354 KD-247 for 30 min at 37 °C. After incubation for 5 h, cells were centrifuged,
355 resuspended in RPMI 1640 medium supplemented with 10% FCS without KD-247. The
356 culture supernatant was harvested on day 6 and used to infect fresh PM1/CCR5 cells for
357 the next round of culture in the presence of increasing concentrations of KD-247. When
358 the virus began to propagate rapidly in the presence of KD-247, the MAb concentration
359 was further increased. After the virus had been passaged in the presence of up to 2,000
360 $\mu$g ml$^{-1}$ of KD-247, the KD-247-resistant virus, HIV-1$_{BaL}$ (2000) p16, was recovered
361 from the cell culture supernatant. After 16 passages with KD-247, we continued
362 culturing the virus for a further 14 passages without KD-247. HIV-1$_{BaL}$ virus was also
363 passaged for the same period in PM1/CCR5 cells in the absence of KD-247, and the
364 resulting virus was designated HIV-1$_{BaL}$ (-) p16. Proviral DNA from infected cells at
365 various passages was subjected to DNA sequencing.

366 Amplification of proviral DNA (pDNA) and nucleotide sequencing

367 Proviral DNA was extracted and nested PCR was performed to amplify the gp120 C1 to
368 C4 coding region as described previously (Wang *et al.*, 2002). The primers used were as
369 follows: for the first-step PCR, 1B (5'-AGA AAG AGC AGA AGA CAG TGG CAA
370 TGA-3') and H (5'-TAG TGC TTC CTG CTG CTC CCA AGA ACC C-3'); for the
371 second-step PCR, 2B (5'-AGC AGA AGA CAG TGG CAA TGA GAG TGA-3') and F

372 (5'-ATA TAA TTC ACT TCT CCA ATT GTC CCT CAT-3'). The PCR products were
373 inserted into a TA vector (Invitrogen) and sequenced.

374 The neutralization-sensitivity assay.

375 The neutralization-sensitivity of each passaged HIV-$1_{BaL}$ virus to KD-247 was
376 determined using TZM-bl cells. Briefly, a virus concentration of 300 $TCID_{50}$ was
377 incubated with various dilutions of KD-247 in duplicate for 30 min at 37 °C in a 96-well
378 flat-bottom culture plate (Corning-Costar). Freshly trypsinized cells ($2 \times 10^4$ cells in 50
379 $\mu$l of 10% FCS/DMEM containing 10 $\mu$g $ml^{-1}$ DEAE-dextran) were added to each well.
380 After incubation for 2 days at 37 °C β-galactosidase activity in each well was measured
381 using Galacto-Star substrate (Applied Biosystems).

382 Construction of mutant envelope expression vectors

383 Proviral DNA isolated from the infected cells at various passages was cloned into
384 envelope expression vectors as previously described (Li *et al.*, 2005; Shibata *et al.*,
385 2007). Briefly, we amplified the full-length gp160 regions from the most frequent
386 clones at the baseline, passage 5 and passage 13 using LA *Taq* (Takara) with primers
387 ENVA (5'-GGC TTA GGC ATC TCC TAT GGC AGG AAG AA-3') and ENVN
388 (5'-CTG CCA ATC AGG GAA GTA GCC TTG TGT-3'), and the PCR products were

389 inserted into the pCR-XL-TOPO vector (Invitrogen) and designated pCR-XL-BaL-WT,
390 pCR-XL-BaL-p5 and pCR-XL-BaL-p13, respectively. Chimeric vectors were generated
391 based on the pCR-XL-BaL-WT by replacing the fragments from pCR-XL-BaL-p5 and
392 pCR-XL-BaL-p13 digested at the restriction enzyme sites indicated below. The
393 *Nde*I-*Sca*I fragment for the pCR-XL-BaL-p5 env gene was subcloned into
394 pCR-XL-BaL-WT, designated pCR-XL-BaL-STA. The *Nde*I-*Sca*I, *Nde*I-*Stu*I and
395 *Stu*I-*Sca*I fragments for the pCR-XL-BaL-p13 env gene were subcloned into the
396 pCR-XL-BaL-WT, designated pCR-XL-BaL-PNGS/SKL, pCR-XL-BaL-PNGS and
397 pCR-XL-BaL-SKL, respectively. Each *EcoR*I fragment of these vectors was ligated into
398 pCXN2 to give pCXN-BaL-WT, pCXN-BaL-STA, pCXN-BaL-PNGS/SKL,
399 pCXN-BaL-PNGS and pCXN-BaL-SKL.

400 Pseudovirus preparation

401 Approximately 5 μg of pSG3$^{\Delta env}$ (Wei *et al.*, 2002) and 0.5 μg of pRSV-Rev (Hope *et*
402 *al.*, 1990), supplied by the ARRRP, and 4.5 μg of the HIV-$1_{BaL}$ env-expressing $pCXN_2$
403 were co-transfected into 293T cells. At 24 h after transfection the
404 pseudovirus-containing supernatants were harvested, filtered and stored at -150 °C.

