independent experiments, and replication rates were averaged. The experimental procedure is illustrated in the Supplemental Material (see **Figure 1 Supplemental Digital Content 1**, http://links.lww.com/QAI/A109).

Resequencing of Recombinant Viral Stocks

For all bulk (quasispecies-containing) recombinant viruses, HIV-1 RNA was extracted from viral culture supernatant (QIAamp viral RNA kit; Qiagen), amplified and sequenced as described in[4] and compared to the original plasma HIV-1 RNA sequences (Fig. 1). All viruses were confirmed as subtype B using RIP (http://www.hiv.lanl.gov). Nucleotide alignments were performed using GeneCutter and maximum-likelihood phylogenetic trees were generated using PHYml[31] (both available at http://www.hiv.lanl.gov). Trees were visualized using Figtree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/). Resistance mutations were identified using the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/). Most controller-derived RT–Integrase sequences were previously deposited in Genbank.[4] Accession numbers for the remaining sequences from controllers and progressors are GQ284657-GQ284730.

Statistical Analysis

Student *T* test was used to compare differences in RC between groups (eg, controllers/progressors; presence/absence of HLA alleles, etc). Spearman and Pearson correlation was used to investigate the relationship between clinical parameters (CD4/pVL) and the presence of HLA-associated escape mutations, respectively, and viral RC. In an exploratory analysis, the Mann-Whitney *U* Test was used to identify specific amino acids in RT–integrase associated with RC; here, *Q* values were used to address multiple tests.[32]

RESULTS

Generation of Recombinant Viruses Expressing RT–Integrase Sequences From Controllers and Progressors

Recombinant viruses were generated using bulk patient plasma-derived PCR amplicons containing RT and integrase sequences and stocks harvested during the early phase of exponential viral spread as described in Methods. For elite controllers (n = 58), the median time to harvest was 25 days

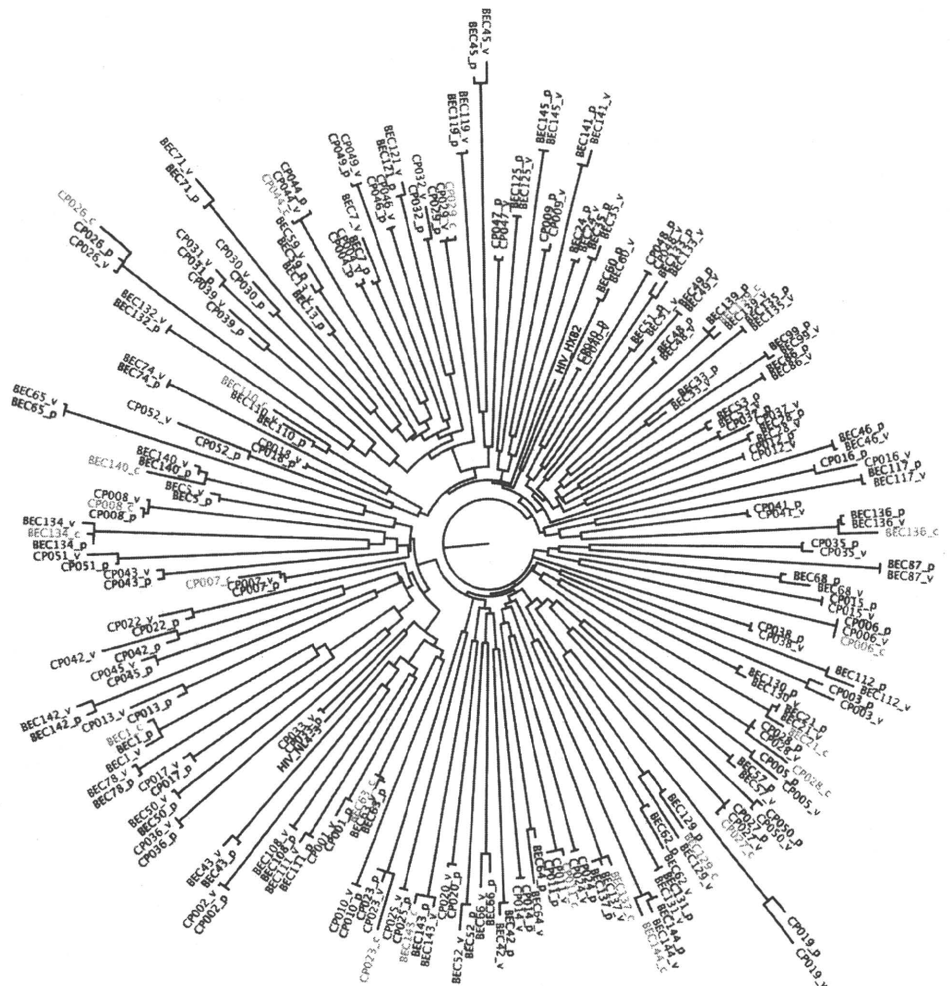

FIGURE 1. Phylogenetic tree illustrating the relationship between HIV-1 RT-Integrase sequences from plasma and recombinant viruses from study subjects. Prefixes "BEC" and "CP" discriminate sequences from controllers and progressors, respectively. Red (suffix "p"), blue (suffix "v"), and green (suffix "c") sequences indicate bulk plasma, bulk recombinant, and clonal recombinant virus sequences, respectively. The sequences of HIV-1 subtype B reference strains HXB2 and NL43 are included for comparison.

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(IQR: 21–28), whereas for progressors (n = 50), it was 19 days (IQR: 15–21). This difference was statistically significant ($P < 0.0001$). The RT–integrase sequences of the recombinant virus and the original plasma HIV RNA were highly concordant (Fig. 1). The median number of full amino acid differences between plasma and recombinant sequences was 1 (IQR: 0–2) of a total of 849 codons spanning RT–integrase, a similarity of 99.9%. In a more conservative analysis where amino acid mixtures were considered full differences, the median number of differences was 6 (IQR: 3–9) (99.3% similarity); values that are comparable to the average interlaboratory nucleotide concordance of sequence-based genotypic drug resistance assays (99.4%).[33] These data indicate that our approach did not result in substantial in vitro selection and that at least some quasispecies diversity was maintained in the recombinant viral stocks.

Reduced RC of RT–Integrase Viruses Derived From Elite Controllers

RC of bulk (quasispecies-containing) recombinants was assayed in 3 independent experiments and results reported as the mean. Concordance between replicates was high [$R = 0.77$, $P < 0.0001$; with a median difference between replicates of 8.03% (IQR: 3.76%–16.4%)].

Controller-derived RT–integrase recombinant viruses constructed using bulk (quasispecies-containing) methods displayed significantly reduced RC compared with those derived from progressors (Figs. 2A, B). The median RC of controller viruses was 0.83 (IQR: 0.63–0.96) compared with 0.98 (0.89–1.07) for progressor viruses ($P < 0.0001$). To assess whether this result may be affected by the diversity of the recombinant quasispecies, we stratified recombinant virus sequences based on the presence or absence of amino acid mixtures in the RT–integrase region, but observed no significant differences in RC between the 2 in either controllers or progressors (Fig. 2C). The overall differences in RC between controllers and progressors remained highly statistically significant regardless of the presence or absence of amino acid mixtures (ANOVA $P < 0.0001$).

To further rule out potential bias due to the quantity and/or diversity of the initial bulk PCR inoculum between controllers and progressors, and the potential existence of minority quasispecies in the recombinant virus stocks, we generated clonal recombinant viruses from a randomly selected subset of 14 controllers and 10 progressors. Consistent with the original findings, median RC of clonal controller viruses was 0.73 (IQR: 0.61–0.98) compared with 0.97 (0.86–1.08) in the progressors ($P = 0.028$; **Figure 2A, Supplemental Digital Content 2**, http://links.lww.com/QAI/A110). In addition, robust concordance was observed between RC of independently constructed clones (n = 10 pairs, $R = 0.84$, $P = 0.002$, not shown) and between the RC of quasispecies-containing versus clonal recombinant viruses ($R = 0.7$, $P = 0.0003$, **Figure 2B, Supplemental Digital Content 2**, http://links.lww.com/QAI/A110).

Taken together, the RT–integrase region from most controllers exhibited reduced function compared with progressors, regardless of whether quasispecies-containing or clonal recombinants were evaluated.

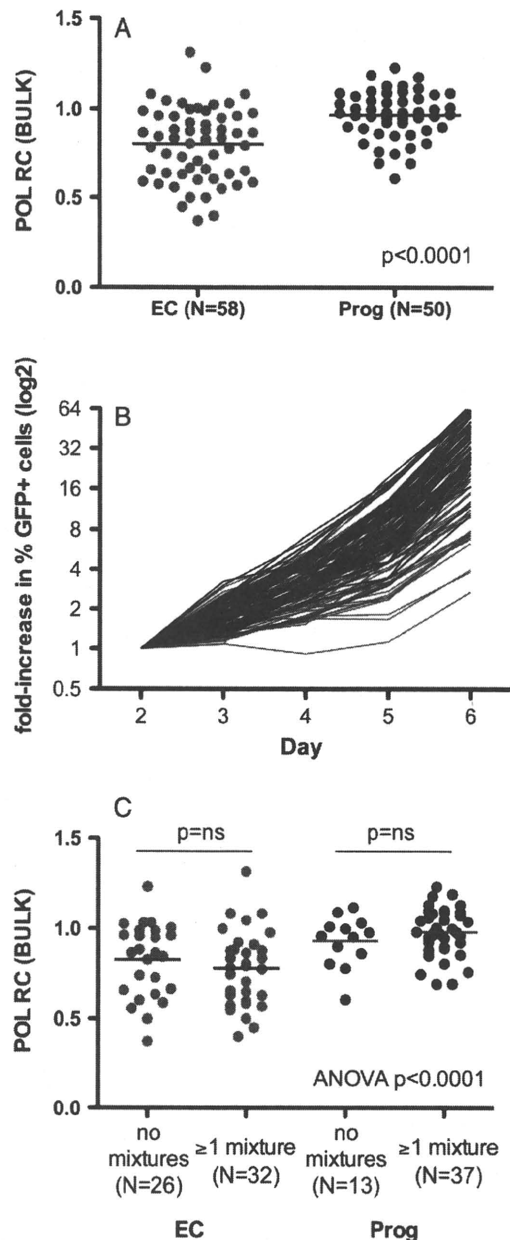

FIGURE 2. Replication capacities of bulk RT-Integrase recombinant viruses derived from elite controller and chronic progressor sequences. A, RC of bulk RT-Integrase recombinant viruses from controllers (EC) and Progressors (Prog) are shown. RC values are normalized to the mean RC of NL4-3, such that an RC of 1.0 indicates equivalent growth to NL4-3, whereas RC >1 and RC <1 indicate faster or slower growth compared with NL4-3, respectively. Results represent the average of three independent experiments. Horizontal bars indicate median values for each group. B, The rate of viral spread in culture (expressed as fold-increase in % GFP+ cells over the assay period) is shown for controller-derived (red lines) vs. progressor-derived (black lines) viruses. C, RC results of bulk RT-Integrase recombinant viruses are stratified by the absence or presence of amino acid mixtures in the recombinant viral stocks. No significant difference in RC was observed between nonmixture–containing vs. mixture-containing recombinant viruses derived from controllers or progressors.

