J, *et al.* **High prevalence of antiretroviral resistance in treated Ugandans infected with nonsubtype B human immunodeficiency virus type 1.** *AIDS Res Hum Retroviruses* 2004; **20**:355–364.
28. Marconi VC, Sunpath H, Lu Z, Gordon M, Koranteng-Apeagyei K, Hampton J, *et al.* **Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa.** *Clin Infect Dis* 2008; **46**:1589–1597.
29. Baxter JD, Schapiro JM, Boucher CA, Hohlbrenner VM, Hall DB, Scherer JR, *et al.* **Genotypic changes in human immunodeficiency virus type 1 protease associated with reduced susceptibility and virologic response to the protease inhibitor tipranavir.** *J Virol* 2006; **80**:10794–10801.
30. Schmitt M, Harrer E, Goldwich A, Bauerle M, Graedner I, Kalden JR, *et al.* **Specific recognition of lamivudine-resistant HIV-1 by cytotoxic T lymphocytes.** *AIDS* 2000; **14**:653–658.
31. Samri A, Haas G, Duntze J, Bouley JM, Calvez V, Katlama C, *et al.* **Immunogenicity of mutations induced by nucleoside reverse transcriptase inhibitors for human immunodeficiency virus type 1-specific cytotoxic T cells.** *J Virol* 2000; **74**:9306–9312.
32. Mason RD, Bowmer MI, Howley CM, Gallant M, Myers JC, Grant MD. **Antiretroviral drug resistance mutations sustain or enhance CTL recognition of common HIV-1 Pol epitopes.** *J Immunol* 2004; **172**:7212–7219.
33. John M, Moore CB, James IR, Mallal SA. **Interactive selective pressures of HLA-restricted immune responses and antiretroviral drugs on HIV-1.** *Antivir Ther* 2005; **10**:551–555.
34. Mahnke L, Clifford D. **Cytotoxic T cell recognition of an HIV-1 reverse transcriptase variant peptide incorporating the K103N drug resistance mutation.** *AIDS Res Ther* 2006; **3**:21.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 12 (2010) 374–381

www.elsevier.com/locate/micinf

Original article

Comparison of CD4$^+$ T-cell subset distribution in chronically infected HIV$^+$ patients with various CD4 nadir counts

Keiko Sakai [a], Hiroyuki Gatanaga [b,c], Hiroshi Takata [a], Shinichi Oka [b,c], Masafumi Takiguchi [a,*]

[a] *Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto-shi, Kumamoto-ken 860-0811, Japan*
[b] *Division of Infectious Disease, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto-shi, Kumamoto-ken 860-0811, Japan*
[c] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*

Received 18 December 2009; accepted 23 January 2010
Available online 1 February 2010

Abstract

Infection with HIV-1 causes CD4$^+$ T-cell dysfunction, including unresponsiveness to antigenic stimuli. To understand the mechanism of virally induced T-cell dysfunction, we investigated changes occurred in functional CD4$^+$ T-cell subsets in the peripheral CD4$^+$ T-cell pool in chronically infected aviremic individuals treated with antiretroviral therapy. We phenotypically defined CD4$^+$ T-cell subsets by surface markers and determined the frequency of each subset by flow cytometry. A substantially low naïve and elevated effector subsets were observed in chronically infected patients with nadir CD4 counts <100 cells/μl. The skewed distribution persisted in these patients even after their CD4 counts increased, and the subset imbalance was still observed in all four subsets after years of successful antiretroviral therapy. They also showed a limited recovery of CD4$^+$ T-cell counts compared to those who maintained at least 250 CD4$^+$ T cells/μl after 3–11 years of successful treatment since CD4 nadir time points. The difference was pronounced in the absolute numbers of naïve and T_{EM} cells. Our results suggested a significant and prolonged impact of nadir CD4 counts on the balanced distribution of the functional CD4$^+$ T-cell subsets and may explain partially why antiretroviral therapy needs to be initiated while patients' CD4 counts remain relatively high.

Keywords: HIV-1; CD4 subset; Nadir CD4 count

1. Introduction

Human CD4$^+$ T lymphocytes are commonly divided into functionally distinct subsets. Two primary categories are cells that are not previously exposed to antigen (naïve) and those that are antigen-experienced (memory). The memory subpopulation is a heterogeneous pool and can be further divided into two broad subsets, central memory (T_{CM}) and effector memory (T_{EM}), based on their functional properties [1–4]. Each subset expresses a characteristic set of surface glycoproteins, which serves as a marker for their functional capacity. These phenotypic markers provide means to examine the prevalence and differentiation/activation state of CD4$^+$ T cells on a cell-by-cell basis, using flow cytometry [3,5–9]. Surface markers used to characterize CD4$^+$ T cells include CD45RA/RO, CCR7, CD27, and CD28 [3,5–7,10–13]. Most studies used either CD27 or CD28 in conjunction with CD45RA/RO and CCR7 to define CD4$^+$ T-cell subsets. However, we recently analyzed CD4$^+$ T cells in detail, using all four markers and characterized the function of each subset based on their ability to produce cytokine upon stimulation [7]. Our study and others showed that the majority of naïve CD4$^+$ T cells exhibited the CD45RA$^+$ CCR7$^+$ CD27$^+$ CD28$^+$ phenotype and sequentially lost CD45RA and CCR7 expression followed by CD27 down regulation as they progressed through stages of differentiation to become T_{CM} (CD45RA$^-$ CCR7$^+$ CD27$^+$ CD28$^+$), T_{EM} (CD45RA$^-$ CCR7$^-$ CD27$^{+/-}$ CD28$^+$), and effector cells (CD45RA$^-$ CCR7$^-$ CD27$^-$ CD28$^-$) [7,10].

Flow cytometric analyses have been performed to examine CD4$^+$ T-cell function in patients infected with human

* Corresponding author. Tel.: +81 96 373 6531; fax: +81 96 373 6532.
E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

1286-4579/$ - see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2010.01.013

immunodeficiency virus type 1 (HIV-1) [14–25]. Numerous studies reported the lack of antigen-specific proliferation and immune responsiveness of $CD4^+$ T cells in HIV^+ patients [24,26–38] and a correlation between prognosis and the CD4 counts at which antiretroviral therapy (ART) was initiated [39–41]. These studies indicated that the functional defects of $CD4^+$ T cells persisted in chronic patients, even though ART restored the number of $CD4^+$ T cells. However, the underlying mechanism of why the regeneration of $CD4^+$ T cells did not lead to the functional recovery of $CD4^+$ T cells is not well understood. To develop a better framework for delineating the mechanism of $CD4^+$ T-cell dysfunction in HIV^+ patients, we examined the homeostatic balance of peripheral $CD4^+$ T-cell subsets in individuals chronically infected with HIV-1. In this study, we phenotypically defined $CD4^+$ T cell into functionally distinct subsets with four surface markers and compared the frequency of each subset in infected patients with that in HIV-negative individuals, using flow cytometry. We also investigated the extent of immune imbalance among patients over time and searched factors that may predict the severity of imbalance.

2. Materials and methods

2.1. Subjects

The study was conducted in accordance with the Declaration of Helsinki and approved by the International Medical Center of Japan and the Kumamoto University Ethical Committee. HIV^+ blood samples were obtained from 34 chronically infected patients who enrolled at the AIDS Clinical Center, International Medical Center of Japan. All the patients were on antiretroviral therapy and aviremic at the time of sample collection. 18 out of 34 patients were selected for longitudinal analysis based on the availability of samples. In some patients, CD4 counts reached their nadir after the initiation of ART, presumably due to the emergence of drug resistant viruses.

2.2. Flow cytometric and statistical analysis of peripheral $CD4^+$ T cells

PBMCs were isolated from the samples by Ficoll–Paque PLUS (GE Healthcare) density gradient centrifugation and stained with the following fluorescently labeled monoclonal antibodies: allophycocyanin-conjugated (APC) CD4 (Dako-Cytomation), phycoerythrin (PE)-Texas Red-conjugated CD28 (Beckman Coulter), APC-Cy7-conjugated CD27 (BD Biosciences), Fluorescein isothiocyanate (FITC)-conjugated CD45RA (BD Biosciences), and PE-Cy7-conjugated CCR7 (BD Biosciences). Cells were first incubated with the antibody cocktail to the CD molecules on ice for 30 min. Subsequently, they were washed with FACS buffer (10% newborn calf serum in phosphate-buffered saline) and stained with CCR7 antibody for 30 min at room temperature. After washed with FACS buffer, cells were fixed with 1% paraformaldehyde for 20 min. Flow cytometric data were collected on a FACScanto II flow cytometer (BD Biosciences) immediately after staining and analyzed using Flowjo software (Tree Star, Inc). For statistical analysis, Student's t-test was used to determine p-value.

3. Results

3.1. Skewed distribution of functional $CD4^+$ T-cell subsets in chronic HIV^+ patients

To understand the mechanism of virally induced $CD4^+$ T-cell dysfunction, we examined the balance of functional populations in peripheral $CD4^+$ T-cells from patients chronically infected with HIV-1 (Supplementary Table 1). Using flow cytometry, we phenotypically characterized $CD4^+$ T cells based on a set of four surface markers (Fig. 1A). $CD4^+$ T cells from 15 healthy individuals were predominantly divided into five subpopulations (Fig. 1B). An average of 55.8% of cells showed the $CD45RA^+$ $CCR7^+$ $CD27^+$ $CD28^+$ naïve phenotype, 16.0% were $CD45RA^-$ $CCR7^+$ $CD27^+$ $CD28^+$ T_{CM} cells, and 13.0% were in the $CD45RA^-$ $CCR7^-$ $CD27^+$ $CD28^+$ population (Fig. 1B), a subset reported to contain primarily Th0 and Th1 T_{EM} cells [5,7]. Another 7.3% belonged to the $CD45RA^-$ $CCR7^-$ $CD27^-$ $CD28^+$ subtype, a heterogeneous population of Th0, Th1, and Th2 T_{EM} cells as well as a small number of Th1 and Th2 effector Tells [7]. A low frequency of cells, approximately 2.5%, displayed the $CD45RA^-$ $CCR7^-$ $CD27^-$ $CD28^-$ phenotype, which mainly consists of Th1 effector [7]. We performed the same analysis on peripheral $CD4^+$ T cells from 34 patients chronically infected with HIV-1 (Supplementary Table 1). All of the patients were on ART and did not have detectable viral loads. In contrast to uninfected individuals, only 31.0% of peripheral $CD4^+$ T cells belonged to the naïve subset in the patients ($p < 0.0001$, Fig. 1B). The $CD4^+$ T-cell pools were skewed away from the naïve subset and, instead, towards T_{CM} (29.4%, $p = 0.0001$) and effector (9.9%, $p < 0.001$, Fig. 1B). A considerable reduction was also observed in patients' T_{EM} (10.1%, $p = 0.005$). These results suggested that the homeostatic balance of peripheral $CD4^+$ T subsets was disturbed in chronically infected patients even after years of ART.

3.2. The impact of nadir CD4 counts on the distribution of the functional $CD4^+$ T-cell subsets

Frequencies of the naïve $CD4^+$ T-cell subset varied considerably among patients, ranging from 3.6% to 71.2% (Fig. 1B). In 25 out of 34 patients, the frequencies were more than two standard deviations below the mean of the uninfected naïve subset ($HIV^+ < 2SD$, shaded circles in Fig. 1B). For the rest of the HIV^+ individuals, the mean percentage of the naïve subset was 57.9% (HIV^+ w/i 2SD, open circles in Fig 1B), approximately the same as that of uninfected group. Compared to the w/i 2SD group, the $HIV^+ < 2SD$ group showed a significantly smaller naïve subset and elevated T_{CM} and effector subsets. To identify factors affecting the phenotypic difference between the two HIV^+ groups, we compared their clinical data. The $HIV^+ < 2SD$ and w/i 2SD groups did not have statistically significant difference in CD4 counts at the time of sample