405 A single-round assay for measuring neutralization of the pseudoviruses

406 A single-round infectivity assay was used to measure the neutralization of HIV-$1_{BaL}$
407 pseudoviruses as described previously (Li *et al.*, 2005). Briefly, reagents including an
408 entry inhibitor, MAbs or rsCD4 at various concentrations and a pseudovirus suspension
409 corresponding to 300 $TCID_{50}$ were pre-incubated for 30 min at 37 °C. The
410 virus-compound mixtures were added to TZM-bl cells in a 96-well plate ($2 \times 10^4$ cells
411 $well^{-1}$). After incubation for 2 days at 37 °C the β-galactosidase activity in each well
412 was measured as described above. The reduction in infectivity was determined by
413 comparing the relative light units in the presence and absence of each compound and
414 was expressed as the percentage of neutralization.

415 Construction of chimeric pWT10/BaL env proviruses

416 Chimeric proviruses were constructed from the pWT/BaL proviral plasmid (from the
417 ARRRP) (Hwang *et al.*, 1991) by replacing the region encoding the envelope gp160.
418 Briefly, the env genes obtained from escaped HIV-$1_{BaL}$ variants or induced by
419 site-directed mutagenesis were substituted into the pWT/BaL vectors after digestion at
420 the restriction enzyme sites *Sal*I and *BamH*I. The resulting replication-competent
421 viruses were designated HX-BaL-X (e.g., HX-BaL-WT, HX-BaL-PNGS/SKL etc.).

422 Preparation of infectious clones and viral replication assays in PM1/CCR5 cells

Approximately 5 μg of the plasmids from the env mutants were transfected into 293T cells using the Effectene transfection reagent (QIAGEN). At 48 h after transfection, the virus-containing supernatants were harvested, filtered and frozen in aliquots at -150 °C. Viral yields were quantified using the HIV-1 p24 antigen ELISA (ZeptoMetrix). PM1/CCR5 cells ($3 \times 10^4$) were exposed to pWT/BaL env chimeric viruses corresponding to 2 ng or 10 ng of p24 for 4 h at 37 °C. Following incubation, cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FCS and cultured for 6 days. Viral replication was monitored by measuring concentration of p24 antigen in culture supernatants.

Statistical analysis

Statistical correlations were analysed using Student's t test. $P$ values $< 0.05$ were considered statistically significant.

Nucleotide sequence accession numbers

The sequence data of env expression vectors from passaged samples have been deposited in the DNA Data Bank of Japan under accession numbers AB521136–AB521148.

439 **Acknowledgments**

440 We thank the Chemo-Sero-Therapeutic Research Institute for kindly providing us with
441 KD-247. The following reagents were obtained through the ARRRP: TZM-bl and
442 pSG3$^{\Delta env}$ from Dr. Kappes and Dr.Wu; pWT/BaL from Dr.Cullen. This work was
443 supported in part by the Program of Founding Research Centres for Emerging and
444 Re-emerging Infectious Diseases and by the Global COE program Global Education
445 and Research Center Aiming at the Control of AIDS and by a grant-in-aid for scientific
446 research (C-21591295) supported by the Ministry of Education, Science, Sports and
447 Culture, Japan; and a grant from the Ministry of Health, Welfare and Labour of Japan
448 (H21-AIDS-010).

449

## References


**Anastassopoulou, C. G., Ketas, T. J., Klasse, P. J. & Moore, J. P. (2009).** Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41. *Proc Natl Acad Sci U S A* **106**, 5318-5323.

**Baba, M., Miyake, H., Wang, X., Okamoto, M. & Takashima, K. (2007).** Isolation and characterization of human immunodeficiency virus type 1 resistant to the small-molecule CCR5 antagonist TAK-652. *Antimicrob Agents Chemother* **51**, 707-715.

**Berro, R., Sanders, R. W., Lu, M., Klasse, P. J. & Moore, J. P. (2009).** Two HIV-1 variants resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. *PLoS Pathog* **5**, e1000548.

**Bontjer, I., Land, A., Eggink, D., Verkade, E., Tuin, K., Baldwin, C., Pollakis, G., Paxton, W. A., Braakman, I., Berkhout, B. & Sanders, R. W. (2009).** Optimization of human immunodeficiency virus type 1 envelope glycoproteins with V1/V2 deleted, using virus evolution. *J Virol* **83**, 368-383.

**Bunnik, E. M., Pisas, L., van Nuenen, A. C. & Schuitemaker, H. (2008).** Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. *J Virol* **82**, 7932-7941.

**Cao, Y., Qin, L., Zhang, L., Safrit, J. & Ho, D. D. (1995).** Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med* **332**, 201-208.

**Cutalo, J. M., Deterding, L. J. & Tomer, K. B. (2004).** Characterization of glycopeptides from HIV-I(SF2) gp120 by liquid chromatography mass spectrometry. *J Am Soc Mass Spectrom* **15**, 1545-1555.

**Deeks, S. G., Schweighardt, B., Wrin, T., Galovich, J., Hoh, R., Sinclair, E., Hunt, P., McCune, J. M., Martin, J. N., Petropoulos, C. J. & Hecht, F. M. (2006).** Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol* **80**, 6155-6164.

**Eda, Y., Takizawa, M., Murakami, T., Maeda, H., Kimachi, K., Yonemura, H., Koyanagi, S., Shiosaki, K., Higuchi, H., Makizumi, K., Nakashima, T., Osatomi, K., Tokiyoshi, S., Matsushita, S., Yamamoto, N. & Honda, M. (2006).** Sequential immunization with V3 peptides from primary human

485 immunodeficiency virus type 1 produces cross-neutralizing antibodies against
486 primary isolates with a matching narrow-neutralization sequence motif. *J Virol*
487 **80**, 5552-5562.
488 **Frost, S. D., Wrin, T., Smith, D. M., Kosakovsky Pond, S. L., Liu, Y., Paxinos, E.,**
489 **Chappey, C., Galovich, J., Beauchaine, J., Petropoulos, C. J., Little, S. J. &**
490 **Richman, D. D. (2005).** Neutralizing antibody responses drive the evolution of
491 human immunodeficiency virus type 1 envelope during recent HIV infection.
492 *Proc Natl Acad Sci U S A* **102**, 18514-18519.
493 **Gartner, S., Markovits, P., Markovitz, D. M., Kaplan, M. H., Gallo, R. C. &**
494 **Popovic, M. (1986).** The role of mononuclear phagocytes in HTLV-III/LAV
495 infection. *Science* **233**, 215-219.
496 **Gorny, M. K., Revesz, K., Williams, C., Volsky, B., Louder, M. K., Anyangwe, C.**
497 **A., Krachmarov, C., Kayman, S. C., Pinter, A., Nadas, A., Nyambi, P. N.,**
498 **Mascola, J. R. & Zolla-Pazner, S. (2004).** The V3 loop is accessible on the
499 surface of most human immunodeficiency virus type 1 primary isolates and serves
500 as a neutralization epitope. *J Virol* **78**, 2394-2404.
501 **Guex, N., Diemand, A. & Peitsch, M. C. (1999).** Protein modelling for all. *Trends*
502 *Biochem Sci* **24**, 364-367.
503 **Hope, T. J., Huang, X. J., McDonald, D. & Parslow, T. G. (1990).** Steroid-receptor
504 fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping
505 cryptic functions of the arginine-rich motif. *Proc Natl Acad Sci U S A* **87**,
506 7787-7791.
507 **Hwang, S. S., Boyle, T. J., Lyerly, H. K. & Cullen, B. R. (1991).** Identification of the
508 envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science*
509 **253**, 71-74.
510 **Johnson, W. E. & Desrosiers, R. C. (2002).** Viral persistence: HIV's strategies of
511 immune system evasion. *Annu Rev Med* **53**, 499-518.
512 **Joos, B., Trkola, A., Kuster, H., Aceto, L., Fischer, M., Stiegler, G., Armbruster,**
513 **C., Vcelar, B., Katinger, H. & Gunthard, H. F. (2006).** Long-term
514 multiple-dose pharmacokinetics of human monoclonal antibodies (MAbs) against
515 human immunodeficiency virus type 1 envelope gp120 (MAb 2G12) and gp41
516 (MAbs 4E10 and 2F5). *Antimicrob Agents Chemother* **50**, 1773-1779.
517 **Krachmarov, C., Pinter, A., Honnen, W. J., Gorny, M. K., Nyambi, P. N.,**
518 **Zolla-Pazner, S. & Kayman, S. C. (2005).** Antibodies that are cross-reactive for
519 human immunodeficiency virus type 1 clade a and clade B V3 domains are

common in patient sera from Cameroon, but their neutralization activity is usually restricted by epitope masking. *J Virol* **79**, 780-790.

**Kuhmann, S. E., Pugach, P., Kunstman, K. J., Taylor, J., Stanfield, R. L., Snyder, A., Strizki, J. M., Riley, J., Baroudy, B. M., Wilson, I. A., Korber, B. T., Wolinsky, S. M. & Moore, J. P. (2004).** Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. *J Virol* **78**, 2790-2807.

**Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., Steenbeke, T. D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P. W., Robinson, J., Van Ryk, D., Wang, L., Burton, D. R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W. A. & Arthos, J. (2002).** HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**, 678-682.

**Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K. M., Bilska, M., Kothe, D. L., Salazar-Gonzalez, J. F., Wei, X., Decker, J. M., Hahn, B. H. & Montefiori, D. C. (2005).** Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol* **79**, 10108-10125.

**Mahalanabis, M., Jayaraman, P., Miura, T., Pereyra, F., Chester, E. M., Richardson, B., Walker, B. & Haigwood, N. L. (2009).** Continuous viral escape and selection by autologous neutralizing antibodies in drug-naive human immunodeficiency virus controllers. *J Virol* **83**, 662-672.

**Manrique, A., Rusert, P., Joos, B., Fischer, M., Kuster, H., Leemann, C., Niederost, B., Weber, R., Stiegler, G., Katinger, H., Gunthard, H. F. & Trkola, A. (2007).** In vivo and in vitro escape from neutralizing antibodies 2G12, 2F5, and 4E10. *J Virol* **81**, 8793-8808.

**Marozsan, A. J., Kuhmann, S. E., Morgan, T., Herrera, C., Rivera-Troche, E., Xu, S., Baroudy, B. M., Strizki, J. & Moore, J. P. (2005).** Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). *Virology* **338**, 182-199.

**Masuda, T., Matsushita, S., Kuroda, M. J., Kannagi, M., Takatsuki, K. & Harada, S. (1990).** Generation of neutralization-resistant HIV-1 in vitro due to amino acid interchanges of third hypervariable env region. *J Immunol* **145**, 3240-3246.

**McCaffrey, R. A., Saunders, C., Hensel, M. & Stamatatos, L. (2004).** N-linked glycosylation of the V3 loop and the immunologically silent face of gp120

protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. *J Virol* **78**, 3279-3295.

**Montefiori, D. C., Hill, T. S., Vo, H. T., Walker, B. D. & Rosenberg, E. S. (2001).** Neutralizing antibodies associated with viremia control in a subset of individuals after treatment of acute human immunodeficiency virus type 1 infection. *J Virol* **75**, 10200-10207.

**Moore, J. P., Cao, Y., Qing, L., Sattentau, Q. J., Pyati, J., Koduri, R., Robinson, J., Barbas, C. F., 3rd, Burton, D. R. & Ho, D. D. (1995).** Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J Virol* **69**, 101-109.

**Nabatov, A. A., Pollakis, G., Linnemann, T., Kliphius, A., Chalaby, M. I. & Paxton, W. A. (2004).** Intrapatient alterations in the human immunodeficiency virus type 1 gp120 V1V2 and V3 regions differentially modulate coreceptor usage, virus inhibition by CC/CXC chemokines, soluble CD4, and the b12 and 2G12 monoclonal antibodies. *J Virol* **78**, 524-530.

**Ogert, R. A., Wojcik, L., Buontempo, C., Ba, L., Buontempo, P., Ralston, R., Strizki, J. & Howe, J. A. (2008).** Mapping resistance to the CCR5 co-receptor antagonist vicriviroc using heterologous chimeric HIV-1 envelope genes reveals key determinants in the C2-V5 domain of gp120. *Virology* **373**, 387-399.

**Park, E. J., Vujcic, L. K., Anand, R., Theodore, T. S. & Quinnan, G. V., Jr. (1998).** Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. *J Virol* **72**, 7099-7107.

**Pinter, A., Honnen, W. J., D'Agostino, P., Gorny, M. K., Zolla-Pazner, S. & Kayman, S. C. (2005).** The C108g epitope in the V2 domain of gp120 functions as a potent neutralization target when introduced into envelope proteins derived from human immunodeficiency virus type 1 primary isolates. *J Virol* **79**, 6909-6917.

**Pinter, A., Honnen, W. J., He, Y., Gorny, M. K., Zolla-Pazner, S. & Kayman, S. C. (2004).** The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. *J Virol* **78**, 5205-5215.

**Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B. & Kabat, D. (1998).** Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol* **72**, 2855-2864.

592 **Pugach, P., Kuhmann, S. E., Taylor, J., Marozsan, A. J., Snyder, A., Ketas, T.,**
593 **Wolinsky, S. M., Korber, B. T. & Moore, J. P. (2004).** The prolonged culture of
594 human immunodeficiency virus type 1 in primary lymphocytes increases its
595 sensitivity to neutralization by soluble CD4. *Virology* **321**, 8-22.
596 **Richman, D. D., Wrin, T., Little, S. J. & Petropoulos, C. J. (2003).** Rapid evolution
597 of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci*
598 *U S A* **100**, 4144-4149.
599 **Sagar, M., Wu, X., Lee, S. & Overbaugh, J. (2006).** Human immunodeficiency virus
600 type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over
601 the course of infection, and these modifications affect antibody neutralization
602 sensitivity. *J Virol* **80**, 9586-9598.
603 **Saunders, C. J., McCaffrey, R. A., Zharkikh, I., Kraft, Z., Malenbaum, S. E.,**
604 **Burke, B., Cheng-Mayer, C. & Stamatatos, L. (2005).** The V1, V2, and V3
605 regions of the human immunodeficiency virus type 1 envelope differentially affect
606 the viral phenotype in an isolate-dependent manner. *J Virol* **79**, 9069-9080.
607 **Shibata, J., Yoshimura, K., Honda, A., Koito, A., Murakami, T. & Matsushita, S.**
608 **(2007).** Impact of V2 mutations on escape from a potent neutralizing anti-V3
609 monoclonal antibody during in vitro selection of a primary human
610 immunodeficiency virus type 1 isolate. *J Virol* **81**, 3757-3768.
611 **Sullivan, N., Thali, M., Furman, C., Ho, D. D. & Sodroski, J. (1993).** Effect of
612 amino acid changes in the V1/V2 region of the human immunodeficiency virus
613 type 1 gp120 glycoprotein on subunit association, syncytium formation, and
614 recognition by a neutralizing antibody. *J Virol* **67**, 3674-3679.
615 **Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique,**
616 **A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B.,**
617 **Katinger, H., Aceto, L. & Gunthard, H. F. (2005).** Delay of HIV-1 rebound
618 after cessation of antiretroviral therapy through passive transfer of human
619 neutralizing antibodies. *Nat Med* **11**, 615-622.
620 **Trkola, A., Kuster, H., Rusert, P., von Wyl, V., Leemann, C., Weber, R., Stiegler,**
621 **G., Katinger, H., Joos, B. & Gunthard, H. F. (2008).** In vivo efficacy of human
622 immunodeficiency virus neutralizing antibodies: estimates for protective titers. *J*
623 *Virol* **82**, 1591-1599.
624 **Wang, F. X., Kimura, T., Nishihara, K., Yoshimura, K., Koito, A. & Matsushita, S.**
625 **(2002).** Emergence of autologous neutralization-resistant variants from
626 preexisting human immunodeficiency virus (HIV) quasi species during virus

627 rebound in HIV type 1-infected patients undergoing highly active antiretroviral
628 therapy. *J Infect Dis* **185**, 608-617.
629 **Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S.,**
630 **Wu, X., Shaw, G. M. & Kappes, J. C. (2002).** Emergence of resistant human
631 immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20)
632 monotherapy. *Antimicrob Agents Chemother* **46**, 1896-1905.
633 **Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez,**
634 **J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M.**
635 **A., Hahn, B. H., Kwong, P. D. & Shaw, G. M. (2003).** Antibody neutralization
636 and escape by HIV-1. *Nature* **422**, 307-312.
637 **Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M.,**
638 **Dorr, P., Ciaramella, G. & Perros, M. (2007).** Reduced maximal inhibition in
639 phenotypic susceptibility assays indicates that viral strains resistant to the CCR5
640 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* **81**,
641 2359-2371.
642 **Yoshimura, K., Shibata, J., Kimura, T., Honda, A., Maeda, Y., Koito, A.,**
643 **Murakami, T., Mitsuya, H. & Matsushita, S. (2006).** Resistance profile of a
644 neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable
645 synergism with anti-CCR5 inhibitors. *AIDS* **20**, 2065-2073.
646 **Yusa, K., Maeda, Y., Fujioka, A., Monde, K. & Harada, S. (2005).** Isolation of
647 TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. *J Biol Chem*
648 **280**, 30083-30090.
649 **Zhu, X., Borchers, C., Bienstock, R. J. & Tomer, K. B. (2000).** Mass spectrometric
650 characterization of the glycosylation pattern of HIV-gp120 expressed in CHO
651 cells. *Biochemistry* **39**, 11194-11204.
652 **Zolla-Pazner, S. (2004).** Identifying epitopes of HIV-1 that induce protective
653 antibodies. *Nat Rev Immunol* **4**, 199-210.
654

655

656 **legends**

657 Fig. 1. Amino acid sequences deduced from the nucleotide sequences of env-encoding

658 regions of proviral DNA isolated at the baseline (A), and indicated passages (p5, p6 and

659 p16) from HIV-$1_{BaL}$ variants selected in the presence of KD-247 and (B) the passage

660 control (p16).The amino acid sequences of the envelope proteins of a clone of HIV-$1_{BaL}$

661 at baseline are shown at the top as a reference. Identity to the sequence at individual

662 amino acid positions is indicated by dots. The numbers of clones with the given amino

663 acid substitutions among a total of 61 clones (A) and 11–20 clones (B) are listed.

664

665 Fig. 2. The gp120 mutation profile of HIV-$1_{BaL}$ evasion variants from KD-247 *in vitro*.

666 The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3

667 regions in gp120 of HIV-$1_{BaL}$ variants were plotted for each passage. The y-axis

668 indicates the percentage of PNGS insertions or mutations in the tested clones and the

669 x-axis shows the concentration of KD-247 ($\mu g\ ml^{-1}$).

670

671 Fig. 3. Schematic representation of recombinant HIV-$1_{BaL}$ env genes used for analysis

672 of the genetic basis for resistance to KD-247.

673 Mutated env genes were amplified from passaged HIV-$1_{BaL}$ virus-infected PM1/CCR5
674 cells in the absence or presence of KD-247. The recombinant env genes were
675 constructed by replacing each region of passaged control with corresponding sequence
676 of escaped variant of HIV-$1_{BaL}$ or by site-directed mutagenesis. The locations and
677 numbers of specific amino acids, based on the HXB2 sequence, are shown above the
678 reference HIV-$1_{BaL}$ sequence.

679

680 Fig. 4. Sensitivities of HIV-1 strains pseudotyped with recombinant HIV-$1_{BaL}$ env genes
681 to KD-247, 2D7, rsCD4 and CCR5 inhibitor.

682 KD-247, 2D7 (anti-CCR5 MAb), rsCD4 and maraviroc (CCR5 inhibitor) were
683 pre-incubated with 300 $TCID_{50}$ of each HIV-$1_{BaL}$ pseudotype virus for 30 min, then
684 added to TZM-bl target cells. Inhibitory effects were determined by measuring
685 β-galactosidase activity on day 2 of culture.

686

687 Fig. 5. Viral infectivity of HIV-1 infectious clones with recombinant HIV-$1_{BaL}$ mutant
688 env genes.

689 HIV-1 infectious clones with the env gene sequences listed in Figure 3 were prepared as
690 described in the Materials and Methods. PM1/CCR5 cells were exposed to the
691 infectious clones [input p24 amount; (A) 2 ng and (B, C) 10 ng ] and cultured for 6 days
692 in the presence or absence of KD-247. The replication of the infectious clones with
693 mutant Env was monitored by measuring the amounts of p24 Gag protein produced in
694 the culture supernatants in the absence (A) or presence (B) of KD-247. The clones with
695 a PNGS and various other mutations were also monitored in the absence of KD-247 (C).

696

697 Fig. 6. The gp120 mutation profile of KD-247 HIV-$1_{BaL}$ evasion variants for 14
698 additional passages without KD-247.

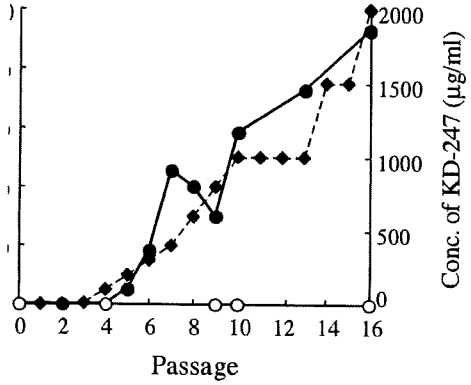
699 The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3 region
700 of HIV-$1_{BaL}$ evasion variants in gp120 were plotted for 16 passages in the presence of
701 KD-247 and an additional 14 passages in the absence of the MAb. The y-axis shows the
702 percentage of PNGS insertions or mutations in the tested clones. The x-axis shows the
703 concentration of KD-247.

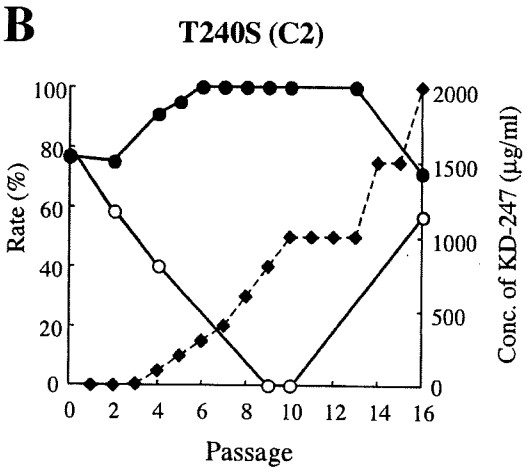
A

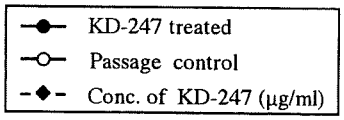
| virus | V2 | C2 | | V3 |
|---|---|---|---|---|
| | 186 | 240 | 283 | 315 317 319 |
| | VPIDN-KIDRY | GPCTNVSTVQ | ENFTNNASKI | IHIGPGRAFYTT |
| 8/61 | .....-..... | .......... | .......... | ..... |
| 6/61 | .....-..... | ...S...... | .......... | ..... |
| 5/61 | .....-..... | ...S...... | .........T | .....L.A. |
| 4/61 | .....-..... | .......... | A..AD..... | ..... |
| 3/61 | .....-..... | .......... | .......... | .....L.A. |
| 3/61 | .....-..... | ...S...... | A..AD..... | .....L.A. |
| 3/61 | .....-..... | ...S...... | .........T | .....A. |
| 3/61 | .....-..... | ...S...... | A..AD..... | ..... |
| 3/61 | .....-..... | ...S...... | .........T | ..... |
| 2/61 | .....-..... | ...S...... | .......... | .....A. |
| 2/61 | .....-..... | ...S...... | .......... | .N.....L... |
| 2/61 | .....-..... | ...S...... | A..AD..... | .....A. |
| 1/61 | .....-..... | ...S...... | ....D..... | ..... |
| 1/61 | ..V..-..... | .......... | .......... | ..... |
| 1/61 | .....-..... | ...S...... | ....S....T | .....A. |
| 1/61 | .....-..... | ...S...... | A..A...... | .....L... |
| 1/61 | .....-..... | ...S...... | A...D..... | ..... |
| 1/61 | .....-..... | .......... | .........T | .N.....L... |
| 1/61 | .....-..... | .......... | .......... | .....L... |
| 1/61 | .....-..... | ...S...... | A...D..... | .....A. |
| 1/61 | .....-..... | ...S...... | .......... | .N.....L... |
| 1/61 | .....-..... | .......... | .........T | M..... |
| 1/61 | .....-..... | ...S...... | .......... | .....L... |
| 1/61 | .....-..... | .......... | .........T | ..... |
| 1/61 | .....-..... | ...S...... | A..AD....T | ..... |
| 1/61 | .....-....K | ...S...... | A..AD..... | ..... |
| 1/61 | ....KSNN... | ...S...... | A..AD..... | .....A. |
| 1/61 | .....-..... | ..RS...... | A..AD....T | .....A. |
| 1/61 | .....-..... | ...S...... | A..AD..... | T.....A. |

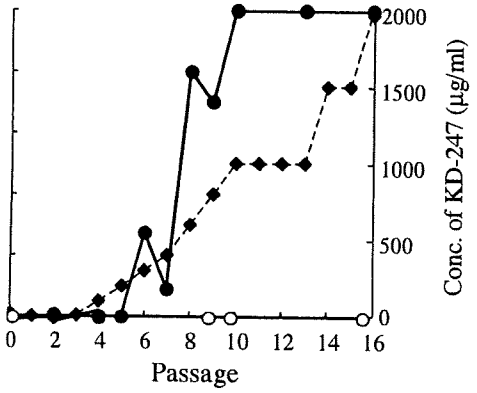
B

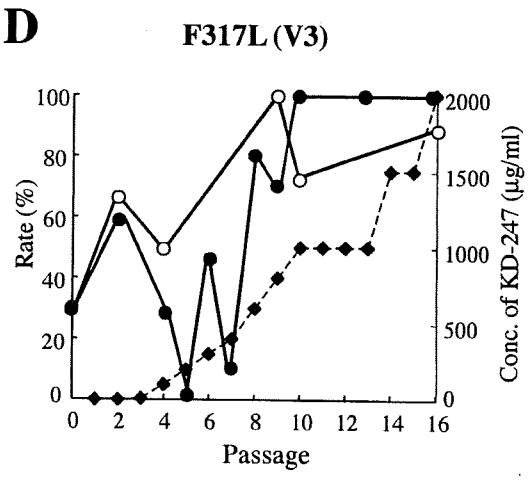
| | | V2 | C2 | | V3 |
|---|---|---|---|---|---|
| | | 186 | 240 | 283 | 315 317 319 |
| | | VPIDN-KIDRY | GPCTNVSTVQ | ENFTNNASKI | IHIGPGRAFYTT |
| 7 selection | | | | | |
| 00) p5 | 15/20 | .....-..... | ...S..... | .....T | .....A. |
| 00) p5 | 1/20 | .....-..... | ...S...I.. | .....T | .....A. |
| 00) p5 | 1/20 | .....-..... | ...S..... | .....T | .N.....L... |
| 00) p5 | 1/20 | .....-..... | ...S..N... | .....T | .....A. |
| 00) p5 | 1/20 | .....NSNN.. | ...S..... | .....T | .....A. |
| 00) p5 | 1/20 | .....-..... | ...S..... | .....T | .N..... |
| 00) p6 | 3/11 | .....-..... | ...S..... | ..... | .....K.L... |
| 00) p6 | 2/11 | .....-..... | ...S..... | .....T | .....A. |
| 00) p6 | 2/11 | .....NSNN.. | ...S..... | ..... | .....A. |
| 00) p6 | 1/11 | .....-..... | ...S..... | ..... | .....L... |
| 00) p6 | 1/11 | .....-..... | ...S..... | ..S.....T | .N....K.L... |
| 00) p6 | 1/11 | .....-..... | ...S..... | .....T | .N..... |
| 00) p6 | 1/11 | .....-..... | ...S..... | ..... | .....L.A. |
| 000) p16 | 4/14 | .....NSNN.. | ...S..... | ..... | .....K.L.A. |
| 000) p16 | 3/14 | .....NSNN.. | ..... | ..... | .....K.L.A. |
| 000) p16 | 2/14 | .....NSNN.. | ...S..... | ..... | .....K.L... |
| 000) p16 | 2/14 | .....NSNN.. | ...S..... | ..... | .....DK.L... |
| 000) p16 | 1/14 | .....NSNN.. | ...S..... | .....T | .....K.L... |
| 000) p16 | 1/14 | .....NSNN.. | ..... | ..... | .....KTL... |
| 000) p16 | 1/14 | .....-..... | ...S..... | ..... | .....K.L... |
| e control | | | | | |
| ) p16 | 4/13 | .....-..... | ..... | ..... | .N.....L... |
| ) p16 | 3/13 | .....-..... | ...S..... | ..... | .N.....L... |
| ) p16 | 1/13 | .....-..... | ...S..G... | ..... | .N.....L... |
| ) p16 | 1/13 | .....-..... | ...S..... | ..... | ..... |
| ) p16 | 1/13 | .....-.N... | ...S..... | ..... | .....L... |
| ) p16 | 1/13 | .....-..... | ..... | ..... | .....L... |
| ) p16 | 1/13 | .....-..... | ..... | ..... | .....L.A. |
| ) p16 | 1/13 | .....-..... | ...S..R... | ..... | .....L... |

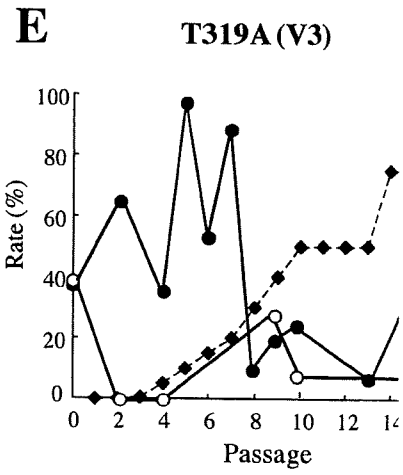

186PNGS (V2)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage
0 2 4 6 8 10 12 14 16
0 500 1000 1500 2000

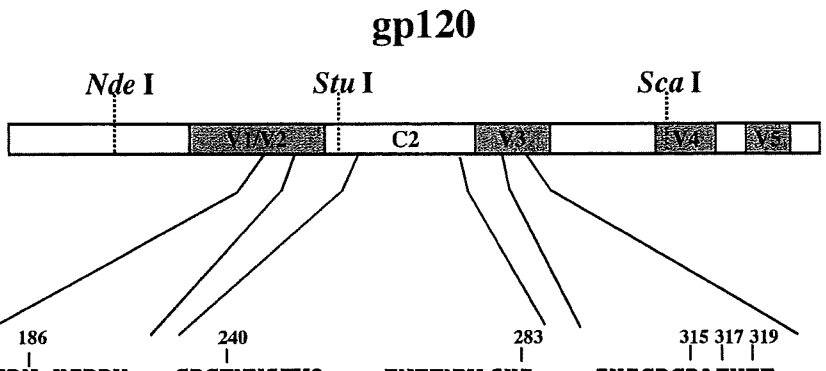

B
T240S (C2)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage
0 2 4 6 8 10 12 14 16
0 20 40 60 80 100
0 500 1000 1500 2000


KD-247 treated
Passage control
Conc. of KD-247 (μg/ml)


R315K (V3)
Conc. of KD-247 (μg/ml)
Passage
0 2 4 6 8 10 12 14 16
0 500 1000 1500 2000

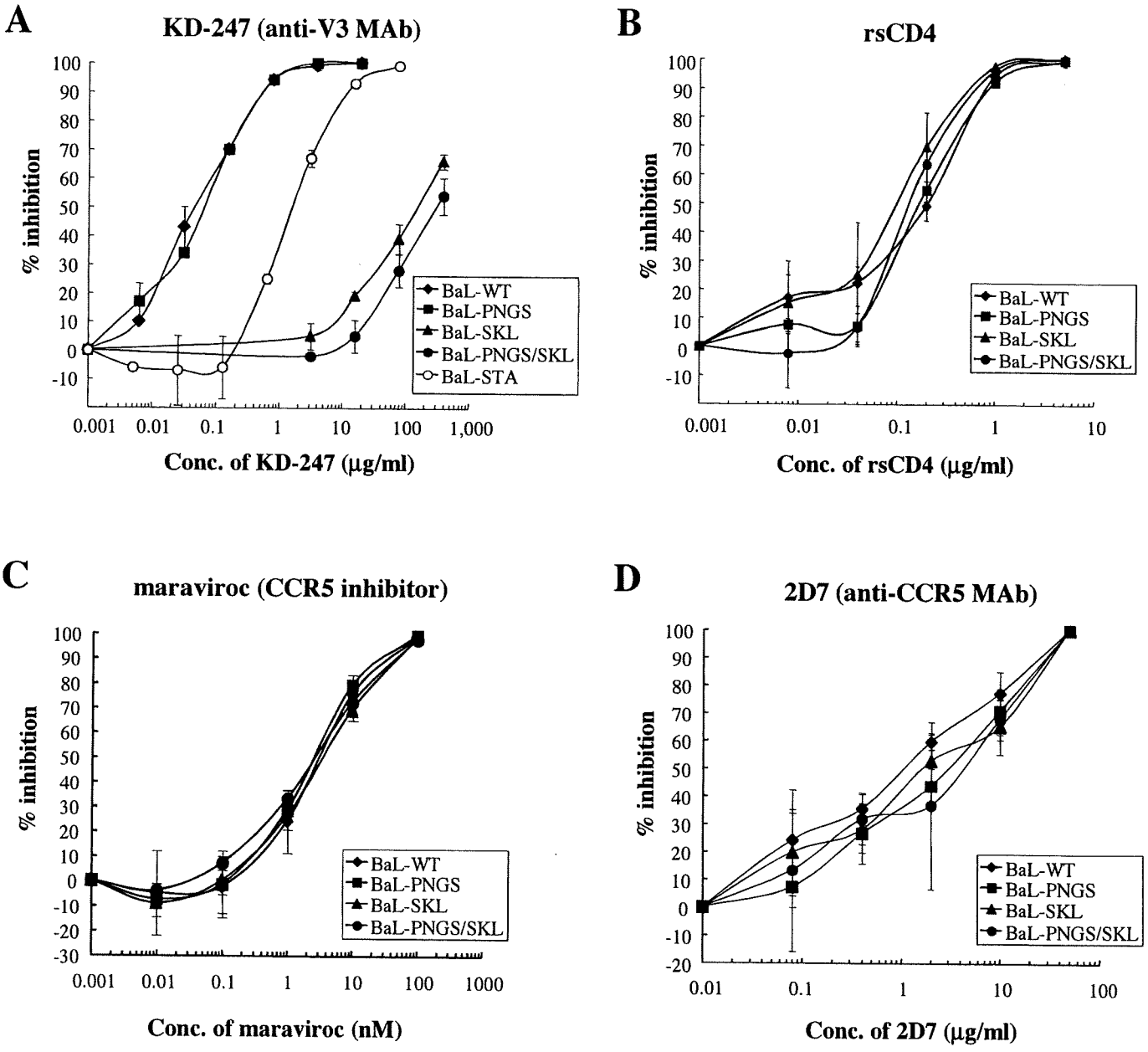

D
F317L (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage
0 2 4 6 8 10 12 14 16
0 20 40 60 80 100
0 500 1000 1500 2000


E
T319A (V3)
Rate (%)
Passage
0 2 4 6 8 10 12
0 20 40 60 80 100




| | | | | | |
|---|---|---|---|---|---|
| WT | VPIDN-KIDRY | GPCTNVSTVQ | ENFTNNASKI | IHIGPGRAFYTT | |
| STA | .....-..... | ...S..... | .....T | .....A. | T240S+I283T+T319A |
| PNGS/SKL | .....NSNN.. | ...S..... | ..... | .....K.L... | 186PNGS+T240S+R315K+ |
| PNGS | .....NSNN.. | ..... | ..... | ..... | 186PNGS |
| SKL | .....-..... | ...S..... | ..... | .....K.L... | T240S+R315K+F317L |
| S | .....-..... | ...S..... | ..... | ..... | T240S |
| K | .....-..... | ..... | ..... | .....K..... | R315K |
| L | .....-..... | ..... | ..... | .....L... | F317L |
| PNGS/S | .....NSNN.. | ...S..... | ..... | ..... | 186PNGS+T240S |
| PNGS/K | .....NSNN.. | ..... | ..... | .....K..... | 186PNGS+R315K |
| PNGS/L | .....NSNN.. | ..... | ..... | .....L... | 186PNGS+F317L |
| Q | ....QNSNN.. | ..... | ..... | ..... | 186Q |
| Q/K | ....QNSNN.. | ..... | ..... | .....K..... | 186Q+R315K |

Insertion and/or mutations


A
KD-247 (anti-V3 MAb)
% inhibition
Conc. of KD-247 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL
BaL-STA
B
rsCD4
% inhibition
Conc. of rsCD4 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL
C
maraviroc (CCR5 inhibitor)
% inhibition
Conc. of maraviroc (nM)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL
D
2D7 (anti-CCR5 MAb)
% inhibition
Conc. of 2D7 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL