が、一旦は単独での耐性度と増殖能の低さのため割合が減少するものの、高濃度存在下で、V3の耐性変異と同時に存在することで増殖可能になったことが示唆された。

次に、PNGS挿入で起こった複製能力の低下を補っているアミノ酸変異を同定するために、我々は、PNGS挿入にC2やV3部の変異を個々に、点突然変異法を用いて、アミノ酸変異を導入し、感染性クローンウイルスを作製した。PNGSとF317Lを同時に持つウイルス (HX-BaL-PNGS/L) は、PNGS単独のものに比べ明らかに増殖能が良かった。PNGSとT240Sの変異を持つウイルス (HX-BaL-PNGS/S) の複製能は、PNGS単独のウイルスよりも同等もしくは、やや低下傾向であった。PNGSとV3-tip変異を持つウイルス (HX-BaL-PNGS/K) は、複製能力の低下が著しかった。しかし、このウイルスの複製能力の低下は、C2変異 (T240S)やV3stem (F317L)の変異を獲得することで改善された(HX-BaL-PNGS/SKL)。

一方、耐性誘導スタート時のHIV-1_{BaL} gp120のシークエンスの結果、T240S (67.2 %) とF317L (29.5 %) は両方とも既に存在していたことがわかった。抗体存在下ではT240Sの割合は、7 passage以降100% になり14 passage目まではそれを持続した。しかし、抗体非存在下で培養を続けたコントロールウイルスでは、C2のT240Sの割合は9 passage目で一旦0% になり、最終16パッセージ目で、スタート時と同等の存在比に戻った (61.5 %)。一方、F317Lは、パッセージコントロール中では、徐々にその割合を増加させ、16パッセージ目で90% を越えた。これらの結果から、C2のT240Sは、PM1/CCR5細胞への適応にはそれほど関与しておらず、KD-247耐性で誘導されたV3変化で引き起こされた複製能力の低下を補うために必要であると考えられ、クローンウイルスでの結果もこれを支持していた。また、V3のF317LはPM1/CCR5細胞への適応のためだけの変異ではなく、V2のPNGS挿入変異による複製能力の低下を補う役割も兼ねていることが考えられた。

D.考察

HIV-1感染初期に、液性免疫からの逃避に関連したHIV-1の進化が見られることが報告されている。しかしながら、これらの報告は、臨床検体から示唆されたものであり、多様性に富んだウイルスと中和抗体が含まれているため、個々の変異やPNGSの挿入が中和逃避とどのように関係しているかは明らかではなかった。今回の研究で我々は、臨床応用が期待されている抗-V3単クローン抗体の耐性誘導実験でHIV-1_{BaL}を用いて中和抵抗性ウイルスを分離し、試験管内でその変異の機能的役割について詳細に検討した。

KD-247低濃度存在下において、C2とV3領域に3つのアミノ酸変化 (T240S/I283T/T319A) が見られた。これらのウイルスは、すでに耐性誘導前に見られたウイルスの1つから増殖したものであった。また、高濃度存在下において、C2 (T240S) とV3 (R315K/F317L) に加えてV2領域に糖鎖付加したウイルスが見られるようになった。V2に糖鎖を持つウイルスは、KD-247濃度を上げていくと徐々にその割合を増やし、最終誘導濃度 (2000 μg/ml) において約90%以上を占めるようになった。シュードタイプおよび感染性ウイルスを用いた中和実験の結果、V2にPNGSの挿入のみを持つHIV-1_{BaL} (BaL-PNGS) は、KD-247に対し感受性を維持していたが、V3の変異とV2の糖鎖挿入を両方持つウイルスのKD-247に対してV3変異単独よりも耐性に傾くことが確認された。増殖動態の比較の結果、R315KやV2のPNGS挿入は、単独では増殖能の著しい低下を招き、PNGS挿入だけでは耐性化も非常に弱かった。しかし、これらの耐性変異が同時に存在しても、C2 (240S) やV3 (F317L) 変異を伴うことにより、野生株と同等の増殖能を示すことがわかった。これらの結果から、ウイルスの複製動態に影響を与える変異や挿入が、他の部位の変異を獲得することで野生株と同等のウイルス複製を持つようになり、ウイルスのレパートリーの中で主要なウイルスになり得たことが示唆された。

E. 結論

我々は、抗V3単クローン抗体 KD-247を用いて高度耐性ウイルスを誘導した。これらの耐性ウイルスは、エピトープ部の変異の他にV2にPNGS挿入が見られた。これらの変異は、強いウイルス複製負荷のかかる変異であったが、その他の部位の変異を獲得することによって、複製能力を野生株とほぼ同等に改善させることが出来るようになることがわかった。つまり、中和逃避に関係した変異は、中和抗体の耐性だけでなく、ウイルス増殖を促進させるような変異も併せ持っていることが示唆された。今回得られた結果は、中和抗体耐性の進化の過程で獲得したそれぞれの変異の相関関係を示唆するものであり、HIV-1に対する有効なワクチン開発に重要な意味を持つと考えられる

F. 健康危険情報:なし

G. 研究発表

1.論文発表

1. Yamada, Y., Ochiai, C., Yoshimura, K., Tanaka, T., Ohashi, N., Narumi T., Nomura, W., Harada, S., Matsushita, S., Tamamura, H. CD4 mimics targeting the mechanism of HIV entry. Bioorganic & Medicinal Chemistry Letters 20: 354–358, 2010.
2. Makiko Hatada, Kazuhisa Yoshimura, Shigeyoshi Harada, Yoko Kawanami, Junji Shibata and Shuzo Matsushita : HIV-1 evasion of a neutralizing anti-V3 antibody involves acquisition of a potential glycosylation site in V2. J. Gen. Virol. 2010 (in press)

2.学会発表

(国内学会)

1. 松下修三:HIV-1 に対する中和単クローン抗体の治療応用に向けた基礎研究. 第9回日本蛋白質化学学会 2009.5.20-22. 熊本.
2. 畑田万紀子、吉村和久、原田惠嘉、松下修三：抗 HIV-1V3 抗体からの逃避過程で挿入される V2 領域の糖鎖が保存されるメカニズム-HIV-1 の進化における耐性度と増殖能のバランスに関する考察- 第 57 回日本ウイルス学会学術集会. 2009.10.25-27 東京.
3. 松下修三：エンベロープの進化と中和抗体. シンポジウム HIV 細胞侵入とその防御機構. 第 23 回に本エイズ学会学術集会・総会. 2009.11.26-28 名古屋.
4. 吉村和久: ケモカインレセプター阻害剤の臨床的研究～臨床分離株を用いたマラビロック耐性誘導～. 第 23 回に本エイズ学会学術集会・総会. 2009.11.26-28 名古屋.
5. 原田 惠嘉, 吉村和久, 松下修三： 最近分離した 7 種の臨床 HIV-1 株を用いた in vitro ラルテグラビル耐性ウイルス誘導. 第 23 回に本エイズ学会学術集会・総会. 2009.11.26-28 名古屋.
6. 石川哲也, 畑田万紀子, 原田 惠嘉, 吉村和久, 松下修三: 実験室 HIV-1 R5 株を用いた in vitro CCR5 阻害薬 (maraviroc) 耐性ウイルス誘導の試み第 23 回に本エイズ学会学術集会・総会. 2009.11.26-28 名古屋.

(国際学会)

1. Matsushita S, Narahara C, Morizono M, Nishida Y, Honda –Shibata A, Harada S., Yoshimura, K,.: Polyclonal antibody response against gp120 including antibodies to V3, CD4bs and CD4i epitopes account or broad neutralization. 5th Conference on HIV Pathogenesis, Treatment and Prevention. 2009.7.19-22, Cape Town, South Africa.
2. Harada S, Yoshimura K., and Matsushita S.: Generation of an integrase inhibitor raltegravir resistant variants using recent primary isolates, X4, R5 and dual/mix HIV-1. 10thKumamoto AIDS Seminar GCOE Joint International Symposium. 2009.9.28-29, Kumamoto
3. Yoshimura K., Harada S, Hatada M. and Matsushita S.: In vitro induction of HIV-1 resistant to a CCR5 antagonist maraviroc. 10th Kumamoto AIDS Seminar GCOE Joint International Symposium. 2009.9.28-29, Kumamoto
4. Ishikawa T., Yoshimura K., Hatada M. Harada S, and Matsushita S.: Mutations in gp120 of R5 HIV-1 laboratory isolate induced by the in vitro selection of maraviroc confer highly sensitive to anti-V3 monoclonal antibody. 10th Kumamoto AIDS Seminar GCOE Joint International Symposium. 2009.9.28-29, Kumamoto
5. Hatada M. Yoshimura K., Harada S, and Matsushita S.:Mechanism of maintaining a glycan-insertion in HIV-1 gp120 V2 region under pressure of a potent neutralizing antibody in vitro. 10th Kumamoto AIDS Seminar GCOE Joint International Symposium. 2009.9.28-29, Kumamoto.
6. Matsushita S. Narahara C, Nishida Y, Honda A., Harada S., Yoshimura K.: Mechanism of maintaining a glycan-insertion in HIV-1 gp120 V2 region under pressure of a potent neutralizing antibody in vitro. 10th Kumamoto AIDS Seminar GCOE Joint International Symposium. 2009.9.28-29, Kumamoto.
7. Yoshimura K, Matsushita S: In vitro induction of HIV-1 resistant to a CCR5. Kumamoto AIDS Seminar GCOE Joint International Symposium- Satellite Symposium. 2009.9.30, Aso, Kumamoto.
8. Matsushita S: Accumulation of multiple

functional mutations in HIV-1 gp120 is involved in the development of neutralization escape under pressure of neutralizing antibody in vitro. Kumamoto AIDS Seminar GCOE Joint International Symposium- Satellite Symposium, 2009.9.30, Aso, Kumamoto.