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Reduced RC Is Not Due to Resistance Mutations

Resistance mutations can affect viral RC.[24–28] Although all patients were untreated at the time of sample collection, 8 of 58 (14%) controller-derived and 8 of 50 (16%) progressor-derived viruses harbored at least 1 major resistance mutation in RT (P = 0.8), most frequently at codons 215 (n = 9) and 219 (n = 6), K70R (n = 5), D67N (n = 4), and others. Of these 16 sequences, 10 encoded ≥2 resistance mutations. After exclusion of the 16 resistant viruses from analysis, the difference in RC between controller and progressor-derived viruses remained statistically significant ($P < 0.0001$, not shown). Furthermore, no significant differences were observed between resistant and nonresistant viruses within each patient group ($P > 0.05$), suggesting that decreased RC in controllers is not due to drug resistance mutations.

Reduced RC Does Not Correlate With Clinical Parameters, or Gag/Protease Function, in Controllers or Progressors

We next addressed whether RT–integrase function correlated with clinical parameters. Among controllers, all pVL were <50 copies/mL, and the median CD4 count was 799 (IQR: 593–1037) cells/mm^3. Among progressors, the median pVL was 4.98 (IQR: 4.51–5.35) $\log_{10}$ HIV RNA copies/mL, and the median CD4 count was 318 (IQR: 61–476) cells/mm^3. In an analysis stratified by patient group, we observed correlation neither between RC and CD4 count in controllers (Spearman $R = -0.03$, $P = 0.8$) or progressors (R = 0.06, $P = 0.7$) nor between replication and pVL in progressors ($R = 0.05$, $P = 0.7$) (not shown).

We also evaluated whether RT–integrase RC correlated with functional RC data for Gag–Protease previously obtained on a subset of these individuals (reported in[20]). Although both the previous and present study report reduced RC among controller-derived viruses, we observed no correlation between RC of Gag–Protease viruses and RT–integrase viruses in an analysis stratified by patient group [controllers (n = 38, $R = -0.09$, $P = 0.6$); progressors (n = 38, $R = 0.13$, $P = 0.4$)], suggesting that these observations are largely independent.

Associations Between RC and HLA Class I Expression

We next investigated associations between RC and HLA class I allele expression, stratified by patient group (Fig. 3). In controllers, no statistically significant associations were observed, however, the poorest replicating viruses originated from HLA-B*51- and B*57-expressing individuals ($P = 0.09$ and $P = 0.08$, respectively). Among progressors, viruses from A*01-expressing and C*02-expressing individuals displayed significantly lower RC ($P = 0.016$ and $P = 0.02$, respectively).

Correlation Between RC and HLA-Associated Sequence Polymorphisms

To further assess the impact of immune selection pressure on RT–integrase function, we investigated potential correlations between the presence of HLA-associated polymorphisms (defined according to a population-based analysis of >1500 subtype-B–infected individuals worldwide[34]) and RC. For each patient, we determined the total possible number of HLA-associated polymorphic sites in RT–integrase according to their HLA class I profile and the number of these sites that exhibited a known HLA-associated polymorphism in the recombinant virus sequence. No significant correlation was observed between RC and the overall burden of HLA-A, B, and C–associated polymorphisms in RT-Integrase assessed in absolute terms ("number of escaped

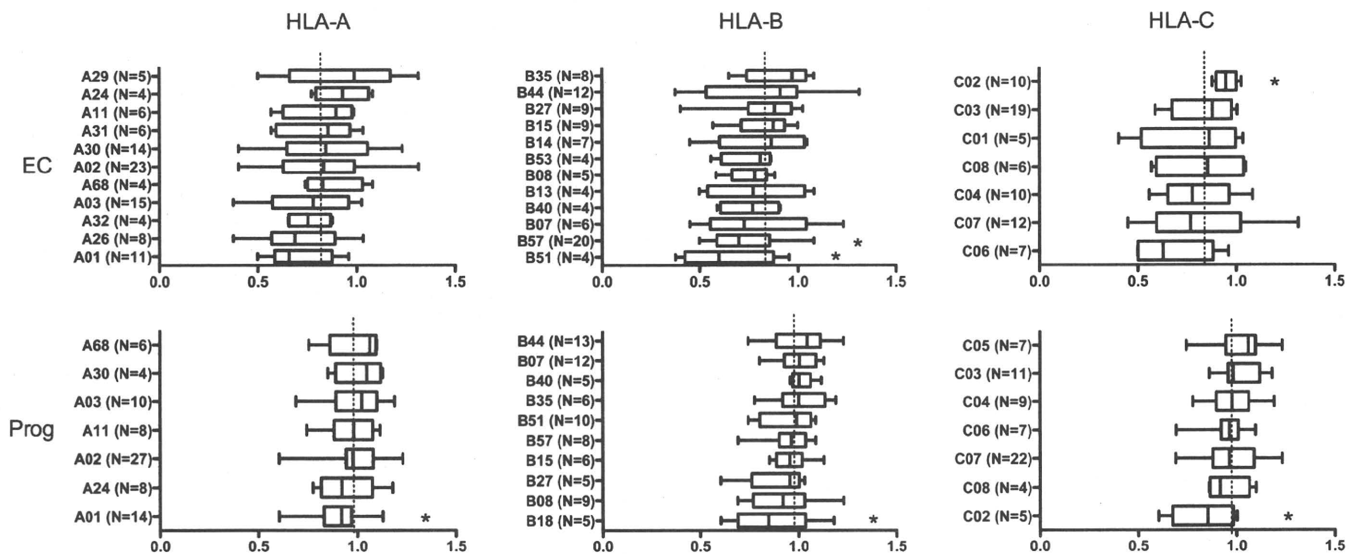

FIGURE 3. Associations between HLA class I allele expression and replication capacities of RT-integrase recombinant viruses. Box and whisker plots indicate the median (line), interquartile range (box), and range (whiskers) of viral RC, stratified by HLA class I alleles expressed. Asterisks indicate observations that trend higher or lower than the population mean, with $P < 0.1$ (Wilcoxon Rank-sum test). Dotted vertical line indicates median replication capacity for each patient group. HLA class I alleles with a minimum of n = 4 observations are shown.

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sites") or relative terms ("proportion of escaped sites") in either controllers or progressors (all $P > 0.05$, not shown).

We next investigated the relationship between HLA-associated polymorphisms and RC in an allele-specific manner, for all alleles with frequencies ≥5. Although no correlations achieved statistical significance in controllers, an inverse relationship was observed between the number of B*57-associated polymorphisms and lower RC in B*57-expressing controllers (n = 20; $R = -0.36$, $P = 0.1$) (Fig. 4A). In B*57-expressing progressors, a strong inverse relationship was observed between the number of B*57-associated RT–integrase polymorphisms and RC (n = 8, $R = -0.89$, $P = 0.003$); no other significant correlations were observed (Fig. 4B). Therefore, although the average RC of viruses derived from B*57-expressing progressors did not significantly differ from the population average, the strong dose-dependent relationship between B*57-associated polymorphisms and decreasing RC suggests that these polymorphisms negatively influence RT–integrase function in a cumulative manner.

No Association Between RC and Transmitted HLA-Associated Polymorphisms

Immune-mediated fitness defects have been reported in individuals acquiring HIV-1 from donors who express protective HLA alleles.[7,8] Therefore, we investigated whether RC correlated with protective allele-associated viral polymorphisms in individuals not expressing these alleles. No significant difference was observed in the average number of polymorphisms associated with protective HLA alleles (defined as HLA-B*13, B*27, B*51, B*57, B*5801, and B*81[35–40]) in controllers (n = 19; mean protective allele-associated polymorphisms/sequence = 8.6) versus progressors (n = 22; mean polymorphisms/sequence = 7.6; $P = 0.25$) not expressing these alleles. Moreover, no correlation was observed between the number of protective HLA-associated polymorphisms and RC in controllers ($R = 0.23$, $P = 0.34$) or progressors ($R = 0.34$, $P = 0.13$) not expressing these alleles. An analysis restricted to B*57-associated mutations in controllers and progressors not expressing this allele also failed to demonstrate significant differences in polymorphism frequency between groups, nor correlations with RC (all $P > 0.1$, not shown). Altogether, these results suggest that the observed reductions in RC seen in controllers were not likely due to transmitted immune escape mutations in RT-integrase.

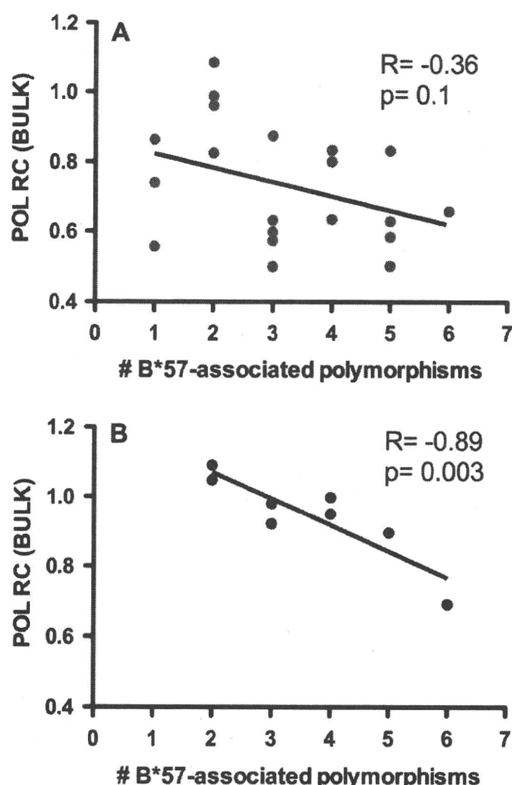

FIGURE 4. Relationship between the number of HLA-B*57-associated polymorphisms in RT-Integrase and viral Replication Capacity. In viruses from the 20 elite controllers (A) and 8 progressors who expressed HLA-B*57 (B), the relationship between the number of HLA-B*57-associated polymorphisms in RT-Integrase and viral RC was characterized using Pearson's correlation. A regression line is drawn to highlight the trend.

Associations Between RC and Specific Amino Acid Residues in RT–Integrase

In an exploratory analysis, the Mann-Whitney *U* test was used to examine associations between RT–integrase amino acid variation and RC. This analysis was performed on the entire dataset, as well as stratified by patient group. In the combined analysis of all polymorphisms occurring at frequencies ≥5, 28 residues (20 in RT, 8 in integrase) were associated with RC at $P < 0.05$ (corresponding Q values 0–0.77; Table 1). The strongest association was observed for integrase codon 265, where the consensus Alanine was associated with significantly higher RC than the polymorphism Valine ($P < 0.0001$, $Q = 0$). Stratification by patient group also identified the codon 265 association as significant in controllers ($P < 0.01$, $Q = 0.4$) but not in progressors (not shown).

DISCUSSION

We recently reported that elite controllers display defects in Gag-Protease function due in part to immune selection by protective HLA alleles, most notably B*57,[20] thus further supporting an impact of Gag escape mutations on viral fitness and HIV disease.[12–17,19,23] However, comparatively little is known about potential immune-mediated attenuation of other HIV-1 proteins and whether this might be relevant to the controller phenotype.

Here, we extend our previous findings and demonstrate that recombinant viruses encoding RT–integrase sequences derived from elite controllers displayed significantly reduced RC compared with viruses derived from untreated chronic progressors. This was true regardless of whether recombinant viruses captured quasispecies diversity or whether they were generated from cloned sequences. The observed defects in controller-derived viruses were not likely due to the presence of drug resistance mutations[24–28] nor the presence of putative transmitted immune escape mutations,[7,8] although the possibility that such mutations were transmitted but then

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TABLE 1. Exploratory Analysis of RT–Integrase Residues Associated With Viral RC

Protein	Codon	Residue	N With	N Without	MEDIAN Replication With	MEDIAN Replication Without	*P*	*Q* Value	Putative HLA/Epitope
RT	211	R	59	49	0.86	0.98	0.003	0.41	A32, A68, B15, B44
	211	K	44	64	0.98	0.87	0.004	0.41	A32, A68, B15, B44
	245	V	69	39	0.95	0.87	0.049	0.77	A29, B57, B58, B57-IW9
	294	P	99	9	0.89	1.01	0.024	0.68	—
	294	T	5	103	1.10	0.90	0.047	0.77	—
	329	I	87	21	0.88	0.96	0.036	0.71	C05
	345	P	101	7	0.88	1.04	0.010	0.48	A11, A11-IK10
	366	K	96	12	0.89	0.99	0.041	0.74	B13, A03-RK11
	366	R	12	96	0.99	0.89	0.041	0.74	B13, A03-RK11
	369	T	96	12	0.89	1.00	0.039	0.74	B13
	376	S	6	102	0.63	0.92	0.036	0.71	B53, B58, C12, B58-IW9
	381	V	102	6	0.92	0.60	0.015	0.48	C12, B58-IW9
	381	I	6	102	0.60	0.92	0.015	0.48	C12, B58-IW9
	390	R	51	57	0.96	0.86	0.032	0.71	B58
	390	K	58	50	0.87	0.97	0.033	0.71	B58
	431	T	6	102	1.04	0.89	0.024	0.68	—
	431	K	101	7	0.88	1.03	0.027	0.68	—
	449	D	5	103	1.00	0.89	0.028	0.68	A26-EY9
	452	I	12	96	0.81	0.93	0.028	0.68	B44, A26-EY9
	517	L	89	19	0.88	0.98	0.027	0.68	—
INT	20	K	6	102	1.03	0.89	0.011	0.48	—
	119	S	80	28	0.88	0.96	0.028	0.68	A01, B39, C05
	156	K	99	9	0.88	1.04	0.005	0.41	B50
	156	N	9	99	1.04	0.88	0.005	0.41	B50
	201	I	34	74	0.98	0.88	0.022	0.68	—
	201	V	75	33	0.88	0.99	0.025	0.68	—
	265	A	81	27	0.95	0.73	0.00008	0	B42-VI9, B15-RY9
	265	V	27	81	0.73	0.95	0.00013	0	B42-VI9, B15-RY9

Results limited to those observed in a minimum of 5 sequences, $P < 0.05$. Putative HLA class I alleles are described in[34]; optimally described HLA-restricted epitopes from http://www.hiv.lanl.gov/content/immunology/tables/optimal_ctl_summary.html.

subsequently reverted cannot be ruled out. The presence of RT drug resistance mutations in a minority of elite controller samples merits mention: previous treatment is not an exclusion criterion for the International HIV controllers study (http://www.hivcontrollers.org/). Alternatively, these mutations may represent transmitted resistance mutations.

Instead, reduced RC of controller-derived recombinant RT–integrase viruses may be explained, at least in part, by the selection of fitness-attenuating mutations that result from effective HLA-restricted CTL responses to the incoming virus. We observed that viruses generated from B*51 and B*57-expressing controllers exhibited the most profound RC defects. Both of these alleles restrict strong CTL responses against epitopes in Pol[41,42] and have been identified as "protective" with respect to HIV disease progression.[36,43–45] Furthermore, among B*57-expressing individuals, the number of B*57-associated polymorphisms correlated inversely with RC, suggesting that the accumulation of B*57-associated mutations in Pol can negatively impact viral RC in a dose-dependent manner. Similar results have been reported previously for HLA-B*5703-selected mutations in the Gag p24/capsid protein.[16] Notably, although both B*57+ controllers and progressors harbored B*57-associated escape mutations, viruses from B*57-expressing controllers displayed further reduced RC than their progressor counterparts, suggesting the presence of additional functional defects in controller sequences beyond those associated with commonly observed primary escape mutations. Previous studies have described rare and/or unique Gag escape mutations associated with fitness costs in controllers,[22] although non-immune mechanisms cannot be ruled out. The selection of compensatory mutations in progressors could also contribute to observed differences, as has been reported in Gag.[13] Observed associations between HLA-A*01 and C*02 and viral RC are also notable and merit further study.

Substantial overlap in the RC distribution of controller-derived and progressor-derived viruses indicates that reduced RT–integrase function is not common to all controllers. Similarly, viruses generated from B*51 and B*57-expressing progressors did not exhibit significantly reduced RC compared with their non-B*51/B*57–derived counterparts. This underscores the observation that expression of a protective allele does not guarantee viral attenuation, and that analysis at the individual sequence level is necessary to elucidate relationships between viral polymorphisms and fitness (indeed, analysis at the sequence-level revealed a significant dose-dependent relationship between the number of B*57-associated escape

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mutations and RC in B*57+ individuals). Furthermore, more than half of controllers expressed neither B*51 nor B*57, yet they still harbored viruses with reduced RC compared with progressors, indicating that immune selection pressure by these 2 alleles does not solely account for the observed effects. We can neither rule out transmission of attenuated viruses in at least some controllers nor the selection of unique mutations, immune-mediated or otherwise that incur fitness costs.

The inherent challenges associated with identifying elite controllers and extracting HIV RNA from individuals with undetectable plasma viral loads limit the size, and thus the power to comprehensively evaluate associations between RC and specific HLA alleles or viral polymorphisms. Despite this, we were able to identify a number of Pol codons that may be associated with reduced RC, including, among others, the B*57-associated V245E mutation in RT (residue 2 of B*57 ISW9 epitope[46,47]) that is selected relatively rapidly after infection in B*57 (and B*58)-expressing individuals.[48] The observation that V245E reverts after transmission to non-B*57/B*58–expressing individuals[48,49] also supports a fitness cost. Integrase 265V was identified as the strongest correlate of lower RC, but mechanisms for this are unclear. This residue lies within described B*15 and B*42-restricted epitopes; however, to our knowledge no HLA-associated polymorphisms have been reported at this position. Although candidate residues were identified in this exploratory analysis, a comprehensive identification of codons associated with Pol RC will require a larger dataset followed by in vitro validation.

In summary, we observed significantly reduced RC of recombinant NL4-3 viruses encoding RT–integrase from elite controllers compared with those from untreated chronic progressors, regardless of whether recombinant viruses captured quasispecies diversity or were generated from cloned sequences. Controller-derived viruses from individuals expressing "protective" HLA-B*51 and B*57 alleles exhibited the lowest overall RC, and a dose-dependent inverse relationship was observed between the number of B*57-associated mutations and viral replication in both controllers and progressors. Although it is well established that envelope is a major determinant of fitness,[2,50] a growing body of evidence indicates that mutations outside envelope mediated by immune[14,16,20,23] or other[27] selective pressures may also result in fitness defects. Results are consistent with functional defects in viruses isolated from HIV-1 elite controllers,[6,20,21] which may arise as a result of immune selection pressures that reduce viral RC.[20]

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CASE REPORT

Long-term successful control of super-multidrug-resistant human immunodeficiency virus type 1 infection by a novel combination therapy of raltegravir, etravirine, and boosted-darunavir

Hitomi Nakamura · Naoko Miyazaki · Noriaki Hosoya · Michiko Koga · Takashi Odawara · Tadashi Kikuchi · Tomohiko Koibuchi · Ai Kawana-Tachikawa · Takeshi Fujii · Toshiyuki Miura · Aikichi Iwamoto

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Abstract Drug-resistant virus infection has been a major hurdle in the management of human immunodeficiency virus type 1 (HIV-1) infection. Recently, three novel antiretrovirals [raltegravir (RAL), etravirine (ETR), and darunavir (DRV)] were introduced into the market almost simultaneously, and salvage regimens containing these three antiretrovirals have been reported to exhibit strong potency against drug-resistant HIV-1 infection. However, the sustainability of such regimens remains unclear, particularly for patients infected with multidrug-resistant viruses. Here we report a case of super-multidrug-resistant HIV-1 infection which has been successfully controlled by novel combination therapy including RAL, ETR, and DRV for over 2 years, indicating that the novel combination could become an ultimate weapon against drug-resistant HIV infection and could alter the landscape of HIV salvage therapy.

Keywords Salvage therapy · Antiretrovirals · Drug resistance · Darunavir · Raltegravir · Etravirine · Human immunodeficiency virus type 1

H. Nakamura · N. Hosoya · M. Koga · T. Odawara · T. Kikuchi · A. Kawana-Tachikawa · T. Miura (✉) · A. Iwamoto
Division of Infectious Disease, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
e-mail: miura523@ims.u-tokyo.ac.jp

N. Miyazaki
Japan Foundation for AIDS Prevention, Tokyo, Japan

N. Miyazaki · T. Koibuchi · T. Fujii · A. Iwamoto
Department of Infectious Diseases and Applied Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Introduction

Human immunodeficiency virus type 1 (HIV-1) was discovered 27 years ago, but the development of HIV vaccines has been unsuccessful. Fortunately, a handful of antiretrovirals have been developed and are widely available, at least in resource-rich settings. Although the eradication of viruses from infected human bodies cannot be achieved at the present time, the emergence of triple combination therapies of antiretrovirals in the late 1990s altered the whole picture of HIV management completely: antiretroviral therapies (ARTs) consisting of 2 nucleoside analogue reverse transcriptase inhibitors (NRTIs) and either one protease inhibitor (PI) or one non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) have enabled viremia suppression to the limit of detection by commercial HIV quantification assay (<30–50 RNA copies/ml plasma). However, viral drug-resistance mutations often evolve under incomplete viremia control [1–3]. Many HIV-infected individuals who had been exposed to single or dual NRTIs before the introduction of triple combination therapy have experienced virologic failure due to the emergence of NRTI-resistant mutants, which, in turn, has facilitated the evolution of mutations resistant to PIs and/or NNRTIs. As a consequence, individuals who acquired viruses in the early days of the HIV epidemic have been suffering from a high level of antiretroviral-resistant HIV infection and progressive disease. Salvage therapies for such patients guided by viral genotypic resistance testing are usually difficult and unsuccessful.

In 2007, three new antiretrovirals were introduced into the market: raltegravir (RAL), an integrase inhibitor that belongs to a novel class of antiretrovirals, inhibiting the viral enzyme integrase [4, 5]; and etravirine (ETR) and

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darunavir (DRV), a novel NNRTI and a novel PI, respectively, both of which were reported to have little cross-resistance to other antiretrovirals [6–11]. We here report a case of super-multidrug-resistant HIV-1 infection in which viremia was successfully controlled by a novel salvage regimen composed of lamivudine, RAL, ETR, and boosted-DRV. Remarkably, the new regimen demonstrated not only strong antiviral potency but also excellent sustainability, even in the presence of viral mutations associated with resistance to ETR and DRV, indicating that regimens including RAL/ETR/boosted-DRV could alter the landscape of salvage therapy for multidrug resistant HIV-1 infection.

Case report

A Japanese man who has sex with men was documented to be infected with HIV-1 in 1996 when he was 29 years old; his CD4+ T-cell count was 81/μl at the time of diagnosis. Shortly after that, he participated in a clinical trial of ART, receiving dual combination treatment composed of zidovudine (AZT) and indinavir (IDV), which raised his CD4+ T cell count from 40/μl to over 300/μl temporarily, but his plasma virus load (pVL) had been only partially controlled and stayed at over 50,000 RNA copies/ml (Fig. 1), despite his good adherence to the medication. A year later, the treatment was switched to a triple combination therapy composed of stavudine/lamivudine/nelfinavir (d4T/3TC/NFV), but this failed to suppress viremia as well. The first drug-resistance genotyping test was performed in 1997, revealing multidrug-resistant mutations in his autologous plasma viral sequence, including those for both NRTIs and PIs (Fig. 1). His treatment regimen had been changed a number of times thereafter; yet none of the regimens could sustain viremia control. Eventually his CD4+ T-cell count fell below 50/μl by the end of 2005. Before the novel combination therapy including RAL/ETR/DRV was implemented in November 2007, he had experienced total of 15 different antiretrovirals, including 6 NRTIs, 7 PIs, a single NNRTI, and a fusion inhibitor (including emtricitabine and fosamprenavir, which are functionally identical to lamivudine and amprenavir, respectively; and excluding ritonavir, which was used for booster purposes). As shown in Fig. 1 and Table 1, an enormous numbers of mutations associated with antiretroviral resistance, including those associated with a fusion inhibitor (enfuvirtide) had accumulated in his plasma autologous viral sequences (Genbank accession No. GU951450-456), indicating that no effective salvage regimens had been left for him according to the availability of antiretrovirals before 2007.

Three antiretrovirals, RAL, ETR, and DRV, were introduced into the market almost simultaneously in 2007. RAL is an integrase inhibitor which has a mechanism of action completely different from that of any other antiretrovirals; and ETR and DRV are a novel NNRTI and a novel PI, respectively, both of which were reported to have little cross-resistance to older drugs. A novel combination therapy composed of 3TC/RAL/ETR/boosted-DRV was initiated for this patient on November 1st 2007. RAL and ETR had been imported from abroad because they were not approved in Japan at the time of the initiation of the salvage therapy. No major adverse events were observed, but mild skin eruption developed shortly after the starting of the new regimen, which was probably attributable to ETR administration [7]. The new regimen exhibited strong potency, reducing pVL from 130,000 to 500 RNA copies/ml over 25 days, and raising his CD4+ T-cell count from 42 to 276 cells/μl over 10 months (Fig. 1). Of importance, the viremia has been suppressed for over 96 weeks (2 years), a period that is often considered as a benchmark of the sustainability of effective antiretroviral regimens.

Although his pVL was well controlled, it had been detectable (fluctuating at around 100–300 RNA copies/ml, measured by the Roche TaqMan® (Roche, Basel, Switzerland) HIV-1 test, which replaced the previous Roche Amplicore® (Roche, Basel, Switzerland) HIV-1 monitor test ver1.5 in early 2008 in Japan). However, as is widely known, it was revealed that the Roche TaqMan® assay was inaccurate at the low end of the dynamic range [12, 13]; moreover, there was a problem with the blood collection tube used for HIV-RNA quantification in Japan, and this was also considered to have contributed to incorrect values for HIV-RNA (reported on the Roche website: http://www.roche-diagnostics.jp/pdf/product/md/taqman_hiv.pdf). Therefore, we repeated HIV-RNA quantification, using the Abbott Real-time HIV-1 test (Abbott, Abbott Park, IL, USA) that had been demonstrated to have an excellent correlation to the Roche Amplicore® HIV-1 monitor test ver1.5 [14, 15]. And we found that the pVL was only 62 RNA copies/ml for the plasma that had been determined as 300 RNA copies/ml by the Roche TaqMan® HIV-1 test; furthermore, after all of the issues related to the Roche TaqMan® HIV-1 test were completely solved, the first HIV-RNA quantification by the Roche TaqMan® assay, performed in February 2010, was less than 40 RNA copies/ml (Fig. 1), these tests collectively indicating that the viremia has been successfully controlled to below or around the detection limit for more than 2 years.

Viral drug-resistance genotyping was performed on a total of 27 occasions since the first ART had been initiated for the present patient in 1997 (Fig. 1). In his autologous plasma viral sequences, a number of resistance-associated mutations for all of the conventional classes of antiretrovirals (NRTIs, NNRTIs, and PIs) had been accumulated [according to the HIV-1 genotypic resistance interpretation algorithm provided by Stanford

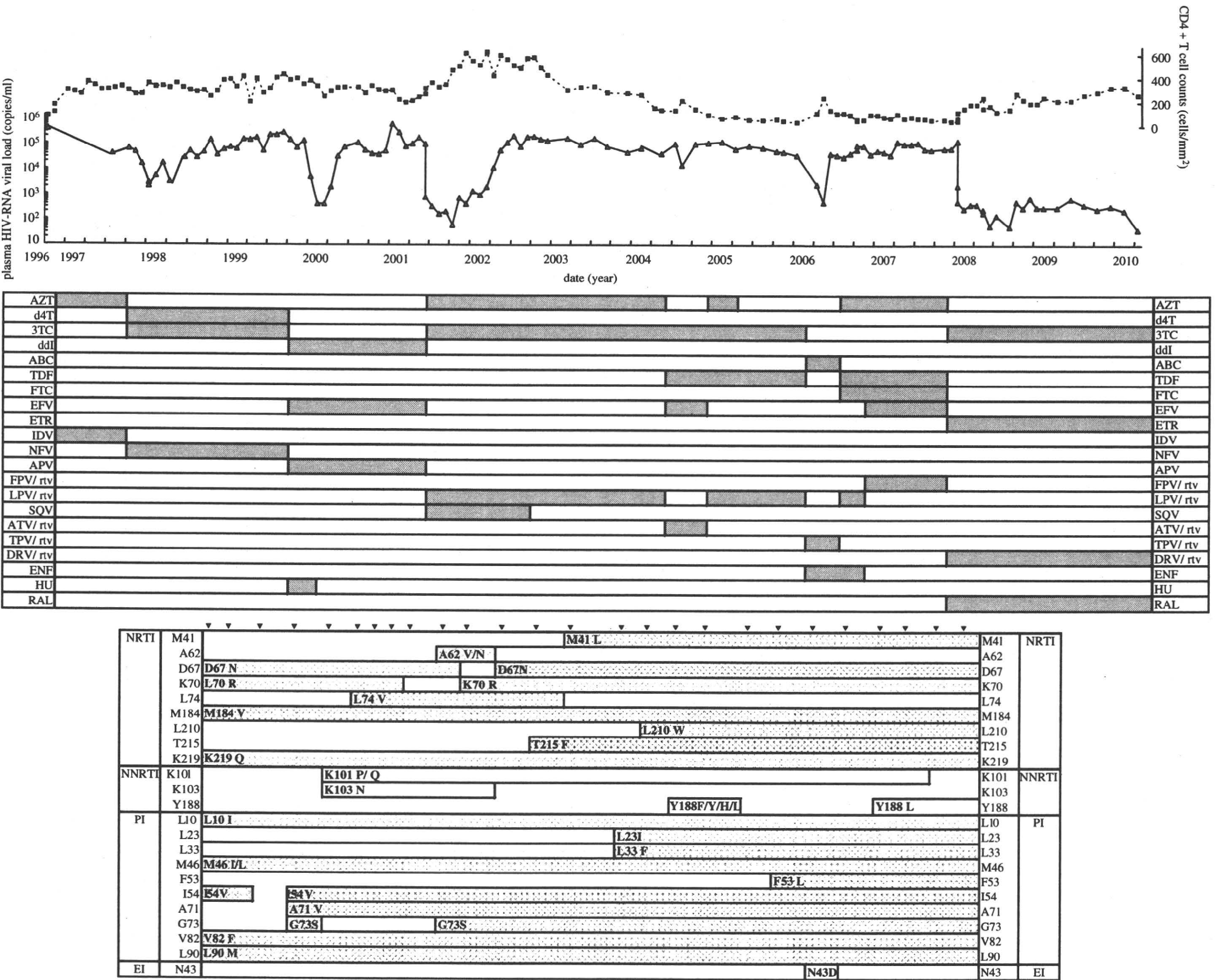

Fig. 1 Clinical course of a Japanese man infected with super-multidrug-resistant human immunodeficiency virus type 1 (the presented case). In the *upper panel*, *solid* and *dashed lines* indicate HIV-RNA level and CD4+ T cell count in the peripheral blood. The *middle panel* shows periods during which each of the antiretrovirals was prescribed; the names of the antiretrovirals follow standard abbreviations (see Table 1), except for *HU* and *ENF*, which indicate hydroxyurea and enfuvirtide, respectively. *Inverted solid triangles* between the middle and lower panels indicate sampling points for drug-resistance genotyping. The *lower panel* displays the major viral resistance mutations detected according to the HIV Drug Resistance Database of Stanford University. *APV* amprenavir, *RAL* raltegravir, *rtv* ritonavir

Table 1 Viral drug resistance interpretation

Class	Drugs	Sensitivity
NRTI[a]	Zidovudine (AZT)	High-level resistance
	Lamivudine (3TC)	High-level resistance
	Stavudine (d4T)	High-level resistance
	Didanosine (ddI)	Intermediate resistance
	Abacavir (ABC)	High-level resistance
	Tenofovir (TDF)	Intermediate resistance
	Emtricitabine (FTC)	High-level resistance
NNRTI[a]	Delavirdine (DLV)	High-level resistance
	Efavirenz (EFV)	High-level resistance
	Etravirine (ETR)	Intermediate resistance
	Nevirapine (NVP)	High-level resistance
PI[a,b]	Atazanavir/r (ATV/r)	High-level resistance
	Darunavir/r (DRV/r)	Low-level resistance
	Fosamprenavir/r (FPV/r)	High-level resistance
	Indinavir/r (IDV/r)	High-level resistance
	Lopinavir/r (LPV/r)	High-level resistance
	Nelfinavir/r (NFV/r)	High-level resistance
	Saquinavir/r (SQV/r)	High-level resistance
	Tipranavir/r (TPV/r)	Intermediate resistance
Fusion inhibitor[c]	Enfuvirtide (ENF)	10–20 fold reduced susceptibility
CCR5 inhibitor[d]	Maraviroc	Potentially harboring X4 virus

Based upon the autologous viral sequence obtained from November 2007

NRTI nucleoside analogue reverse transcriptase inhibitor, *NNRTI* non-nucleoside analogue reverse transcriptase inhibitor, *PI* protease inhibitor

[a] According to HIV-1 genotypic resistance interpretation algorithm provided by Stanford University (http://hivdb.stanford.edu/pages/algs/HIVdb.html)

[b] Small letter 'r' indicates ritonavir for booster purposes

[c] According to Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/)

[d] According to the geno2pheno algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de/)

University (http://hivdb.stanford.edu/pages/algs/HIVdb.html)]. The virus was also found to be resistant to a fusion inhibitor (enfuvirtide). Notably, the autologous plasma viral sequences in the presented case were carrying some resistance-mutations to both DRV and ETR at the time of initiation of the new salvage regimen (Table 1): V11I and L33F substitutions were observed that had been reported as DRV resistance mutations in the POWER/DUET study (6th European HIV Drug Resistance Workshop, 2008); likewise, a K101P substitution was found in the RT sequence known to reduce susceptibility to ETR by sixfold [16]; and finally Y188L, which confers low-level resistance to ETR, was also detected. These findings indicated that although only RAL was a fully active antiretroviral in the presented case (RAL resistance mutations are rarely seen in RAL-naïve patients), virus replication has been successfully controlled by the novel regimen for over 2 years.

Discussion

The patient in the case presented here had experienced almost all of the conventional antiretrovirals, including a fusion inhibitor, prior to the initiation of the new salvage regimen. Multidrug-resistance mutations had accumulated in his autologous plasma viral sequences, indicating that no effective regimens were left for this case before 2007; therefore, the infection could be called a "super-multidrug-resistant HIV infection". The successful viremia control for over 2 years by the novel salvage regimen including three recently approved antiretrovirals, RAL, ETR, and DRV, strongly indicated that this novel combination could become an ultimate weapon against drug-resistant HIV-1 infection.

The strong potency of a regimen containing RAL/ETR/DRV has been reported recently in the French TRIO study, which demonstrated successful viremia control in

individuals infected with multidrug-resistant viruses [17]. However, the duration of the follow-up of the study was limited to 48 weeks, and the enrolled patients seemed not to have very advanced diseases: the median CD4+ T-cell count was relatively high (255/μl), and the median pVL was relatively low (4.2 $\log_{10}$ RNA copies/ml). Furthermore, the median numbers of viral drug-resistance mutations observed in the studied subjects were limited [4 for PIs (only primary mutations were counted), 6 for NRTIs, and 1 for NNRTIs]. Therefore, the durability of the regimen containing these three newly approved antiretrovirals had remained unanswered, particularly for patients with advanced cases infected with viruses that had low to intermediate resistance to ETR and/or DRV. The patient presented here had an advanced disease, having a very low CD4+ T-cell count (<50/μl) and a high pVL (50,000–100,000 RNA copies/ml), even under AZT/tenofovir (TDF)/emtricitabine (FTC)/efavirenz (EFV)/fosamprenavir (FPV)/ritonavir (rtv) treatment (Fig. 1), and harboring viruses carrying numerous resistance mutations [7 for PIs (only primary mutations), 9 for NRTIs, and 3 for NNRTIs], though displaying no AIDS-defining illness. The over 2 years of sustained viremia control and immunological improvement observed in this patient demonstrated robust control of super-multidrug-resistant HIV infection by this novel combination therapy.

The majority of viral mutations selected for under ART pressure confer cross-resistance to other antiretrovirals, and this has been hampering HIV salvage therapy for a long time. Notably, the presented case suggested that a low to intermediate level of viral resistance to some of the components of this novel regimen may not necessarily result in treatment failure.

In the presented case, lamivudine (3TC) was added to the salvage regimen, in the expectation of maintaining the M184V RT mutation, which is known to be selected for under lamivudine pressure and to impair viral replication capacity considerably [18, 19]. However, as of now, it has not been elucidated whether NRTIs should be added to the combination of RAL/ETR/DRV. Thus, it would be warranted to investigate whether the addition of NRTIs would have beneficial effects for long-term viremia control; likewise, it would be warranted to investigate whether there are advantages in continuing with antiretrovirals to which viruses have already become resistant, aiming to maintain fitness-reducing viral mutations as a dominant form in vivo.

Another novel-class antiretroviral, maraviroc, which blocks HIV binding to the coreceptor CCR5, could have been used as a component of the salvage therapy for the present patient; however, to date, the prescription of maraviroc requires a demonstration of the nonexistence of CXCR4-tropic viruses in peripheral blood. We retrospectively examined the V3-loop sequences of the viral envelope gene at the time of initiation of the novel salvage therapy and found that one of the 5 clones obtained was a CXCR4-tropic virus according to the 'geno2pheno' algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de/), which is widely accepted as a predictor of viral coreceptor usage [20, 21], thereby suggesting that maraviroc would not be a useful option for the treatment of the present patient (Genbank accession No: GU951445-449).

In conclusion, a salvage antiretroviral regimen including RAL in combination with ETR and boosted-DRV could become an ultimate weapon against multidrug-resistant HIV-1 infection and could change the landscape of HIV salvage therapy in the near future.

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HLA-Associated Immune Pressure on Gag Protein in CRF01_AE-Infected Individuals and Its Association with Plasma Viral Load

Goragoch Gesprasert[1], Nuanjun Wichukchinda[2], Masahiko Mori[3], Teiichiro Shiino[4], Wattana Auwanit[2], Busarawan Sriwanthana[2], Panita Pathipvanich[5], Pathom Sawanpanyalert[2], Toshiyuki Miura[6], Prasert Auewarakul[7], Arunee Thitithanyanont[1]*, Koya Ariyoshi[3,8]

1 Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, **2** Department of Medical Sciences, National Institute of Health, Ministry of Public Health, Nonthaburi, Thailand, **3** Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan, **4** AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan, **5** Day Care Center, Lampang Hospital, Lampang, Thailand, **6** Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, **7** Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **8** Global COE Program, Nagasaki University, Nagasaki, Japan

Abstract

Background: The human leukocyte antigen (HLA)-restricted cytotoxic T-lymphocyte (CTL) immune response is one of the major factors determining the genetic diversity of human immunodeficiency virus (HIV). There are few population-based analyses of the amino acid variations associated with the host HLA type and their clinical relevance for the Asian population. Here, we identified HLA-associated polymorphisms in the HIV-1 CRF01_AE Gag protein in infected married couples, and examined the consequences of these HLA-selected mutations after transmission to HLA-unmatched recipients.

Methodology/Principal Findings: One hundred sixteen HIV-1-infected couples were recruited at a government hospital in northern Thailand. The 1.7-kb *gag* gene was amplified and directly sequenced. We identified 56 associations between amino acid variations in Gag and HLA alleles. Of those amino acid variations, 35 (62.5%) were located within or adjacent to regions reported to be HIV-specific CTL epitopes restricted by the relevant HLA. Interestingly, a significant number of HLA-associated amino acid variations appear to be unique to the CRF01_AE-infected Thai population. Variations in the capsid protein (p24) had the strongest associations with the viral load and CD4 cell count. The mutation and reversion rates after transmission to a host with a different HLA environment varied considerably. The p24 T242N variant escape from B57/58 CTL had a significant impact on the HIV-1 viral load of CRF01_AE-infected patients.

Conclusions/Significance: HLA-associated amino acid mutations and the CTL selection pressures on the p24 antigen appear to have the most significant impact on HIV replication in a CRF01_AE-infected Asian population. HLA-associated mutations with a low reversion rate accumulated as a footprint in this Thai population. The novel HLA-associated mutations identified in this study encourage us to acquire more extensive information about the viral dynamics of HLA-associated amino acid polymorphisms in a given population as effective CTL vaccine targets.

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* E-mail: scaty@mahidol.ac.th

Introduction

Accumulating evidence indicates that cytotoxic T lymphocytes (CTLs) play a central role in controlling human immunodeficiency virus (HIV) replication *in vivo*, and a number of CTL-inducing vaccines have been developed [1,2]. All trials of CTL-inducing vaccines against HIV have been unsatisfactory including the most recent trial conducted in Thailand [3,4,5]. Genetic polymorphisms in the human leukocyte antigens (HLAs) are key factors contributing to the complexity of developing CTL-inducing vaccines [6,7]. HLA class I molecules play a critical role in defining the epitopes of CTLs, which probably influence their antiviral efficacy. The extraordinary capacity of this virus to generate genetic diversity is another important factor contributing to this complexity. To date, 13 prototype HIV clades and 43 circulating recombinant forms have been described worldwide and HIV diversity appears to be increasing as the infection spreads [8].

Once the virus infects a host, it rapidly evolves and evades the host cellular immune response. Viral adaptation to the HLA-restricted immune response and the selection of viral mutations associated with the loss of the antiviral immune response have been described in both acute and chronic HIV-1 infections at the

individual level [9,10]. Recently, viral adaptations to HLA have also been reported at the population level [11,12]. Therefore, there is a growing concern that HIV may evolve to reduce the availability of key CTL epitopes that are associated with the control of HIV infection at the population level. This in turn would greatly affect the clinical outcomes of HIV/AIDS. Therefore, these associations are becoming increasingly important for effective CTL-based vaccine strategies.

Several studies have attempted to define HLA-associated mutations in a given population using a large number of HIV genome sequences and to determine their influence on clinical outcomes [13,14]. These studies have identified HLA polymorphisms in the HIV-1 Gag protein and this association continues to be reinforced [15]. However, most information has been derived from studies of subtype-B-HIV-infected Caucasian and subtype-C-HIV-infected African populations, and very little information is available on the CRF01_AE virus, the predominant clade circulating in southeast Asia [16,17].

Therefore, in this study, we investigated the amino acid variations in the HIV-1 CRF01_AE Gag protein among HIV-1-infected people with known HLA alleles in Thailand, with the primary objective of identifying the amino acid mutations associated with the host HLA class I types and their influence on clinical outcomes. Moreover, because our cohort included dozens of discordant couples (viral transmission pairs), we took advantage of this point and further analyzed the fate of these HLA-selected mutations after transmission to HLA-unmatched recipients.

Methods

Ethical statement

This study was approved by the Ethics Committee of the Thai Ministry of Public Health and was conducted in accordance with the set guidelines for research. All patients provided their written informed consent for the collection of the samples and their subsequent analysis.

Population and samples

We recruited 116 chronically HIV-1-infected Thai couples (219 patients in total) at a government referral hospital in northern Thailand between 6 July 2000 and 15 October 2002. The cohort has been described in detail elsewhere [18]. We obtained two sequential blood samples from each patient, with an interval of 6–27 months (mean interval 19.75 months, mode 24 months) between the two collections. The majority of patients were naïve to antiretroviral therapy, except for 27 individuals who were receiving treatment with single or dual nucleoside reverse transcriptase inhibitors. However, no patient was receiving highly active antiretroviral therapy. The median (interquartile range, IQR) $CD4^+$ cell count in our study population was 163 (23, 370) cells/μL, and the median (IQR) plasma viral load was 5.20 (4.54, 5.63) $\log_{10}$ RNA copies/mL. Peripheral blood mononuclear cells (PBMCs) were separated with a commercially available cell-separation tube (CPT® Cell Preparation Tube with Sodium Citrate, BD, Franklin Lakes, NJ, USA) and used in this study.

HLA class I typing

Genomic DNA was extracted from patient PBMCs with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. HLA class I typing for the A and B loci was performed using a PCR microtiter plate hybridization method (*WAKFlow*® HLA typing kit) (Wakunaga Co. Ltd., Hiroshima, Japan), according to the manufacturer's instructions. For statistical analysis, each HLA allele of each individual was assigned a two-digit designation.

PCR amplification of HIV *gag* and sequencing

Genomic DNA was extracted from patient PBMCs as described above, and nested PCR was performed. First, the 9.1-kb nearly full-length HIV genome was amplified using Takara *LA Taq* DNA polymerase (Takara, Shiga, Japan) and the following primers, which bind to both the long terminal repeat (LTR) regions of the HIV genome: sense outer primer, MSF12b 5′-AAATCTCTAGCAGTGGCGCCCGAACAG-3′, and antisense outer primer, OFMR1 5′-TGAGGGATCTCTAGTTACCAGAGTC-3′. The PCR conditions were as follows: melting at 95°C for 5 min; 30 cycles each of 95°C for 10 s, 65°C for 30 s, and 68°C for 8 min; and a final extension at 68°C for 7 min. The 1.7-kb fragment containing the entire *gag* gene was amplified from the first round PCR product using Qiagen *Taq* DNA polymerase (Qiagen): sense inner primer, Gag-F1 5′-TCTCGACGCAGGACTCGGCTTGCT-3′, and antisense inner primer, Gag-R2 5′-CCTCCAATTCCCCCTATCATTTTTGG-3′. The thermocycling conditions for the second round of PCR were as follows: melting at 95°C for 2 min; 30 cycles each of 95°C for 30 s, 60°C for 30 s, and 68°C for 90 s; and a final extension at 68°C for 7 min. The PCR product was analyzed by gel electrophoresis. The appropriate PCR products were directly sequenced by Macrogen Inc., Korea.

Sequence analysis

The HIV nucleic acid sequences were analyzed to identify their subtypes, using the RIP 2.0 software (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). All CRF01_AE sequences were submitted to GenBank (accession number GU458430–GU458799). Only the sequences that included the complete *gag* open reading frame were selected for sequence analysis. The sequences were aligned and translated using the MEGA 3.1 software [19]. A consensus sequence was created from the most abundant amino acid at each position in the cohort. HIV transmission between spouses was confirmed by constructing a neighbor-joining phylogenetic tree using the entire *gag* nucleotide sequences derived from the whole sample. If the viruses derived from a husband and wife clustered on the same branch, the couple's viruses were regarded as having a common ancestor, implying that the virus was transmitted between them. On this basis, we identified 68 such couples. Each member of the remaining couples was considered to be infected with virus distinct from that infecting his/her spouse. The direction of transmission was determined by in-depth interviews with field workers. The associations between the sequence polymorphisms and the HLA types were analyzed with Fisher's exact test with a 95% confidence interval (CI), using only the patients who were source of virus in the couples (index cases), and was limited to the HLA alleles shared by at least five subjects to ensure sufficient statistical power. Amino acids that were identical to the consensus sequence were considered to be "dominant" amino acids, and any difference from the consensus sequence was classified as "non-dominant". An amino acid position was declared an "HLA-associated variable site" if a significant HLA association was identified in the sequence at both times of sample collection. The HLA-associated variable site was mapped in relation to the best-defined CTL epitopes published in the Los Alamos HIV Databases [http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html, accessed Dec. 2009].

For detecting adaptive evolution in protein-coding sequences under natural selection in the population, the branch lengths and nucleotide substitution rate parameter was estimated to approx-

imate the analogous parameters of the codon model. The MG94 codon model, which estimates synonymous and non-synonymous rate independently for every amino-acid, was performed. The estimating site-by-site variation rate was evaluated by single likelihood ancestor counting (SLAC) and fixed-effected likelihood (FEL) methods. The adaptive evolution study was done by HyPhy 2.0 software [20,21].

Statistical analysis

All statistical analyses were performed with Excel 2007. Fisher's exact test with a 95% CI was used to detect HLA-associated dominant or non-dominant sites, and Spearman's correlation test was used to determine the number of HLA-associated non-dominant sites and for the viral load correlation analysis. We also used one-way ANOVA to test the differences in viral load among the T242X mutations with or without compensatory mutations in the HLA_B*57/*58-positive or -negative groups.

Results

Sequencing results, study population, and HLA allele frequencies

By subtype analysis, nine individuals were found to be infected with subtype B or a CRF01_AE/subtype B recombinant form of virus, so they were excluded from further analysis. Then, 370 CRF01_AE Gag sequences were determined in the 209 and 161 samples at the first and second time points, respectively, obtained from 219 individuals (116 couples). The numbers of CRF01_AE-infected individuals carrying specific HLA class I alleles are shown in Figure 1. The most frequent HLA_A allele was A*11, followed by A*02, A*24, and A*33. The allele B*15 was the most frequent HLA_B allele, followed by B*40, B*46, and B*13. Clearly, the HLA distribution in Thailand differs from those in North American and African countries. We also analyzed the linkage disequilibrium. Strong linkage was found between A*33 and B*58 ($p = 1.39 \times 10^{-12}$), as previously reported elsewhere [22,23].

HLA-associated amino acid variations

To identify HLA-associated amino acid variations, we analyzed the Gag amino acid sequences in relation to the HLA types. Phylogenetic analysis identified 68 couples in which the CRF01_AE virus transmission between the spouses was confirmed (Figure S1). In the remaining couples, the spouses were considered to be infected with distinct viruses. To minimize the lineage effect that might result from sampling viruses from concordant couples, we included only one spouse from each couple in the analysis. After removing the contact cases from these 68 concordant couples, 144 first samples and 122 second samples were used for further analysis. We found 44 amino acid site variations (among the known 498 amino acid positions) in the Gag region. All these

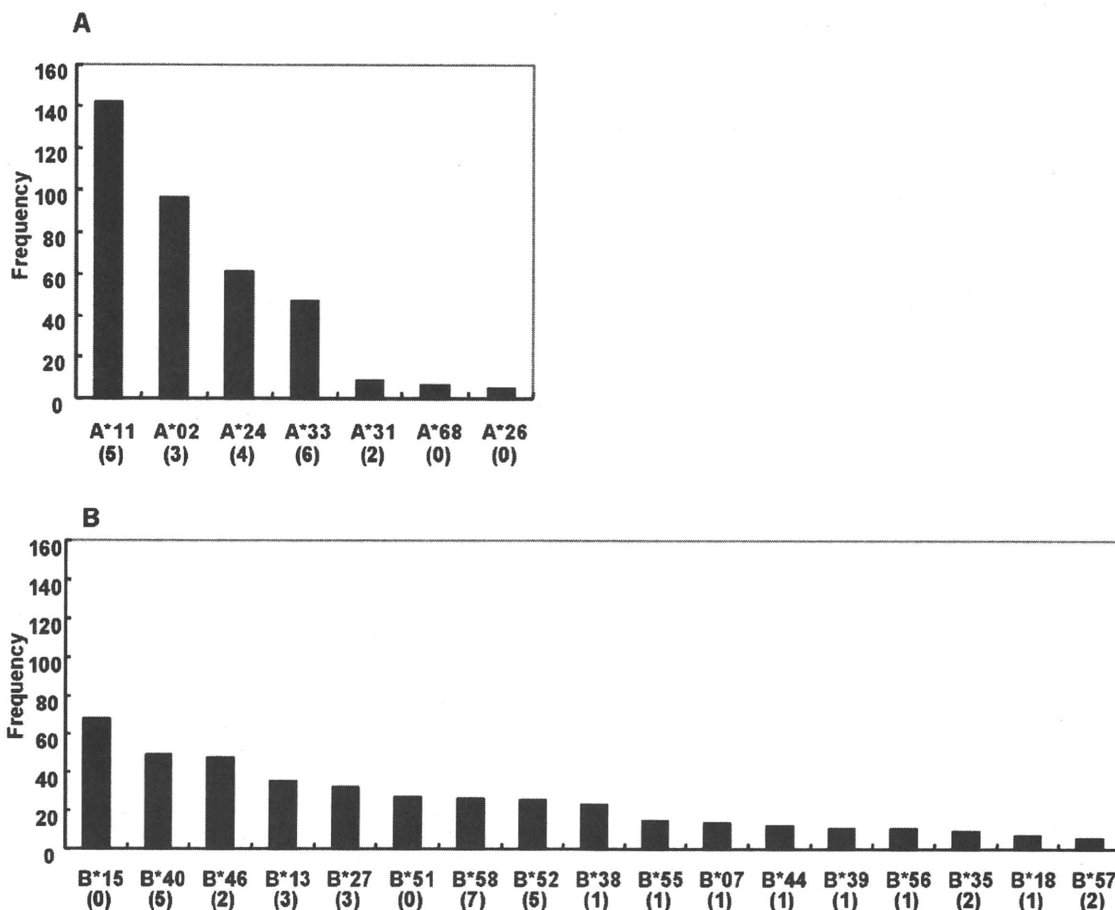

Figure 1. HLA_A and HLA_B allele distributions among patients. HLA allele distributions: the frequencies of HLA_A and _B alleles occurring in at least five or more individuals are shown in (A) and (B), respectively. The number of HLA-associated amino acid variations is indicated in brackets under each HLA allele.
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variations showed statistically significant associations with some of the HLA types ($p<0.05$) and these are described below.

In total, 56 associations between the HLA types and amino acid variations were identified: 20 associations with five HLA_A alleles (A*02, *11, *24, *31, and *33) and 36 associations with 15 HLA_B alleles (all except B*15 and *51). Seventeen positions (23 associations) were in p17, 16 positions (21 associations) in p24, and the remaining 11 positions (12 associations) were in p2/7/1/6 (Table 1). The associations in p17 were restricted more by HLA_A alleles (13/23 associations), whereas the associations in p24 were restricted more by HLA_B alleles (16/21 associations).

The number of HLA-associated amino acid variations did not necessarily correlate with the frequency of the allele. More than five amino acid variations were associated with B*58 and B*52, despite the relative infrequency of these alleles, whereas no variation was significantly associated with one of the most frequent alleles, B*15 (Figure 1B). Among the 56 HLA-associated amino acid variations, 49 (87.5%) were selected by non-dominant amino acids in the presence of the specific HLA type. The remaining seven (12.5%) variations were selected by dominant amino acids in the presence of a specific HLA type. Six amino acid variations caused by negative selection were located in p17, whereas only one was located in p24. Dominant amino acid selection was always associated with frequent HLA alleles: five variations were associated with A alleles (A*11, A*02, A*24, or A*33) and two were associated with B alleles (B*46 or B*27).

We also found that 35 (62.5%) HLA-associated amino acid variations were located within or adjacent to the best-defined HIV-specific CTL epitopes, restricted by the relevant HLA allele [24] (Table 1). Some HLA-associated amino acid variations were located at anchor positions of binding peptides: A*24-associated F79X, B*40-associated E93X, and B*58-associated V485X (Table 1). Odds ratios were widely variable, ranging from 2.60 to 90.0, with a median (IQR) of 7.87 (4.48, 13.3). The odds ratio was highest by far at B*58-associated T242.

The codon-based analysis revealed a large number of significant selection sties in the Gag protein, mostly purifying selection; among the 498 Gag amino acid positions, 270 (54.2%) sites and 52 (10.4%) sites were identified by either SLAC or FEL method as purifying selection and positive selection sites, respectively (Table S1). Interestingly 19 (36.5%) out of the 52 positive selection sites located at the sites of HLA-associated amino acid variations, whereas only 6 (2.2%) out of 270 purifying selection sites located at the sites of HLA-associated amino acid variations (Table 1). This implies that HLA-pressure is one of major factors driving the positive amino acid selection among Gag protein.

Associations between numbers of HLA-associated amino acid variants and clinical outcomes

After defining the HLA-associated amino acid variation sites in CRF01_AE in the analysis described above, we counted the numbers of HLA-associated variations in autologous viral sequences for each patient, and plotted them on the X axis, and plotted the plasma viral loads and $CD4^+$ cell counts on the Y axis. We found significant associations between the numbers of HLA-associated amino acid variations and the $CD4^+$ cell counts or viral loads. Patients with a higher number of HLA-associated amino acid variants tended to have a higher plasma viral load and lower $CD4^+$ cell counts (Figure 2A). We further analyzed these associations according to the subregions of Gag in which the variations occurred. Intriguingly, these correlations were mainly driven by the associations with variations in the p24 region (Figure 2B).

Amino acid variations in a recipient host with different HLA alleles

With in-depth interviews conducted by designated field workers, the index and contact cases were determined among the 65 concordant couples. Looking at the viral sequences in a pairwise manner, we noted that the frequencies of *de novo* HLA-associated mutations and reversions after viral transmission to contact cases with distinct HLA profile varied considerably, depending on the amino acid positions involved. Mutations and reversions of each HLA-associated amino acid variant were studied whenever data for at least five couples were available. When the virus was transmitted to a contact case with a different HLA environment, as confirmed by sequencing, the rate of reversion or mutation for each HLA-associated amino acid variant was calculated and was plotted on a scatter graph (Figure 3). To avoid overestimation of the mutation or reversion rate, we counted only HLA-associated sites with p values of <0.01 with a 99% CI and with a denominator of more than one when we calculated their rates. In total, 30 HLA-associated amino acid variation sites were listed. For instance, at the S9X site restricted by B*13, which was selected by non-dominant amino acid, the mutation rate was calculated as 5/11 (=0.45), five S9T–B*13-positive contact cases divided by 11 S9S–B*13-negative index cases. Its reversion rate was calculated as 4/6 (=0.67), four S9S–B*13-negative contact cases divided by six S9T–B*13-positive index cases (see supplementary data for details of the mutation and reversion sequence variations, Table S2). For dominant amino acid selection sites, the mutation rate and reversion rate were calculated in the opposite way. At the K76X site restricted by A*02, the mutation rate was calculated as 3/3 (=1.0), three K76K–A*02-positive contact cases divided by three K76R–A*02-negative index cases. The reversion rate was calculated as 1/13 (=0.077), one K76R–A*02-negative contact case divided by 13 K76K–A*02-positive index cases. The average reversion rate was 0.42 and the average mutation rate was 0.33. There was a rough inverse relationship between the reversion and mutation rates. P255X (A*11) and I223X (B*13) scored reversion rates of 1.00 and both had low mutation rates. Conversely, F79X (A*24), K76X (A*02), and T242X (B*58) had mutation rates of 1.00 and the former two had low reversion rates. Interestingly, T242X (B*58) was outstanding in that both its mutation rate and reversion rate were very high. This indicates that the rate of accumulation of CTL escape mutations in a given population varies considerably among mutations and restricting HLA types.

T242N mutations

As described above, T242X had a high reversion rate. The vast majority of T242X mutations were T242N, known as an escape mutation from CTL (TSTLQEQIGW: TW10), restricted by the protective HLA alleles B*57 and B*5801 in the setting of clade B and C infections. This mutation emerges almost universally in B*57/*5801-positive subjects. Several studies have demonstrated that the T242N substitution affects viral replicative fitness *in vitro* and it is believed to contribute to the protective effect of these alleles against the progression of HIV disease [25,26]. Moreover, several mutations within the cyclophilin A binding loop, such as H219 and M228, have been shown to compensate to some extent for the reduced viral replicative capacity caused by T242N [27,28]. However, the roles of T242N and the compensatory mutations in CRF01_AE infections are unknown. HLA_B*5801 is known to present the same epitopes as B*57 [29], and our unpublished data indicate that the vast majority of B*58 alleles in Thailand are B*5801. There were no statistically significant differences in the plasma virus loads of the B*57/*58-positive and -negative populations in our cohort (data not shown). Five of the 23

Table 1. HLA-associated amino acid variations.

Part	Position	Selection*	Restricted HLA	HLA	non-dominant	dominant	Odds rario	95% CI range	p value(Fisher)	Reported CTL epitope
p17	V7X	Pos	B*44	+	5	6	5.69	1.56	0.0132	
				−	17	116		20.69		
	S9X	n.s	B*13	+	12	7	22.10	6.98	8.98E-08	
				−	9	116		69.98		
	K18X	n.s	A*33	+	1	36	0.10	0.01	0.00488	
				−	24	83		0.74		
	R30X	Pos	A*11	+	52	36	2.60	1.30	0.010045622	**(Flanking) KIRLRPGGK**
				−	20	36		5.20		
			A*24	+	10	35	0.17	0.08	1.10356E-05	**A*2402: KYKLKHIVW**
				−	62	37		0.38		
	V35X	n.s	B*52	+	2	13	19.69	1.67	0.0282	
				−	1	128		232.20		
	T53X	Pos	A*02	+	16	51	4.52	1.56	0.004090448	A2: GLLESSEGC
				−	5	72		13.12		
	A54X	Pos	A*02	+	12	55	8.18	1.76	0.00332653	A2: GLLESSEGC
				−	2	75		38.05		
	S66X	Pos	B*18	+	4	1	28.71	3.03	0.001572746	
				−	17	122		272.17		
	L75X	Pos	A*33	+	7	30	3.33	1.08	0.04825	
				−	7	100		10.26		
			B*57	+	2	1	21.50	1.81	0.02502	(Flanking) B57: RSLYNTVATLY
				−	12	129		254.77		
			B*58	+	7	13	9.00	2.73	0.000653882	(Flanking) B58: RSLYNTVATLY
				−	7	117		29.71		
	K76X	Pos	A*02	+	7	60	0.33	0.13	0.01934	**(Flanking) A*02: SLYNTVATL**
				−	20	57		0.85		
	F79X	Pos	A*24	+	6	39	0.25	0.10	0.003083254	(Flanking) A24: LYNTVATL
				−	38	61		0.64		
	T81X	Pos	B*58	+	8	12	4.84	1.70	0.004583808	B58: RSLYNTVATLY
				−	15	109		13.77		
	V82X	Pos	A*24	+	24	21	4.51	2.10	0.000153966	(Flanking) A24: LYNTVATL
				−	20	79		9.69		
			B*58	+	11	9	3.37	1.28	0.0171	B58: RSLYNTVATLY
				−	33	91		8.86		
	V83X	Pos	A*11	+	22	66	0.09	0.04	2.48E-10	**IATLWCVHQR**
				−	44	12		0.20		
			A*24	+	30	15	3.50	1.67	0.001063416	A24: LYNTVATL
				−	36	63		7.36		
	E93X	n.s	B*40	+	21	14	13.36	5.33	8.96E-09	B*4001: IEIKDTKEAL
				−	11	98		33.52		
	I104X	Pos	A*11	+	64	24	4.44	2.17	5.18849E-05	A11: KIEEEQNKSK
				−	21	35		9.10		
			B*27	+	4	9	0.27	0.08	0.0392	
				−	81	50		0.94		
	S125X	n.s	A*11	+	15	73	3.63	1.00	0.0419	
				−	3	53		13.18		
p24	M186X	Purify	B*35	+	1	4			0.0347	
				−	0	139				
	A196X	n.s	B*38	+	4	13	38.77	4.03	0.000641253	B38: GHQAAMQML

Table 1. Cont.

Part	Position	Selection*	Restricted HLA	HLA	non-dominant	dominant	Odds rario	95% CI range	p value(Fisher)	Reported CTL epitope
				−	1	126		373.21		
	E203X	n.s	B*52	+	4	11	6.34	1.60	0.01658	(Flanking) B52: HQAAMQMLK
				−	7	122		25.06		
	I223X	n.s	B*13	+	4	15	6.40	1.55	0.0181	(Flanking) B13: GQMREPRGSDI
				−	5	120		26.48		
	M228X	n.s	B*13	+	9	10	5.35	1.91	0.002052971	B13: GQ**M**REPRGSDI
				−	18	107		14.98		
	T242X	Pos	A*33	+	14	23	12.42	4.06	2.88923E-06	
				−	5	102		37.94		
			B*46	+	0	34	0.00		0.007061705	
				−	19	91				
			B*57	+	3	0			0.001988329	B*5701, 5703: TS**T**LQEQIGW
				−	16	125				
			B*58	+	15	5	90.00	21.75	6.06E-13	B*5801: TS**T**LQEQIGW
				−	4	120		372.40		
	G248X	n.s	A*33	+	8	29	3.94	1.32	0.0238	
				−	7	100		11.78		
			B*58	+	8	12	11.14	21.75	0.000120432	B*5801: TSTLQEQI**G**W
				−	7	117		372.40		
	P255X	Pos	A*11	+	20	68	7.94	1.78	0.001603328	
				−	2	54		35.48		
	V280X	Pos	B*46	+	12	22	14.45	4.26	4.9055E-06	
				−	4	106		49.02		
	S281X	n.s	B*52	+	10	5	21.45	6.22	9.32E-07	B*5201: RMYSPT**S**I
				−	11	118		74.04		
	R286X	n.s	B*52	+	7	8	13.23	3.82	0.000122838	(Flanking)B*5201: RMYSPTSI
				−	8	121		45.79		
	D295X	Purify	B*39	+	2	7	38.29	3.09	0.010144785	
				−	1	134		474.84		
	T310X	n.s	A*33	+	13	24	3.92	1.61	0.005101645	A*33 motif: VDRFYKLTRAEQA**S**
				−	13	94		9.54		
			B*58	+	8	12	3.93	1.41	0.011165919	B*5801: QA**T**QDVKNW
				−	18	106		10.94		
	N315X	Purify	A*33	+	8	29	5.63	1.71	0.004581687	(Flanking)A*33: VDRFYKLTRAEQAS
				−	5	102		18.52		
	T348X	n.s	B*35	+	1	4			0.0347	
				−	0	139				
	S357X	Purify	B*07	+	2	8	10.92	1.59	0.039	B*0702: GP**S**HKARVL
				−	3	131		74.94		
p2	R387X	Pos	A*31	+	4	2	8.22	1.43	0.0197	
				−	27	111		47.26		
			B*27	+	6	7	3.63	1.12	0.0345	
				−	25	106		11.76		
p7	HXB2 R403X	Pos	A*31	+	2	4	16.75	2.34	0.020250589	**A*3101: LARNCRAPRK**
	(R401X)			−	4	134		119.78		
p6	HXB2 P453X	Pos	B*55	+	6	3	18.77	4.19	0.000158687	
	(P451X)			−	13	122		84.06		

Table 1. Cont.

Part	Position	Selection*	Restricted HLA	HLA	non-dominant	dominant	Odds rario	95% CI range	p value(Fisher)	Reported CTL epitope
	HXB2 T456X	Pos	B*56	+	5	3	5.89	1.33	0.02086	
	(T454X)			–	30	106		26.07		
	M463X	n.s	B*27	+	6	7	5.38	1.62	0.008955598	
				–	18	113		17.84		
	HXB2 Q476X	n.s	B*52	+	4	11	15.27	3.03	0.002287463	
	(Q473X)			–	3	126		77.09		
	HXB2 E480X	Purify	B*40	+	11	24	7.87	2.65	0.000183624	B*4001: K**E**LYPLTSL
	(E479X)			–	6	103		23.39		
	HXB2 L483X	n.s	B*40	+	22	13	4.90	2.18	0.000166254	B*4001: KELYPLTSL
	(H480X)			–	28	81		11.00		
	HXB2 485X	n.s	B*40	+	7	28	3.64	1.18	0.0424	B*4001: KELY**P**LTSL
	(P482X)			–	7	102		11.25		
	HXB2 485X	Purify	B*40	+	7	28	3.64	1.18	0.008631568	B*4001: KELY**P**LTSL
	(P483X)			–	7	102		11.25		
	HXB2 T487X	n.s	B*58	+	7	13	6.88	2.20	0.0018202	B58: LASLRSLF
	(V485X)			–	9	115		21.56		

Foot note for Table 1: *Pos. positive selection, n.s. not significant; P483X was an insertion mutation.

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B*57/*58-positive subjects did not carry T242X, and there was no statistically significant difference in their plasma viral loads or CD4 cell counts, i.e., in terms of the presence or absence of T242X in these patients (data not shown). We then stratified the B57*/*58-positive patients with T242X according to the presence/absence of the described compensatory mutations. Interestingly, we found that B*57/*58-positive patients with the compensatory mutations had significantly higher viral loads and lower CD4 cell counts than those without the compensatory mutations (Figure 3), indicating that the proposed mechanism of virus attenuation by the escape mutation and its restoration by the compensatory mutations at the B*57/*5801 TW10 epitope is applicable in the context of CRF01_AE infections.

T242N mutations and transmission

It was recently reported that the transmission of viruses with attenuating CTL escape mutations, particularly T242N from B*57-restricted CTL, is associated with better early clinical outcomes in HLA-unmatched recipients [30,31]. However, the long-term effects of the transmission of these viruses to HLA-unmatched recipients remain unknown. We summarized the amino acid variations around the TW10 epitope in B*57/*58-negative contact cases who had contracted the virus from B*57/*58-positive spouses and their clinical features (Table 2). Only two B*57/*58-negative spouses carried the T242N mutation at the time of sampling. Both had very high CD4 cell counts of >500 cell/μL and very low viral loads of less than 10^4 copies/mL, which is in distinct contrast to the remaining six B*57/*58-negative spouses who lacked T242N (median plasma viral load, 5.39 log copies/mL), and supports the results of the recent study by Chopera et al. [28]. However, because the T242N escape mutation is known to emerge within the first three months of infection in B*57-positive subjects [32], it is unlikely that these six contact cases had acquired the wild-type T242 virus, but instead, the transmitted T242N probably reverted after its transmission to these recipients. These data suggest that the majority of the recipients from B*57/*58-positive donors do not receive the benefit conferred by the transmission of the attenuated virus after many years of infection, although we did not know the duration of the infection in each patient in the present study. We identified three other patients without the B*57/B*58 alleles who carried viruses with T242N, and they all had very low viral loads of less than 10^4 copies/mL (data not shown). We presume that they contracted the virus from B*57/B*58-positive patients, although we could not identify their index cases in our study population. Taken together, these results imply that the transmission of CTL-selected attenuated viruses might confer a survival advantage on HLA-unmatched recipients, at least during the early stage of infection, and that this advantage is not limited to infection with a particular clade of virus. However, this effect may not be retained for an extended period of time.

Discussion

This is the first published study that systematically analyzes variations in the Gag sequence and their associations with HLA in HIV-1 CRF01_AE infections. We identified 56 amino acid variations at 44 amino acid positions, which were significantly associated with a particular HLA class I type. We found that a substantial number of HLA-associated amino acid variations appeared to be unique to this CRF01_AE-infected Thai population. However, despite these distinct variants, we confirmed that the capsid protein (p24) is probably the preferred target of CTLs in CRF01_AE infections. We also found that the