9. Narahara C, Hatada M, Harada S., Yoshimura, K,. Matsushita S, : A primary R5 isolate undergoes different escape pathway during in vitro selection with low or high concentration of an anti-V3 monoclonal antibody. AIDS Vaccine 2009, 2009.10.19-22, Paris.

H.知的財産権の出願・登録状況（予定を含む）；なし

Ⅲ. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

研究分担者　滝口雅文

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Murakoshi, H., Kitano, M., Akahoshi, T., Kawashima, Y., Dohki, S., Oka, S., and Takiguchi, M.	Identification and characterization of 2 HIV-1 Gag immunodominant epitopes restricted by Asian HLA allele HLA-B*4801.	Hum. Immunol.	70	170-174	2009
Kawashima, Y., Pfafferott, K., Frater, J., Matthews, P., Payne, R., Addo, M., Gatanaga, H., Fujiwara, M., Hachiya A., Koizumi, H., Kuse, N., Oka, S., 他29名, Takiguchi, M.*, and Goulder, P.* (*equally contributed).	Adaptation of HIV-1 to HLA I.	Nature	458	641-645	2009
Koizumi, H., Iwatani, T., Tanuma, J., Fujiwara, M., Izumi, T., Oka, S., and Takiguchi, M.	Escape mutation selected by Gag28-36-specific cytotoxic T cells in HLA-A*2402-positive HIV-1-infected donors.	Microbes Infect.	11	198-204	2009
Zheng, N., Fujiwara, M., Ueno, T., Oka, S., and Takiguchi, M.	Strong ability of Nef-specific CD4+ cytotoxic T cells to suppress HIV-1 replication in HIV-1-infected CD4+ T cells and macrophages.	J. Virol.	83	7668 -7677	2009
Motozono, C., Yanaka, S., Tsumoto, K., Takiguchi, M., and Ueno, T.	Impacts of intrinsic cooperative thermodynamics of peptide-MHC complexes on antiviral activity of HIV-specific cytotoxic T lymphocytes.	J. Immunol	182	5528 -5536	2009
Hashimoto, M., Kitano, M., Honda, K., Koizumi, H., Dohki, S., Oka, S., and Takiguchi, M.	Selection of escape mutation by Pol154-162-specific cytotoxic T cells among chronically HIV-1-infected HLA-B*5401-positive individuals.	Hum. Immunol.	71	123-127	2010
Gatanaga, H., Ode, H., Atsuko Hachiya, A., Hayashida, T., Sato, H., Takiguchi, M., and Oka, S.	Impact of human leukocyte antigen-B*51-restricted cytotoxic T-lymphocyte pressure on mutation patterns of nonnucleoside reverse transcriptase inhibitor resistance.	AIDS	in press		
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.	Microbes Infect.	in press		
Koizumi, H., Hashimoto, M., Fujiwara, M., Murakoshi, H., Chikata, T., Borghan M. A., 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.	in press.		

研究成果の刊行に関する一覧表

研究分担者　潟永 博之

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Kawashima Y, Pfafferott K, Frater J, Matthews P, Payne R, Addo M, Gatanaga H, Fujiwara M, Hachiya A, Koizumi H, Kuse N, Oka S, Duda A, Prendergast A, Crawford H, Leslie A, Brumme Z, Brumme C, Allen T, Brander C, Kaslow R, Tang J, Hunter E, Allen S, Mulenga J, Branch S, Roach T, John M, Mallal S, Ogwu A, Shapiro R, Prado JG, Fidler S, Weber J, Pybus OG, Klenerman P, Ndung'u T, Phillips R, Heckerman D, Harrigan PR, Walker BD, Takiguchi M, Goulder P.	Adaptation of HIV-1 to human leukocyte antigen class I.	Nature	458	641-645	2009
Hachiya A, Shimane K, Sarafianos SG, Kodama EN, Sakagami Y, Negishi F, Koizumi H, Gatanaga H, Matsuoka M, Takiguchi M, Oka S.	Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients.	Antiviral Res	82	115-121	2009
Davaalkham J, Unenchimeg P, Baigalmaa Ch, Oyunbileg B, Tsuchiya K, Hachiya A, Gatanaga H, Nyamkhuu D, Oka S.	High-risk status of HIV-1 infection in the very low epidemic country, Mongolia, 2007.	Int. J. STD AIDS	20	391-394	2009
Honda H, Gatanaga H, Matsumura J, Kamimura M, Goto K, Tsukada K, Honda M, Teruya K, Kikuchi Y, Oka S.	Favorable use of non-boosted fosamprenavir in patients treated with warfarin.	Int. J. STD AIDS	20	441	2009
Watanabe T, Yasuoka A, Honda H, Tanuma J, Yazaki H, Tsukada K, Honda M, Teruya K, Gatanaga H, Kikuchi Y, Oka S.	Serum (1→3) β-D-glucan as a non-invasive useful adjunctive diagnostic marker for Pneumocystis pneumonia in patients with human immunodeficiency virus.	Clin. Infect. Dis.	49	1128-1131	2009
Tsukada K, Teruya K, Tasato D, Gatanaga H, Kikuchi Y, Oka S. Raltegravir-associated perihepatitis and peritonitis: a single case report.	Raltegravir-associated perihepatitis and peritonitis: a single case report.	AIDS	24	160-161	2010

研究成果の刊行に関する一覧表

研究分担者　満屋裕明

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakata, H., Kruhlak, M., Kamata, W., Ogata-Aoki, H., Li, J., Maeda, K., Ghosh, AK., and Mitsuya, H.	Effects of CCR5 Inhibitors on the Dynamics of CCR5 and CC-chemokine-CCR5 interactions. *A*	Antiviral Therapy (in press)			2010
Ghosh, AK, Gemma S, Simoni E, Baldridge A, Walters, DE, Ide K, Tojo Y, Koh Y, Amano M, and Mitsuya H.	Synthesis and biological evaluation of novel allophenyl- norstatine-based HIV-1 protease inhibitors incorporating high affinity P2-ligands.	Bioorg. Med. Chem .Lett.	20	1241-6	2010
Ghosh AK, Leshchenko-Yashchuk S, Anderson DD, Baldridge A, Noetzel M, Miller HB, Tie Y, Wang YF, Koh Y, Weber IT, Mitsuya H.	Design of HIV-1 protease inhibitors with pyrrolidinones and oxazolidinones as novel P1'- ligands to enhance backbone-binding interactions with protease: synthesis, biological evaluation, and protein-ligand X-ray studies.	J. Med. Chem.	52	3902-14	2009
Fujimoto H, Higuchi M, Watanabe H, Koh Y, Ghosh AK, Mitsuya H, Tanoue N, Hamada A, and Saito H.	P-glycoprotein mediates efflux transport of darunavir in human intestinal Caco-2 and ABCB1 gene-transfected renal LLC-PK1 cell lines.	Biol. Pharm. Bull.	32	1588-93	2009
Ghosh AK, Sarang Kulkarni S, Anderson DD, Hong L, Baldridge A, Wang Y-F, Chumanevich AA, Kovalevsky AY, Tojo Y, Koh Y, Tang J, Weber IT, and Mitsuya H.	Design, synthesis, protein- ligand X-ray structures and biological evaluation of a series of novel macrocyclic HIV-1 protease inhibitors to combat drug-resistance.	J. Med. Chem.	52	7689-705	2009
Hattori S, Ide K, Nakata H, Harada H, Suzu S, Ashida N, Kohgo S, Hayakawa H, Mitsuya H, and Okada S.	Potent activity of a nucleoside reverse transcriptase inhibitor,4'-ethynyl-2-fluoro-2'-deoxyadenosine, against HIV-1 infection in Hu-PBMC-NOD/ SCID/JAK3null (NOJ) mouse model.	Antimicrob. Agents Chemother.	53	3887-93	2009
Das D, Koh Y, Tojo Y, Ghosh AK, and Mitsuya H.	Prediction of Potency of Protease Inhibitors Using Free Energy Simulations with Polarizable Quantum Mechanics-Based Ligand Charges and a Hybrid Water Model.	J. Chem. Inf. Model	49	2851-62	2009
Michailidis E, Bruno Marchand B, Ei-Ichi Kodama E-I, Kamlendra Singh K, Matsuoka M, Ashida N, Kirby K, Ryan EM, Sawani AM, 1, Eva Nagy E, Mitsuya H, Parniak MP, and Sarafianos SG.	Mechanism of inhibition of HIV-1 reverse transcriptase by 4'-ethynyl-2-fluoro-deoxyadenosine triphosphate, a translocation defective reverse transcriptase inhibitor.	J. Biol. Chem.	284	35681-91	2009
Aoki M, Venzon DJ, Koh Y, Aoki-Ogata H, Miyakawa T, Yoshimura K, Maeda K, Mitsuya H.	Non-cleavage Site Gag Mutations in Amprenavir-resistant HIV-1 Predispose HIV-1 to Rapid Acquisition of Amprenavir Resistance But Delays Development of Resistance to Other Protease Inhibitors.	J Virol.	83	3059-68	2009
Koh Y, Das D, Leschenko S, Nakata H, Ogata-Aoki H, Amano M, Nakayama M, Ghosh AK, Mitsuya H.	GRL-02031: A Novel Nonpeptidic Protease Inhibitor (PI) Containing A Stereochemically Defined Fused Cyclopentanyl- tetrahydrofuran (Cp-THF) Potent Against Multi-PI-Resistant HIV-1 In Vitro.	Antimicrob. Agents Chemother	53	997-1006	2009

別紙４

研究成果の刊行に関する一覧表

研究分担者　馬場 昌範

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Masanori Baba	Entry inhibitors of human immuno-deficiency virus	Robert L. LaFemina	Antiviral Research: Strategy in Antiviral Drug Discovery	ASM Press	Washington, DC	2009	19-32

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Minyi Shi, Xin Wang, Mika Okamoto, Sonshin Takao, Masanori Baba	Inhibition of porcine endogenous retrovirus (PERV) replication by HIV-1 gene expression inhibitors	Antiviral Research	83 (2)	201-204	2009

別紙４

研究成果の刊行に関する一覧表

研究分担者　松岡　雅雄

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Izumi K, Kodama E, Shimura K, Sakagami Y, Watanabe K, Ito S, Watabe T, Terakawa Y, Nishikawa H, Sarafianos S. G, Kitaura K, Oishi S, Fujii N, Matsuoka M.	Design of peptide-based inhibitors of HIV-1 strains resistant to T-20	J. Biol. Chem.	284(8)	4914-4920	2009
Ueno M, Kodama E.N, Shimura, K, Sakurai Y, Kajiwara K, Sakagami, Y, Oishi S, Fujii N, Matsuoka M.	Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide.	Antiviral Res.	82(1)	67-72	2009
Naito T, Izumi K, Kodama E, Sakagami Y, Kajiwara K, Nishikawa H, Watanabe K, Sarafianos S.G, Oishi S, Fujii N, Matsuoka M.	SC29EK, a peptide fusion inhibitor with enhanced α-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide.	Antimicrob. Agents Chemother.	53(3)	1013-1018	2009

別紙４

研究成果の刊行に関する一覧表

研究分担者　松下修三

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Yamada, Y., Ochiai, C., Yoshimura, K., Tanaka, T., Ohashi, N., Narumi T., Nomura, W., Harada, S., Matsushita, S., Tamamura, H.	CD4 mimics targeting the mechanism of HIV entry	Bioorganic & Medicinal Chemistry Letters	20	354–358	2010
Hatada, M., Yoshimura, K., Harada, S., Kawanami, Y., Shibata, J., Matsushita S.	HIV-1 evasion of a neutralizing anti-V3 antibody involves acquisition of a potential glycosylation site in V2.	J. Gen. Virol	in press		2010

Ⅳ. 研究成果の刊行物・別刷

Human Immunology 70 (2009) 170-174

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Brief communication

Identification and characterization of 2 HIV-1 Gag immunodominant epitopes restricted by Asian HLA allele HLA-B*4801

Hayato Murakoshi [a], Mitsutaka Kitano [a], Tomohiro Akahoshi [a], Yuka Kawashima [a], Sachi Dohki [a], Shinichi Oka [b,c], Masafumi Takiguchi [a,*]

[a] *Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan*
[b] *Division of Infectious Disease, Center for AIDS Research, Kumamoto University, Kumamoto, Japan*
[c] *AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan*

ARTICLE INFO

Article history:
Received 22 October 2008
Accepted 18 December 2008
Available online 22 January 2009

Keywords:
HIV-1
Cytotoxic T lymphocytes
HLA-B*4801
Epitopes

ABSTRACT

HLA-B*4801 is frequently found in Asian populations but rarely in Caucasian or African populations. Although HLA-B*4801-restricted human immunodeficiency virus-1 (HIV-1) epitopes would be useful for acquired immune deficiency syndrome (AIDS) vaccine development in Asia, they have not been reported so far. In the present study, we sought to identify HLA-B*4801-restricted HIV-1 epitopes by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef as well as 8- to11-mer truncated peptides, and thereby identified two HLA-B*4801-restricted Gag epitopes. These epitope-specific $CD8^+$ T cells strongly responded to HIV-1-infected cells expressing HLA-B*4801, confirming that these Gag epitopes were endogenously presented by HLA-B*4801. These epitope-specific $CD8^+$ T cells were elicited in five of the seven tested chronically HIV-1-infected individuals with HLA-B*4801, suggesting them to be immunodominant epitopes. These epitopes will be useful for the studies of AIDS immunopathogenesis and the development of an HIV-1 vaccine in Asia.

© 2009 Society. Published by Elsevier Inc. All rights reserved.

1. Introduction

In human immunodeficiency virus type-1 (HIV-1) infection, cytotoxic T lymphocytes (CTLs) are elicited to control HIV-1 replication. HIV-1-specific CTLs strongly respond to HIV-1-infected cells in long-term non-progressors and slow progressors [1,2]. When monkeys whose CTLs are deleted *in vivo* are infected with simian immunodeficiency virus (SIV), they fail to control the virus [3,4]. Thus, HIV-1-specific or SIV-specific CTLs play an important role in the control of HIV-1 or SIV infection. On the other hand, HIV-1-specific CTLs cannot completely eradicate HIV-1 from infected individuals because HIV-1 escapes the host immune system. There are several proposed mechanisms that would allow HIV-1-infected cells to avoid being killed by HIV-1-specific $CD8^+$ T cells [5–10]. A mutation within CTL epitopes and the flanking region of them is one of the mechanisms for CTL escape [5,6]. Therefore, identification and characterization of HIV-1 CTL epitopes are very important for HIV-1 vaccine development.

To identify epitopes, we previously used the strategy of reverse immunogenetics based on the motif of HLA class I-binding peptides [11–13]. However, some epitopes may not be identified by this method in cases in which the epitopes are inconsistent with the motif of HLA class I-binding peptide [14,15]. The use of overlapping peptides is another useful method for identification of CTL epitopes [16–18]. This method has the advantage of identifying epitopes that are inconsistent with HLA class I-binding motifs. We recently identified HLA-B*5401-restricted HIV-1-specific CTL epitopes by using such overlapping peptides [19].

HLA-B48 is found in Asian populations but rarely in Caucasian or African populations [20–22]. The phenotypic frequencies of HLA-B*48 in Japanese, Chinese, Korean, Mogolian, and Thai populations are 6.4%, 3.8%, 8.0%, 9.4%, and 2.2%, respectively [20,23]. HLA-B*4801 is the only genotype of this allele in Japan [24]. Therefore, the identification of HLA-B*4801-restricted HIV-1 epitopes is important for studies of acquired immune deficiency syndrome (AIDS) immunopathogenesis and AIDS vaccine development in Japan and other Asian countries. So far, no HLA-B*4801-restricted HIV-1 epitopes have been reported.

In the present study, we used 17-mer overlapping peptides spanning Pol, Gag, and Nef to identify HLA-B*4801-restricted HIV-1 epitopes. We investigated the recognition of HIV-1-infected HLA-B*4801$^+$ cells by epitope-specific CTLs to clarify whether the identified epitopes were naturally occurring peptides. The induction of these epitope-specific $CD8^+$ T cells in chronically HIV-1-infected HLA-B*4801$^+$ individuals was further investigated to clarify the immunodominancy of these epitopes.

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

0198-8859/09/$32.00 - see front matter © 2009 Society. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.humimm.2008.12.011

2. Subjects and methods

2.1. Patients

Blood samples were obtained from HIV-1–seropositive Japanese individuals carrying HLA-B*4801. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

2.2. Cells

Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from peripheral blood mononuclear cells (PBMC) of laboratory volunteers and an HIV-1–seropositive individual as described previously [19]. C1R cells expressing HLA-B*4801 (C1R-B*4801) were generated by transfecting C1R cells with the HLA-B*4801 gene. For generation of .221 cells expressing CD4 (.221-CD4), .221 cells were transfected with the CD4 gene. Such cells expressing HLA-B*4801 (.221-CD4-B*4801) were subsequently generated by transfecting .221-CD4 cells with the HLA-B*4801 gene.

2.3. Synthetic peptides

We designed a panel of 281 overlapping peptides consisting of 17 amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B sequences. Each 17-mer peptide was overlapped by at least 11 amino acids. The 281 peptides were synthesized by using an automated multiple peptide synthesizer. All peptides were purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.4. Induction of peptide-specific T cells

The peptide-specific T cells were induced from PBMCs of HIV-1-seropositive individuals carrying HLA-B*4801. PBMCs were cultured with each peptide (1 μmol/l) in culture medium (RPMI-1640 containing 10% fetal calf serum (FCS) and 200 U/ml interleukin-2). Two weeks later, they were used in intracellular interferon (IFN)–γ staining assays.

2.5. Infection of .221-CD4-B*4801 cells with HIV-1

HIV-1 clones, NL-432, were produced as described previously [25,26]. The .221-CD4- B*4801 or .221-CD4 cells were incubated with HIV-1 clones for 4 days at 37°C. The cells were then harvested to determine the percentage of HIV-1–infected cells. They were fixed with 4% paraformaldehyde and then permeabilized with PBS containing 10% FCS and 0.1% saponin (permeabilizing buffer). Thereafter the cells were stained with fluorescein isothiocyanate (FITC)- conjugated anti–HIV-1 p24 MAb KC-57 (Beckman Coulter, Miami, FL). The percentage of cells positive for intracellular HIV-1 p24 was determined by flow cytometry.

2.6. Intracellular IFN-γ staining assay

After B-LCL, C1R-B*4801, or C1R cells had been incubated for 60 minutes with each peptide (1 μmol/l), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed, HIV-1-infected .221-CD4- B*4801, or .221-CD4 cells (2 $\times$ 10^5 cells per well) and the cultured PBMCs (1 $\times$ 10^5 cells per well) were added to a 96-well round-bottomed plate. Subsequently, Brefeldin A (10 μg/ml) was added, and these cells were incubated for 6 hours. After the cells had been stained with anti-CD8 mAb (DAKO, Glostrup, Denmark), they were fixed with 4% paraformaldehyde and then permeabilized with the permeabilizing buffer. Thereafter the cells were stained with anti–IFN-γ mAb (BD Bioscience, CA). The percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by flow cytometry.

3. Results

3.1. Identification of optimal epitope peptides

We previously showed that 8 Gag, 8 Pol, and 4 Nef 17-mer overlapping peptides could elicit the peptide-specific $CD8^+$ T cells among PBMCs from a chronically HIV-1–infected individual KI-119 carrying HLA-A*0206/A*0206 and HLA-B*5401/B*4801 [19]. To determine HLA restriction of these T-cell responses, we investigated the ability of the peptide-stimulated bulk cultured cells to produce IFN-γ in response to a panel of B-LCLs sharing one HLA class I allele with KI-119. The $CD8^+$ T cells in the bulk cultures produced IFN-γ only after stimulation with autologous or HLA-B*4801–positive B-LCLs (U-37) pre-pulsed with five (Gag37-53, Gag43-59, Gag313-329, Gag361-377, and Gag421-437) of the 20 17-mer peptides. The Gag44-53 peptide-specific $CD8^+$ T cells could recognize the peptide-pulsed $B*4801^+$ cells but not HIV-1–infected ones (data not shown), although both Gag37-53 and Gag43-59 17-mer peptides include this 10-mer peptide. The Gag361-377–specific $CD8^+$ T cell response was restricted by HLA-Cw*0801 (data not shown). Therefore, these results suggest that only Gag313-329 and Gag421-437 peptides include HLA-B*4801–restricted epitopes (Fig. 1A).

To identify the optimal epitope recognized by $CD8^+$ T cells specific for these two peptides, we first designed 11-mer peptides that overlapped 9 amino acids in the sequence of the 17-mer peptides and then investigated whether the 17-mer peptide-induced $CD8^+$ T cells could recognize these 11-mer peptides. The Gag313-329 (VKNWMTETLLVQNANPD)-induced $CD8^+$ T cells could not recognize any of these 11-mer overlapping peptides (Fig. 1B). Since HLA-B*4801-binding peptides have 2 anchor residues, Lys or Gln at position 2 and Leu at the C-terminus [27], 2K and 9L or 10L in the Gag313-329 peptide would be expected to serve as an anchor for HLA-B*4801. Therefore, to identify the optimal peptide, we generated 2 truncated peptides, Gag313-322 (VKNWMTETLL) and Gag313-321 (VKNWMTETL), and investigated whether Gag313-329–induced $CD8^+$ T cells could recognize these peptides. They recognized Gag313-321 but not Gag313-322 peptide (Fig. 1C), suggesting that L at the C-terminus of Gag313-322 affected the binding to HLA-B*4801 or the recognition by the T cells. To investigate whether L at the C-terminus of Gag313-321 was necessary for the specific CTL response, we generated truncated Gag313-320 (VKNWMTET). Gag313-329–induced $CD8^+$ T cells did not recognize the Gag313-320 peptide (data not shown). These results confirmed the Gag313-321 peptide to be an optimal epitope.

On the other hand, Gag421-437 (HQMKDCTERQANFLGKI)–induced $CD8^+$ T cells recognized Gag427-437 (TERQANFLGKI) peptide but not the three other 11-mers (Fig. 1D). Because Gln is also an anchor at position 2 (P2) for HLA-B*4801 [27], 4Q in this 11-mer peptide would be expected to be the P2 anchor for HLA-B*4801. We therefore generated three truncated peptides, Gag428-437 (ERQANFLGKI), Gag429-437 (RQANFLGKI), and Gag429-436 (RQANFLGK). Gag421-437–induced $CD8^+$ T cells recognized both Gag428-437 and Gag429-437 but not Gag429-436 (Fig. 1E). When we assayed the responsivenesses of the specific CTLs to Gag429-437 or Gag428-437 at various concentrations, they recognized lower concentrations of Gag429-437 peptides but only higher concentrations of Gag428-437 (Fig. 1F). These findings indicate that Gag429-437 is the optimal epitope.

3.2. Recognition of HIV-1-infected cells by specific CTLs

To confirm the restriction molecule of these two epitope-specific CTLs, we generated CTL clones specific for each peptide. We investigated the ability of the CTL clones to produce IFN-γ after stimulation of them with HLA-B*4801-positive and -negative C1R cells pre-pulsed with each peptide. The two clones specific for each peptide induced the IFN-γ-producing cells after stimulation with

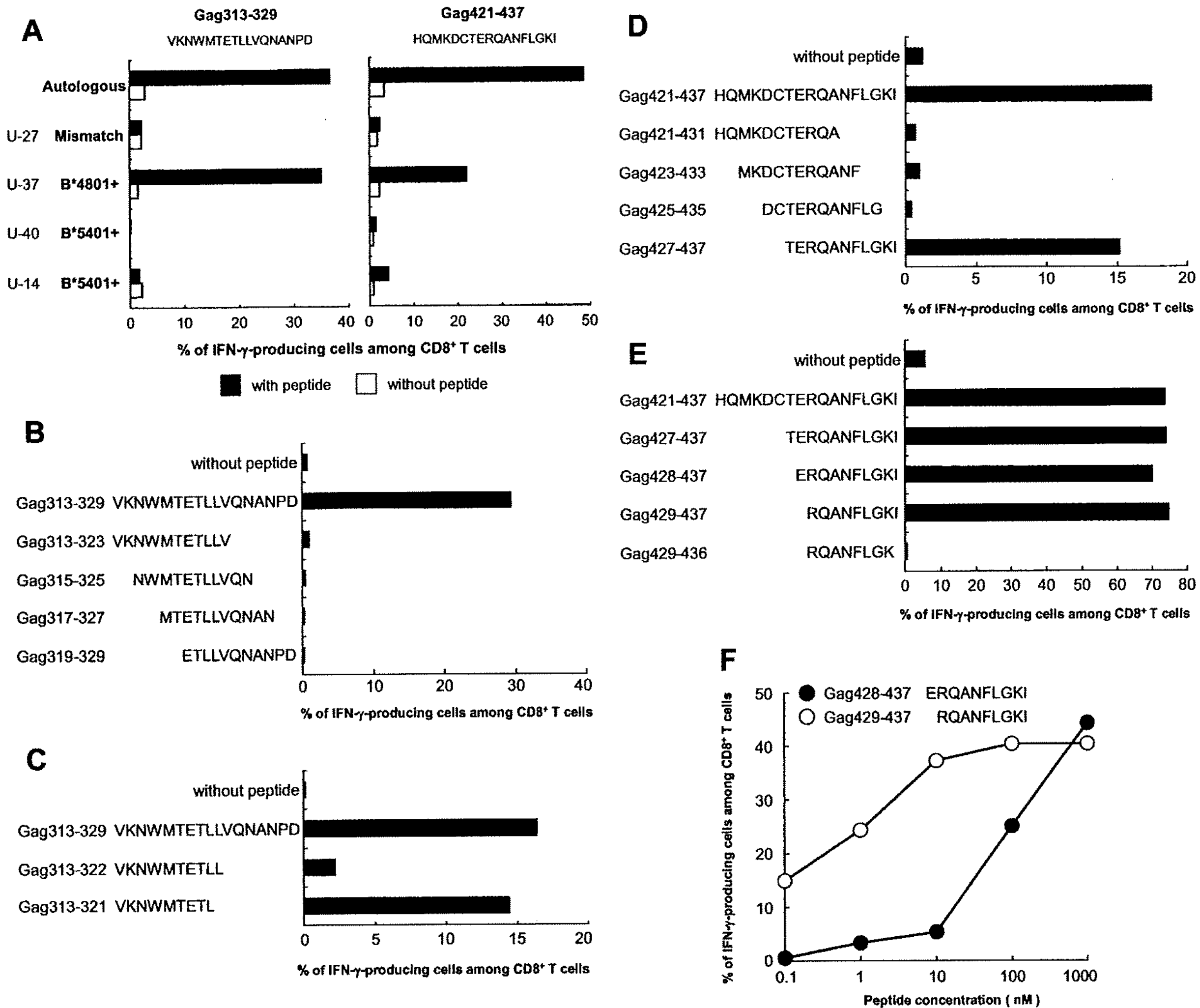

Fig. 1. Identification of optimal epitope peptides presented by HLA-B*4801. PBMCs from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Gag313-329 or Gag421-437 peptide and then cultured for 2 weeks. The cultured cells were stimulated with Gag313-329 or Gag421-437–pulsed autologous B-LCL or B-LCLs sharing one HLA class I allele with KI-119 (A). Gag313-329–specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide (B) or 9- to 10-mer truncated peptide (C) at a concentration of 1000 nmol/l. Gag421-437–specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide (D) or 8- to 10-mer truncated peptide (E) at a concentration of 1000 nmol/l. The stimulator cells were pulsed with the 9- or 10-mer truncated peptide at concentrations from 0.1 to 1000 nmol/l (F). After the cultured cells were stimulated with each peptide-pulsed B-LCL for 6 hours, interferon (IFN)-γ–producing CD8$^+$ T cells specific for each peptide were measured by flow cytometry.

HLA-B*4801–positive C1R cells but not with HLA-B*4801-negative ones (Fig. 2A), confirming that the restriction molecule of Gag313-321–specific and Gag429-437–specific CTLs was indeed HLA-B*4801.

To clarify whether Gag313-321 and Gag429-437 epitopes are naturally occurring, we investigated the response of these peptide-specific CD8$^+$ T cells toward HLA-B*4801-expressing .221-CD4 cell lines infected with HIV-1 (NL-432) by using the intracellular IFN-γ staining assay. The .221 cells were infected with NL-432, and then incubated at 37°C for 4 days. The percentage of the HIV-1–infected cells was measured by using intracellular HIV-1 p24 staining (Fig. 2B). The Gag313-321–specific and Gag429-437–specific CTL clones responded to .221-CD4-B*4801 cells infected with HIV-1 but not to uninfected .221-CD4-B*4801 cells or to HLA-B*4801–negative.221-CD4 infected with HIV-1 (Fig. 2C). These results indicate that Gag313-321 and Gag429-437 peptides were naturally processed and presented by HLA-B*4801.

3.3. *HLA-B*4801–restricted HIV-1–specific CD8$^+$ T cell responses in chronically HIV-1–infected individuals with HLA-B*4801*

To clarify whether these HLA-B*4801–restricted CD8$^+$ T cells were predominantly induced in chronically HIV-1–infected individuals bearing HLA-B*4801, we investigated the induction of the specific CD8$^+$ T cells in PBMCs from seven chronically HIV-1–infected HLA-B*4801–positive individuals by stimulating them with either of these two epitope peptides. The cells were cultured for 14 days, and the frequency of the specific CD8$^+$ T cells in the cultured cells was determined by using intracellular IFN-γ production assay. Both Gag313-321- and Gag429-437–specific CD8$^+$ T cells were found in five of the seven HIV-1–infected individuals (Table 1), indicating that these two Gag epitopes were immunodominant epitopes.

4. Discussion

A previous study analyzing the pool sequences of endogenously bound peptides showed that primary anchor residues for HLA-

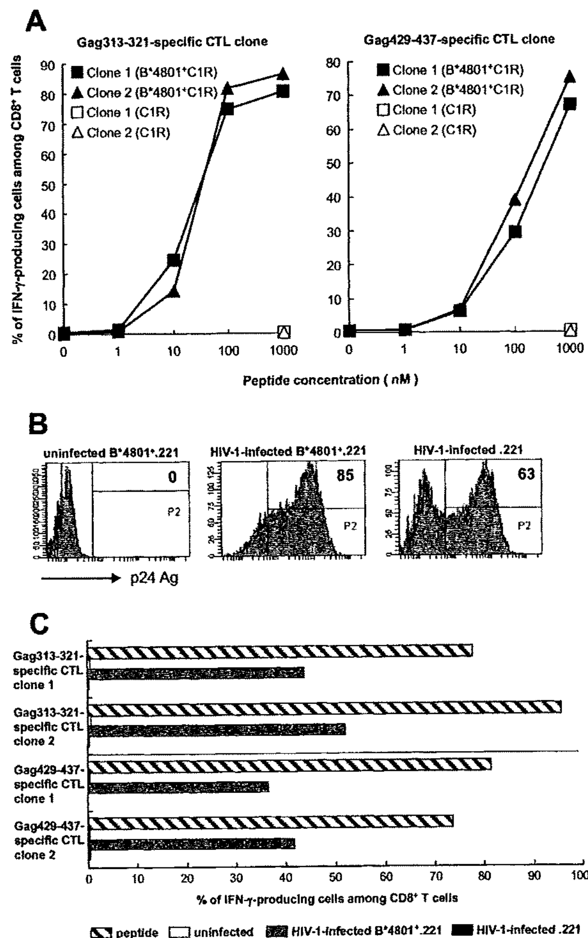

Fig. 2. HLA-B*4801-restricted recognition of HIV-1-infected cells by the Gag313-321 or Gag429-437-specific CTLs. (A) Confirmation of HLA-B*4801 restriction in Gag313-321- or Gag429-437-specific CTLs. The responsiveness of each epitope peptide-specific CTL clones to B*4801$^+$C1R cell lines pre-pulsed with the corresponding peptide at concentrations from 1 to 1000 nmol/l was determined by performing the intracellular IFN-γ staining assay. (B) The 221-CD4 cell lines were infected with HIV-1 (NL-432) in incubation at 37°C for 4 days. The HIV-1-infected cells were determined by using intracellular p24 staining with anti-p24 MAb. The percentage of HIV-1-infected cells is shown in each figure. (C) The activities of each peptide-specific CTLs toward B*4801$^+$.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1 μmol/l) were measured by using the intracellular IFN-γ staining assay.

B*4801 were Lys or Gln at P2 and Leu at the C-terminus [27]. In the present study, we identified 2 HLA-B*4801–restricted HIV-1 Gag-specific CTL epitopes carrying Lys or Gln at P2 and Leu or Ile at the C-terminus. Thus these results confirm Lys and Gln as P2 anchor as well as Leu as the C-terminal anchor for HLA-B*4801 and identified an additional C-terminal anchor Ile. Leu, at the C-terminus of Gag313-322 (VKNWMTETLL), affected the recognition by specific T cells, suggesting that this additional Leu may reduce the affinity of the peptide for HLA-B*4801. However, it remains unknown why the 17-mer peptide Gag313-329 can be recognized by the T cells.

HLA-B*1302 and HLA-B*3902 have the same peptide motif as HLA-B*4801 [28,29]. In fact, five HLA-B*1302–restricted HIV-1 epitopes were found to have the same motif [28], although no HLA-B*3902–restricted HIV-1 epitopes have been identified. Interestingly, the Gag429-437 epitope is presented by both HLA-B*1302 and HLA-B*4801 [28]. As a previous study showed that the viral load of HLA-B*13-positive subjects is significantly lower than that of B*13-negative subjects [28], we speculated that HLA-B*4801 is associated with successful immune control.

The two Gag epitope-specific CD8$^+$ T cells were detected in five of seven chronically HIV-1–infected individuals with HLA-B*4801. These findings indicate that these epitopes are recognized as immunodominant ones in chronically HIV-1–infected individuals with HLA-B*4801. The sequences of Gag313-321 (VKNWMTETL) and Gag429-437 (RQANFLGKI) were found in 117 (93%) and 107 (85%) of 126 HIV-1 clade B isolates, respectively, and in 95 (17%) and 457 (83%) of 554 HIV-1 clade C ones, respectively, in reported HIV-1 sequences (Los Alamos National Laboratory HIV Molecular Immunology Database), indicating that both sequences are relatively conserved in clade B but only Gag429-437 in clade C. CTLs specific for these epitopes were frequently induced in HIV-1–infected individuals with HLA-B*4801. In addition, Gag313-321 and Gag429-437–specific CTL clones strongly responded to HIV-1–infected .221-CD4 expressing HLA-B*4801, suggesting that these specific CTLs may effectively kill HIV-1–infected cells. Thus, our findings suggest that these HLA-B*4801 epitopes may be useful in developing an HIV-1 vaccine to effectively induce specific CTLs.

A recent study reported that Gag-specific CTLs play a critical role in the control of HIV-1 replication [30]. These results suggest that HLA-B*4801–restricted Gag-specific CTLs may contribute to control of HIV-1 replication. A previous study reported that the affinity of binding of HLA-B*4801 to the CD8 co-receptor is weaker than that of other HLA class I molecules because of the mutation to threonine at position 245 in the α3 domain of HLA class I [27]. However we showed that the HIV-1 epitope-specific CTL clones strongly recognized HIV-1–infected .221-CD4 cells expressing HLA-B*4801, suggesting that these CTLs can recognize HIV-1–infected cells *in vivo*. Further studies are required to clarify the role of these CTLs *in vivo*.

HLA-B*4801 is found only in Asia. Therefore, this allele had not been analyzed for its effect on the progression to AIDS in Caucasian or African cohorts. In addition, there is no cohort study of HLA-B*4801 on an Asian cohort. Further analyses are required to clarify the effect of this allele in an Asian cohort.

We previously identified five HLA-B*5401–restricted CTL epitopes from Nef and Pol by using PBMCs from the same chronically HIV-1–infected individual, KI-119 [19]. In contrast, two HLA-B*4801–restricted CTL epitopes were identified from Gag by using PBMCs from the same individual. If Gag-specific CTLs are the most effective T cells to control HIV-1 replication, HLA-B*4801 and HLA-B*5401 may be associated with slow and rapid progression to AIDS, respectively. Both alleles are common ones in Asia, but the association of these alleles with progression to AIDS has not been analyzed.

In summary, we identified two novel HLA-B*4801–restricted HIV-1 Gag–specific CTL epitopes by using 17-mer overlapping peptides in this study. These epitopes were relatively conserved, and the specific T cells were predominantly induced in HIV-1–infected

Table 1
Induction of epitope-specific CD8$^+$ T cells among PBMCs from HLA-B*4801$^+$ HIV-1–infected individuals.

Patients[a]	Viral load[b]	CD4[c]	CD8[c]	% of IFN-γ-producing cells in CD8$^+$ T cells	
				Gag313-321	Gag429-437
KI-119	3.0×10^3	536	1268	26.0	50.5
KI-034	9.6×10^4	235	1239	56.1	65.8
KI-092	2.2×10^2	971	1492	23.1	5.2
KI-179	1.4×10^3	202	668	0.0	0.0
KI-067	8.9×10^4	234	1198	4.9	50.2
KI-134	1.1×10^5	335	1155	32.3	0.0
KI-169	1.6×10^5	63	930	0.0	79.1

[a] HIV-1–infected individuals with HLA-B*4801.
[b] Copies/ml.
[c] Cells/μl.

individuals carrying HLA-B*4801. Thus these findings suggest that these epitopes are useful for the development of an HIV-1 vaccine and for analysis of the immunopathogenesis of AIDS.

Acknowledgments

The authors thank Sachiko Sakai for secretarial assistance. This research was supported by a grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of the government of Japan and by a grant from the Japan Health Science Foundation. H.M. is a JAIDS Foundation Research Fellow.

References

[1] Barker E, Mackewicz CE, Reyes-Teran G, Sato A, Stranford SA, Fujimura SH, et al. Virological and immunological features of long-term human immunodeficiency virus-infected individuals who have remained asymptomatic compared with those who have progressed to acquired immunodeficiency syndrome. Blood 1998;92:3105-14.

[2] Klein MR, van Baalen CA, Holwerda AM, Kerkhof Garde SR, Bende RJ, Keet IP, et al. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med 1995;181:1365-72.

[3] McMichael AJ, Rowland-Jones SL. Cellular immune responses to HIV. Nature 2001;410:980-7.

[4] Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999;283:857-60.

[5] Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997;3:205-11.

[6] Goulder PJ, Philips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T lymphocyte response associated with progression to AIDS. Nat Med 1997;3:212-7.

[7] Collins KL, Chen BK, Kalams SA, Walker BD, Blatimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 1998;391:397-401.

[8] Xu XN, Laffert B, Screaton GR, Kraft M, Wolf D, Kolanus W, et al. Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J Exp Med 1999;189:1489-96.

[9] Mueller YM, De Rosa SC, Hutton JA, Witek J, Roederer M, Altman JD, et al. Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells. Immunity 2001;15:871-82.

[10] Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 2001; 410:106-11.

[11] Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: Strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242-52.

[12] Kawashima Y, Satoh M, Oka S, Takiguchi M. Identification and characterization of HIV-1 epitopes presented by HLA-A*2603: Comparison between HIV-1 epitopes presented by A*2601 and A*2603. Hum Immunol 2005;66:1155-66.

[13] Tomiyama H, Miwa K, Shiga H, Ikeda-Moore Y, Oka S, Iwamoto A, et al. Evidence of presentation of multiple HIV-1 cytotoxic T lymphocyte epitopes by HLA-B*3501 molecules that are associated with the accelerated progression of AIDS. J Immunol 1997;158:5026-34.

[14] Dong T, Boyd D, Rosenberg W, Alp N, Takiguchi M, McMichael AJ, et al. An HLA-B35-restricted epitope modified at an anchor residue results in an antagonist peptide. Eur J Immunol 1996;26:335-39.

[15] Goulder PJ, Reid SW, Price DA, O'Callaghan CA, McMichael AJ, Phillips RE, et al. Combined structural and immunological refinement of HIV-1 HLA-B8-restricted cytotoxic T lymphocyte epitopes. Eur J Immunol 1997;27:1515-21.

[16] Addo MM, Altfeld M, Rosenberg ES, Eldridge RL, Philips MN, Habeeb K, et al. The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. Proc Natl Acad Sci U S A 2001;98:1781-86.

[17] Goulder PJ, Addo MM, Altfeld MA, Rosenberg ES, Tang Y, Govender U, et al. Rapid definition of five novel HLA-A*3002-restricted human immunodeficiency virus-specific cytotoxic T-lymphocyte epitopes by ELISPOT and intracellular cytokine staining assays. J Virol 2001;75:1339-47.

[18] Draenert R, Altfeld M, Brander C, Basgoz N, Corcoran C, Wurcel AG, et al. Comparison of overlapping peptide sets for detection of antiviral CD8 and CD4 T cell responses. J Immunol Methods 2003;275:19-29.

[19] Kitano M, Kobayashi N, Kawashima Y, Akahoshi T, Nokihara K, Oka S, et al. Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes. Microbes Infect 2008;10:764-72.

[20] Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, editors. HLA 1991: Proceedings of the Eleventh International Histocompatibility Workshop and Conference. Oxford, Oxford University Press, 1992:1065-220.

[21] Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernandez-Vina MA. Analysis of the frequencies of HLA-A, -B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum Immunol 2001;62:1009-30.

[22] Cao K, Hollenbach JA, Shi XJ, Shi WX, Chopek M, Fernandez-Vina MA. HLA-A, -B and -Cw allele frequencies in a Caucasian population from the USA. Hum Immunol 2004;65:1196-8.

[23] Shaw CK, Chen LL, Lee A, Lee TD. Distribution of HLA gene and haplotype frequencies in Taiwan: A comparative study among Min-nan, Hakka, Aborigines and Mainland Chinese. Tissue Antigens 1999;53:51-64.

[24] Itoh Y, Mizuki N, Shimada T, Azuma F, Itakura M, Kashiwase K, et al. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. Immunogenetics 2005; 57:717-29.

[25] Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J Virol 2000;74:2907-12.

[26] Tomiyama H, Akari H, Adachi A, Takiguchi M. 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 2002;76:7535-43.

[27] Martinez-Naves E, Barber LD, Madrigal JA, Vullo CM, Clayberger C, Lyu SC, et al. Interactions of HLA-B*4801 with peptide and CD8. Tissue Antigens 1997;50: 258-64.

[28] Honeyborne I, Prendergast A, Pereyra F, Leslie A, Crawford H, Payne R, et al. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple Gag-specific CD8+ T-cell epitopes. J Virol 2007;81: 3667-72.

[29] Falk K, Rötzschke O, Takiguchi M, Gnau V, Stevanović S, Jung G, et al. Peptide motifs of HLA-B38 and B39 molecules. Immunogenetics 1995;41:162-4.

[30] Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007;13:46-53.

International weekly journal of science

nature

Adaptation of HIV-1 to human leukocyte antigen class I

Yuka Kawashima[1], Katja Pfafferott[3,6], John Frater[4,5], Philippa Matthews[3], Rebecca Payne[3], Marylyn Addo[7], Hiroyuki Gatanaga[2,8], Mamoru Fujiwara[1], Atsuko Hachiya[1,8], Hirokazu Koizumi[1], Nozomi Kuse[1], Shinichi Oka[2,8], Anna Duda[4,5], Andrew Prendergast[3], Hayley Crawford[3], Alasdair Leslie[3], Zabrina Brumme[7], Chanson Brumme[7], Todd Allen[7], Christian Brander[7,9], Richard Kaslow[10], James Tang[10], Eric Hunter[11], Susan Allen[12], Joseph Mulenga[12], Songee Branch[13], Tim Roach[13], Mina John[6], Simon Mallal[6], Anthony Ogwu[14], Roger Shapiro[14], Julia G. Prado[3], Sarah Fidler[15], Jonathan Weber[15], Oliver G. Pybus[16], Paul Klenerman[4,5], Thumbi Ndung'u[17], Rodney Phillips[4,5], David Heckerman[19], P. Richard Harrigan[18], Bruce D. Walker[7,17,20], Masafumi Takiguchi1 & Philip Goulder[3,6,17]

[1]Divisions of Viral Immunology and [2]Infectious Disease, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. [3]Department of Paediatrics, [4]Nuffield Department of Clinical Medicine and [5]The James Martin 21st Century School, Peter Medawar Building for Pathogen Research, South Parks Road, Oxford OX1 3SY, UK. [6]Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia 6000, Australia. [7]Partners AIDS Research Center, Massachusetts General Hospital, 13th Street, Building 149, Charlestown, Boston, Massachusetts 02129, USA. [8]AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. [9]Fundació IrsiCaixa-HIVACAT, Hospital Germans Trias i Pujol, Badalona and Institucio Catalana de Recerca i Estudis Avancats (ICREA), Barcelona 08916, Spain. [10]University of Alabama at Birmingham, Birmingham, Alabama 35294, USA. [11]Emory University Vaccine Center and Yerkes National Primate Research Center, Atlanta, Georgia 30329, USA. [12]Zambia Emory HIV Research Project, and the Zambia Blood Transfusion Service, Lusaka, Zambia. [13]Ladymeade Reference Unit, University of West Indies, Bridgetown BB11156, Barbados. [14]Botswana-Harvard School of Public Health AIDS Initiative Partnership, Gaborone, Botswana. [15]Division of Medicine, Wright Fleming Institute, Imperial College, St Mary's Hospital, Norfolk Place, Paddington, London W2 1PG, UK. [16]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3SY, UK. [17]HIV Pathogenesis Programme, The Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban 4013, South Africa. [18]Miscrosoft Research, One Microsoft Way, Redmond, Washington 9805, USA. [19]BC Centre for Excellence in HIV/AIDS, Vancouver, British Columbia V6Z 1Y6, Canada. [20]Howard Hughes Medical Institute, Chevy Chase, Maryland 20185, USA.

Reprinted from Nature, Vol. 458, No. 7238, pp. 641–645, 2 April 2009

© Nature Publishing Group, 2009

LETTERS

Adaptation of HIV-1 to human leukocyte antigen class I

Yuka Kawashima[1], Katja Pfafferott[3,6], John Frater[4,5], Philippa Matthews[3], Rebecca Payne[3], Marylyn Addo[7], Hiroyuki Gatanaga[2,8], Mamoru Fujiwara[1], Atsuko Hachiya[1,8], Hirokazu Koizumi[1], Nozomi Kuse[1], Shinichi Oka[2,8], Anna Duda[4,5], Andrew Prendergast[3], Hayley Crawford[3], Alasdair Leslie[3], Zabrina Brumme[7], Chanson Brumme[7], Todd Allen[7], Christian Brander[7,9], Richard Kaslow[10], James Tang[10], Eric Hunter[11], Susan Allen[12], Joseph Mulenga[12], Songee Branch[13], Tim Roach[13], Mina John[6], Simon Mallal[6], Anthony Ogwu[14], Roger Shapiro[14], Julia G. Prado[3], Sarah Fidler[15], Jonathan Weber[15], Oliver G. Pybus[16], Paul Klenerman[4,5], Thumbi Ndung'u[17], Rodney Phillips[4,5], David Heckerman[19], P. Richard Harrigan[18], Bruce D. Walker[7,17,20], Masafumi Takiguchi[1] & Philip Goulder[3,6,17]

The rapid and extensive spread of the human immunodeficiency virus (HIV) epidemic provides a rare opportunity to witness host–pathogen co-evolution involving humans. A focal point is the interaction between genes encoding human leukocyte antigen (HLA) and those encoding HIV proteins. HLA molecules present fragments (epitopes) of HIV proteins on the surface of infected cells to enable immune recognition and killing by CD8$^+$ T cells; particular HLA molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, are more likely to mediate successful control of HIV infection[1]. Mutation within these epitopes can allow viral escape from CD8$^+$ T-cell recognition. Here we analysed viral sequences and HLA alleles from >2,800 subjects, drawn from 9 distinct study cohorts spanning 5 continents. Initial analysis of the HLA-B*51-restricted epitope, TAFTIPSI (reverse transcriptase residues 128–135), showed a strong correlation between the frequency of the escape mutation I135X and HLA-B*51 prevalence in the 9 study cohorts ($P = 0.0001$). Extending these analyses to incorporate other well-defined CD8$^+$ T-cell epitopes, including those restricted by HLA-B*57 and HLA-B*27, showed that the frequency of these epitope variants (n = 14) was consistently correlated with the prevalence of the restricting HLA allele in the different cohorts (together, $P < 0.0001$), demonstrating strong evidence of HIV adaptation to HLA at a population level. This process of viral adaptation may dismantle the well-established HLA associations with control of HIV infection that are linked to the availability of key epitopes, and highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.

The extent to which HIV is evolving at the population level in response to immune selection pressure is under debate[2–6]. Resolving the impact of HLA class I alleles on viral evolution is problematic because it can be obscured by other influences, such as founder effect[6] (polymorphisms present within the early strains establishing the epidemic in a group). In addition, most HLA alleles do not drive significant selection pressure on HIV, a proportion of escape mutations revert to wild type after transmission, and different HLA alleles may drive the identical escape mutation[7].

To test the hypothesis that the frequency of escape mutations in a given population is correlated with the prevalence of the relevant HLA allele in that population, we studied nine distinct cohorts from North America, the Caribbean, Europe, sub-Saharan Africa, Australia and Japan, in which we performed HLA typing, and defined the viral mutations arising within CD8$^+$ T-cell epitopes. We focused initially on a well-characterized mutation, I135X, within the HLA-B*51-restricted epitope, TAFTIPSI (RT 128–135)[8], because it arises in acute infection, non-HLA-B*51 alleles do not also select this mutation[7,9], and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects[9]. Thus, if highly prevalent HLA alleles drive a high frequency of escape mutations in the population, this would be most obvious in relation to HLA-B*51 and the escape mutant I135X. We then considered an additional 13 well-defined escape mutations, including those known to reduce viral fitness and therefore liable to revert after transmission.

I135X was selected in 205 of 213 (96%) HLA-B*51-positive individuals analysed (Figs 1 and 2, and Supplementary Fig. 1). The I135X variants do not significantly affect viral replicative capacity *in vitro*, other than the rare I135V mutation. This was the only variant observed to revert to wild-type *in vivo* during a 3-year follow-up of 38 HLA-B*51-negative subjects identified during acute HIV infection who carried I135X mutant viruses at transmission (Fig. 1e). The I135X mutants substantially affect HLA binding, and therefore also recognition by CD8$^+$ T cells (Fig. 1f–h). Thus, HIV transmission from HLA-B*51-positive subjects would probably involve transmission of I135X, which would persist in the new host. Newly infected HLA-B*51-positive subjects receiving an I135X mutant would be unable to generate an HLA-B*51-TAFTIPSI-specific response.

To test the hypothesis that the population frequency of I135X is correlated with HLA-B*51 prevalence, HIV sequence and HLA data were collated from the nine study cohorts. One cohort comprised subjects with acute/early HIV infection; the remaining cohorts comprised chronically infected subjects. In all cohorts the odds ratio strongly favoured I135X in the HLA-B*51-positive subjects, even in the acute cohort where I135X was selected sufficiently early to be already over-represented in HLA-B*51-positive subjects (odds ratio 1.65, $P = 0.07$, Fig. 2a). In Japan, where HLA-B*51 is highly

[1]Divisions of Viral Immunology and [2]Infectious Disease, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. [3]Department of Paediatrics, [4]Nuffield Department of Clinical Medicine and [5]The James Martin 21st Century School, Peter Medawar Building for Pathogen Research, South Parks Road, Oxford OX1 3SY, UK. [6]Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia 6000, Australia. [7]Partners AIDS Research Center, Massachusetts General Hospital, 13th Street, Building 149, Charlestown, Boston, Massachusetts 02129, USA. [8]AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. [9]Fundació IrsiCaixa-HIVACAT, Hospital Germans Trias i Pujol, Badalona and Institucio Catalana de Recerca i Estudis Avancats (ICREA), Barcelona 08916, Spain. [10]University of Alabama at Birmingham, Birmingham, Alabama 35294, USA. [11]Emory University Vaccine Center and Yerkes National Primate Research Center, Atlanta, Georgia 30329, USA. [12]Zambia Emory HIV Research Project, and the Zambia Blood Transfusion Service, Lusaka, Zambia. [13]Ladymeade Reference Unit, University of West Indies, Bridgetown BB11156, Barbados. [14]Botswana-Harvard School of Public Health AIDS Initiative Partnership, Gaborone, Botswana. [15]Division of Medicine, Wright Fleming Institute, Imperial College, St Mary's Hospital, Norfolk Place, Paddington, London W2 1PG, UK. [16]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3SY, UK. [17]HIV Pathogenesis Programme, The Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban 4013, South Africa. [18]Miscrosoft Research, One Microsoft Way, Redmond, Washington 9805, USA. [19]BC Centre for Excellence in HIV/AIDS, Vancouver, British Columbia V6Z 1Y6, Canada. [20]Howard Hughes Medical Institute, Chevy Chase, Maryland 20185, USA.

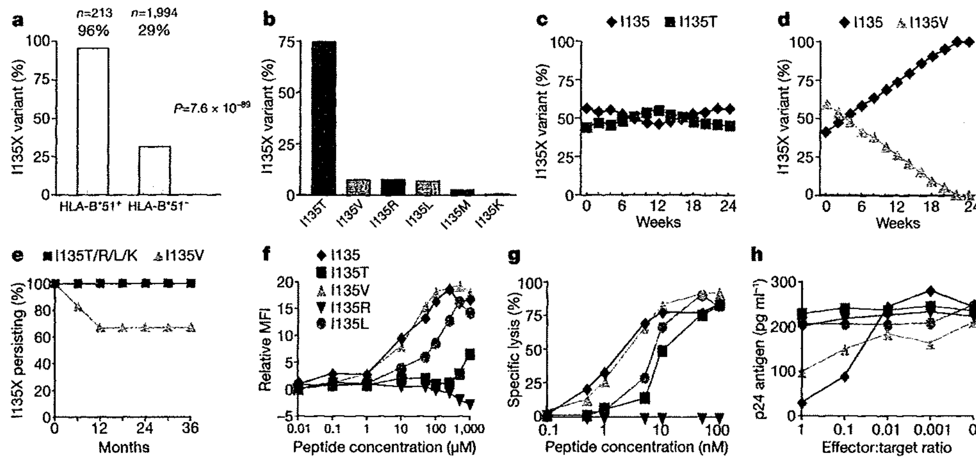

Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51–TAFTIPSI (RT 128–135)-specific CD8+ T cells. **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition assays between NL4-3 wild-type virus and I135X viral variants (I135T (**c**) and I135V (**d**)). I135R and I135L showed no fitness cost (not shown). **e**, Persistence of I135X mutants in 38 HLA-B*51-negative subjects followed from acute infection. **f**, TAFTIPSI variant binding to HLA-B*51 (see Methods). MFI, mean fluorescence intensity. **g**, **h**, Recognition of peptide-pulsed HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8+ T-cell clones.

prevalent[10] (21.9% of the study cohort), the frequency of I135X was >50%, and overall across all cohorts the I135X frequency was strongly correlated with HLA-B*51 prevalence ($P = 0.0001$, Fig. 2b). To control for the possibility that disproportionately more virus sequences from HLA-B*51-positive subjects were analysed, the same analysis comparing I135X frequency in HLA-B*51-negative subjects only was undertaken, with similar findings (Fig. 2c, $P = 0.0006$). These data suggest that HIV may be adapting to HLA-B*51 with respect to the HLA-B*51–TAFTIPSI response in localities where HLA-B*51 is at high prevalence.

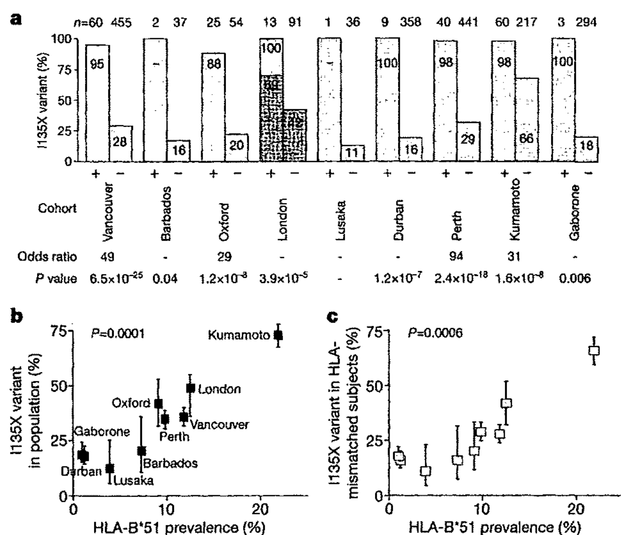

Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts. **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (−) subjects within nine study cohorts. In the acute cohort (London) 69% of HLA-B*51-positive subjects expressed I135X mutant at enrolment, 100% within 2 years of baseline (Supplementary Fig. 1). **b**, Correlation between frequency of I135X mutation and HLA-B*51 prevalence in the nine study populations. Logistic regression $P = 0.0001$ (Supplementary Table 1). **c**, Correlation between I135X frequency in HLA-B*51-negative subjects and HLA-B*51 prevalence in nine study populations. Error bars represent 95% confidence limits, obtained using a binomial error distribution.

Additional evidence that I135X is accumulating in Japan comes from the observation that only 3 of 14 (21%) HLA-B*51-negative Japanese haemophiliacs infected in 1983 carried I135X, compared with 30 of 43 (70%) HLA-B*51-negative subjects infected between 1997 and 2008 ($P = 0.002$). Furthermore, HLA-B*51 does not protect against disease progression in Japanese subjects infected between 1997 and 2008, whereas HLA-B*51-positive haemophiliacs infected in 1983 had lower viraemia levels and higher CD4 counts than HLA-B*51-negative haemophiliacs (Supplementary Fig. 2). These data are consistent with fewer HLA-B*51-positive subjects targeting TAFTIPSI during 1997–2008, owing to a population-level increase in the HLA-B*51 I135X escape mutation over this 14–25-year period.

To investigate HIV adaptation to other HLA alleles, we initially examined other escape mutations shown previously to persist stably after transmission[5,7]. We selected the three non-reverting Gag polymorphisms that, from analysis of 673 study subjects in Durban, South Africa[7], were most strongly associated with the relevant restricting allele ($P < 10^{-6}$ after phylogenetic correction), namely, S357X, D260X and D312X within epitopes restricted, respectively, by HLA-B*07 (GPSHKARVL, Gag 355–363), HLA-B*35 (PPIPVGDIY, Gag 254–262) and HLA-B*44 (AEQATQDVKNW, Gag, 306–316). In addition, we analysed a non-reverting I31V variant (LPPIVAKEI, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence[5]. These additional polymorphisms show a similar relationship to that between I135X and HLA-B*51, overall showing a strongly significant correlation between variant frequency and prevalence of the restricting HLA allele (Figs 3 and 4a, and Supplementary Fig. 3).

The spectrum of HLA-associated polymorphisms also includes mutations reducing viral fitness[1]. These either revert to wild type after transmission, or persist in the presence of compensatory mutations. We extended these analyses to include epitopes restricted by HLA-B*27 and HLA-B*57, alleles strongly associated with successful immune control of HIV[11,12]. The mutations analysed themselves are associated with precipitating loss of immune control[13–16] and all inflict a documented viral fitness cost, either demonstrated by *in vitro* fitness studies and/or *in vivo* reversion[7,14,17–21] (data not shown for V168I).

Again, a strong correlation between escape mutant frequency and prevalence of the restricting HLA allele was observed (Figs 3c–f and 4b, and Supplementary Fig. 3; overall, for these nine variants affecting viral fitness, $r = 0.69$, $P < 0.0001$). Unexpectedly, this correlation

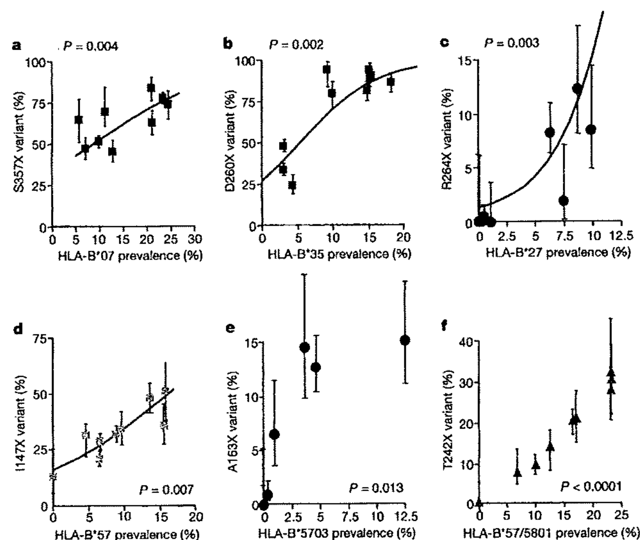

Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b**, Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDIY (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263–272). **d**, Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e**, Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f**, Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting[18,20] mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan[10], 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific CD8$^+$ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted CD8$^+$ T-cell responses. Non-reverting variants[5,7], as well as those previously shown to arise at a fitness cost[7,14,16–21], were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms[7,20]. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those

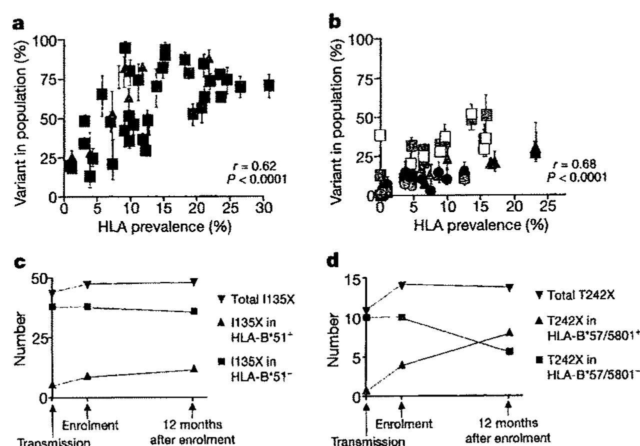

Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. **a**, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares. A146X; sky-blue squares, V168I; yellow circles, I247X). **c**, **d**, Data from acute London cohort. **c**, Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d**, The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

believed to contribute significantly to containment of HIV[4,7,13–19]. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms[6]. Phylogenetic correction of sequence data excludes founder effect as a confounder[6,7,9], and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain CD8$^+$ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV