(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.

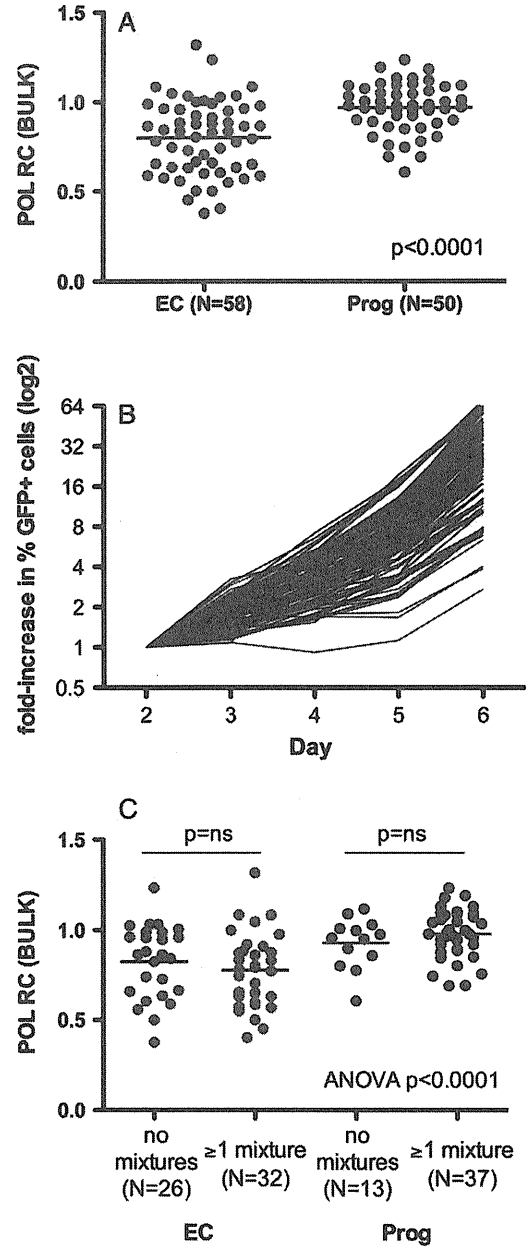

FIGURE 2. Replication capacities of bulk RT-Integrase recombinant viruses derived from elite controller and chronic progressor sequences. A, RC of bulk RT-Integrase recombinant viruses from controllers (EC) and Progressors (Prog) are shown. RC values are normalized to the mean RC of NL4-3, such that 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.

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

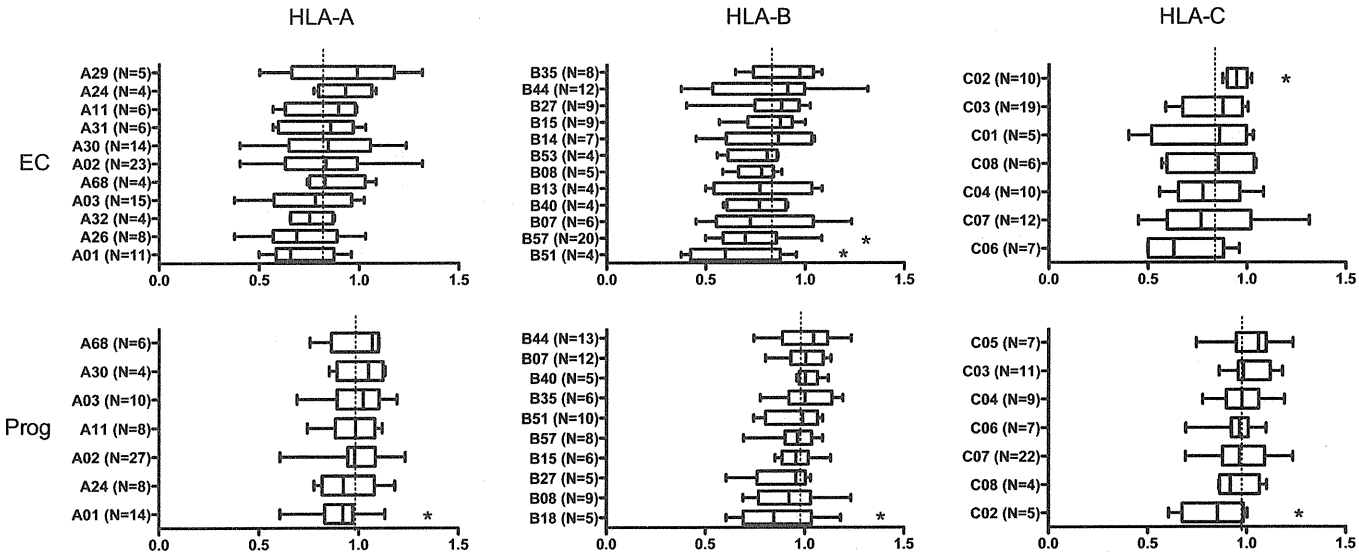

FIGURE 3. Associations between HLA class I allele expression and replication capacities of RT-integrase recombinant viruses. Box and whisker plots indicate the median (line), interquartile range (box), and range (whiskers) of viral RC, stratified by HLA class I alleles expressed. Asterisks indicate observations 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.

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.

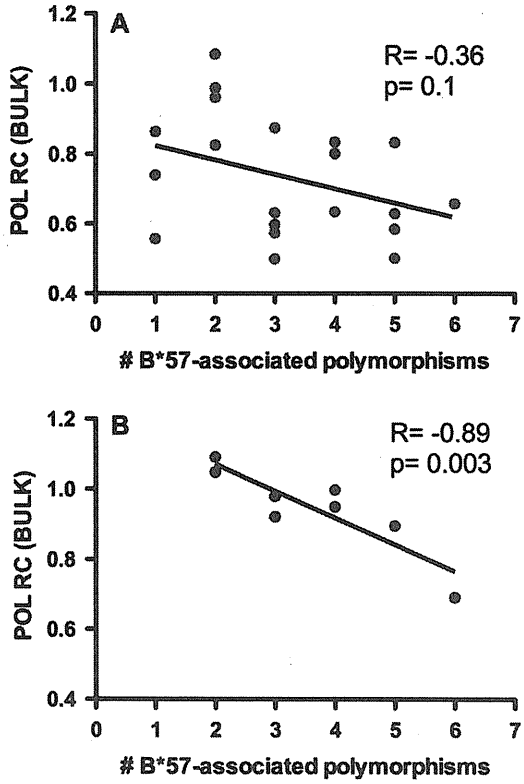

FIGURE 4. Relationship between the number of HLA-B*57-associated polymorphisms in RT-Integrase and viral Replication Capacity. In viruses from the 20 elite controllers (A) and 8 progressors who expressed HLA-B*57 (B), the relationship between the number of HLA-B*57-associated polymorphisms in RT-Integrase 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

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

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

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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

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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

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

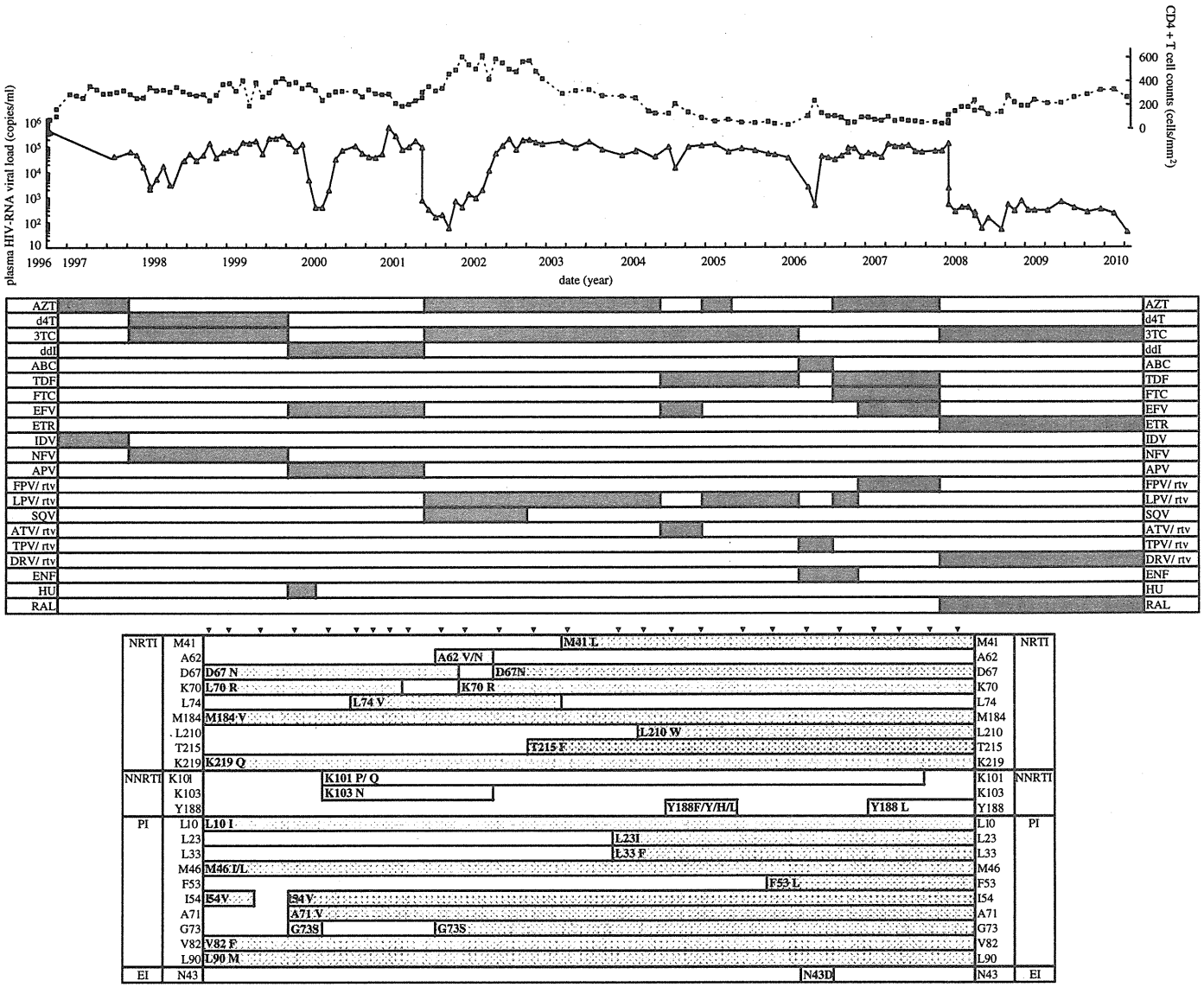

Fig. 1 Clinical course of a Japanese man infected with super-multidrug-resistant human immunodeficiency virus type 1 (the presented case). In the *upper panel*, *solid* and *dashed lines* indicate HIV-RNA level and CD4+ T cell count in the peripheral blood. The *middle panel* shows periods during which 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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PLoS one

Unique CRF01_AE Gag CTL Epitopes Associated with Lower HIV-Viral Load and Delayed Disease Progression in a Cohort of HIV-Infected Thais

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Abstract

Cytotoxic T Lymphocytes (CTLs) play a central role in controlling HIV-replication. Although numerous CTL epitopes have been described, most are in subtype B or C infection. Little is known about CTL responses in CRF01_AE infection. Gag CTL responses were investigated in a cohort of 137 treatment-naïve HIV-1 infected Thai patients with high CD4+ T cell counts, using gIFN Enzyme-Linked Immunospot (ELISpot) assays with 15-mer overlapping peptides (OLPs) derived from locally dominant CRF01_AE Gag sequences. 44 OLPs were recognized in 112 (81.8%) individuals. Both the breadth and magnitude of the CTL response, particularly against the p24 region, positively correlated with CD4+ T cell count and inversely correlated with HIV viral load. The breadth of OLP response was also associated with slower progression to antiretroviral therapy initiation. Statistical analysis and single peptide ELISpot assay identified at least 17 significant associations between reactive OLP and HLA in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these contained new CTL Gag epitopes. A substantial proportion of CTL epitopes in CRF01_AE infection differ from subtype B or C. However, the pattern of protective CTL responses is similar; Gag CTL responses, particularly against p24, control viral replication and slow clinical progression.

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Introduction

Cytotoxic T-Lymphocytes (CTLs) are an important component of the adaptive immune system which mediate control of HIV replication during acute infection and consequent viral set point [1]. Numerous CTL epitopes have been reported across the HIV proteome. However, the influence of CTL on clinical outcome varies, as their recognition of viral antigen is restricted by highly polymorphic class I Human Leukocyte Antigen (HLA) molecules [2,3]. Furthermore, the tremendous degree of viral diversity increases this complexity; to date, 13 prototype HIV clades and 43 circulating recombinant forms (CRF) have been described [4]. Some epitopes have been reported in a single clade; others have been reported in multiple clades (cross-clade) [5,6]. No reported epitope to date universally covers all HIV subtypes, or overcomes the global variation in HLA allele distribution (CTL Epitopes. Los Alamos National Lab. http://www.hiv.lanl.gov/).

Gag CTL responses, but not other CTL responses, have consistently been reported to have a significant association with viral control and clinical outcome [7]. However these findings were derived mainly from African or Caucasian populations infected with subtype C or B HIV, respectively; data from Asian populations infected with subtypes circulating in south-east Asia, such as CRF01_AE, have not yet been reported. To determine whether a similar association exists in south-east Asian subtypes, CTL epitope information is essential. However, as of April 2011, only 26 of 420 known Gag epitopes have been reported in CRF01_AE infection. Recently, the first successful phase III HIV vaccine trial was reported from Thailand [8], although its efficacy was marginal. For the development of a more effective vaccine, we believe it is crucial to accurately understand the influence of sequence variation amongst HIV subtypes, and HLA diversity amongst ethnic groups. To provide more information about CTL epitopes in CRF01_AE infection, we investigated cellular immune responses to Gag overlapping peptides in an HIV-1 CRF01_AE-infected Thai population and evaluated their impact on clinical outcome.

Methods

Subjects

This study was approved by the Thai Ministry of Public Health Ethics Committee and was conducted according to set guidelines for research. Written informed consent was obtained after

explaining the purpose and expected consequences of the study. Patients were eligible for inclusion if they were chronically HIV-infected and antiretroviral-naïve, with a CD4+ T cell count >200 cells/ul. A total of 137 HIV-1 CRF01_AE infected individuals were recruited at a government referral hospital in Thailand from October 2003 to May 2009. Study subjects were requested to visit the clinic every 3 months and CTL responses were evaluated every 6 months. The study endpoint was initiation of antiretroviral therapy, when their CD4+ T cell count declined below 200 cells/ul.

Synthetic HIV-1 Gag overlapping peptides

Fifteen-mer overlapping peptides (OLPs) of locally dominant CRF01_AE Gag sequences were designed based on 125 *gag* clonal sequences derived from 45 CRF01_AE infected individuals attending the clinic. All deduced amino-acid sequence data were aligned and the most frequent 15-mer amino-acid sequence was used as the dominant sequence.

Peptides were synthesized by Sigma Genosys (Hokkaido, Japan) with a high purity of >90% as determined by high-pressure liquid chromatography. In total, 98 peptides were synthesized and 20 pools were made by mixing 10 peptides per pool in a 10×10 matrix design so that a single responsible peptide could be identified by detecting the common peptide between two reactive pools, as described previously [9–11]. When more than one peptide was recognized, we further confirmed the responsible peptide recognition by individually testing candidate peptides, which were suspected by the matrix method.

ELISpot assay

1×10^5 fresh PBMC/well were plated onto multiScreen plates (MAHA54510; Millipore) that had been coated overnight at 4°C with 50 µl of anti-gIFN capture Ab 1-D1-K (2 µg/ml; Mabtech, Ohio, USA). Peptides were added directly to wells at a final concentration of 1 µM in 50 µl of R10 and incubated at 37°C in 5% CO_2 for 24 hrs. PBMC were stimulated with either medium alone for negative control, 10 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich) for positive control or peptide (1 µM final concentration) for 24 hrs at 37°C. Plates were washed extensively with wash buffer (PBS/Tween20 0.001%), followed by incubation with biotinylated anti-human gIFN mAb (0.5 µg/ml; clone 7-B6-1; Mabtech) in PBS/10% FBS for 2 hrs at 37°C. Following six further washes with wash buffer, 2 µg/ml streptavidin HRP (Mabtech) was added to wells with 1 hr incubation at room temperature. Spots were visualized using BCIP/NBT substrate (Chemicon, Australia) and were counted using an Automated Enzyme-Linked Immunospot (ELISpot) Reader System with KS 4.3 software by an independent scientist in a blinded fashion. Each assay was undertaken in triplicate. Spot forming units (SFU) were counted and expressed as SFU per million PBMCs, using the average result from triplicate wells followed by subtraction of the negative control values. A response was defined as positive if it was three times higher than the negative control and greater than 150 SFU/1×10^6 PBMC. The breadth of response was defined as the total number of peptides recognized by each subject. The magnitude of response for an individual was defined as the sum of all positive peptide responses (in SFU/1×10^6 PBMC). To avoid overestimation of breadth or magnitude, two adjacent positive overlapping peptides were counted as one response, using the higher of the two responses.

HLA class I typing

Genomic DNA was extracted from buffy coat using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) and 4-digit HLA class I typing for A, B and Cw loci was undertaken by bead-based array hybridization (WAKFlow HLA typing kit, Wakunaga Pharmaceutical, Hiroshima, Japan) according to manufacturer's instructions at a commercial laboratory (Kyoto HLA Laboratory, Kyoto, Japan).

Statistical analysis

Statistical analysis was performed using EXCEL 2007 and SPSS. We first selected viral loads (VL) in the lowest (=q1) and highest (=q4) quartiles (n=34 for each) and compared the number of individuals with positive ELISpot responses to p17, p24 and p15 proteins, using Fisher's exact test to compare groups. We then analyzed the association between breadth and clinical outcome (CD4+ T cell count and VL), using the Kruskal-Wallis test, and between magnitude and clinical outcome (CD4+ T cell count and VL) using Spearman's correlation test. We also performed a longitudinal analysis of the effect of breadth on Highly Active Anti-Retroviral Therapy (HAART) initiation, using the log rank test and Cox regression. For this analysis, the first individual was enrolled on 6 July 2000 and the last individual on 4 September 2007, with a censoring date of 31 May 2009. Analysis of OLP-HLA associations was undertaken using Fisher's exact test with 95% confidential intervals (CI). To have enough statistical power, we analyzed OLP-HLA associations when OLPs were recognized by 3 or more individuals with relevant HLA alleles and at least in one individual, the OLP recognition was confirmed by single peptide ELISpot experiments.

Results

Individuals' background, including HLA distribution

Of 137 individuals recruited, 107 were female and 30 were male. Median age was 31 years (range 16–56), CD4+ T cell count 461 cells/ul (range 204–1,191), and VL 4.22 log copies/ml (range 2.60–5.88). No individual had any HIV-related symptoms at the time of enrollment. In total, 87 variations of HLA alleles were found: 23 variations in HLA_A, 46 in HLA_B and 18 in HLA_Cw in four digits (Table S1). Median duration of follow-up was 22 months (range 0–60) and ELISpot experiments were repeated median 4 times (range 1–11) per individual. The peptide recognition pattern was confirmed to be consistent on at least two occasions for all except 24 individuals, in whom ELISpot assays were undertaken only once. During the follow-up period, the peptide recognition pattern did not change in any individual.

Gag OLP recognition and clinical outcome

Among 137 individuals, 112 (81.8%) recognized at least one OLP. Of 98 OLPs, 44 (44.9%) were recognized by at least one individual (Figure 1A): 12 peptides in p17, 26 in p24 and 6 in p15. The second half of p24 (HXB2 261–360; OLP 52–69), was the most highly targeted protein region; the first half of p17 (HXB2 5–60; OLP 1–9) was the second most highly targeted region. 14 OLPs were recognized in one individual and the other 30 OLPs were recognized in more than one individual. The most frequently recognized peptides were all located in the second half of p24: OLP 54 (HXB2 271–285), was recognized by 27 individuals; OLP 59 (HXB2 296–310) by 23 individuals; and OLP 66 (HXB2 331–345) by 22 individuals.

To further elucidate the peptide recognition pattern that best contributes to viral control, we next compared ELISpot responses between two extreme VL groups: the lowest quartile (=q1) (median VL 3.27 log copies/ml (range 2.60–3.71)) and the highest quartile (=q4) (median 5.09 log copies/ml (range 4.76–5.88)) (Figure 1B). Median CD4+ T cell count was 515 cells/ul (range

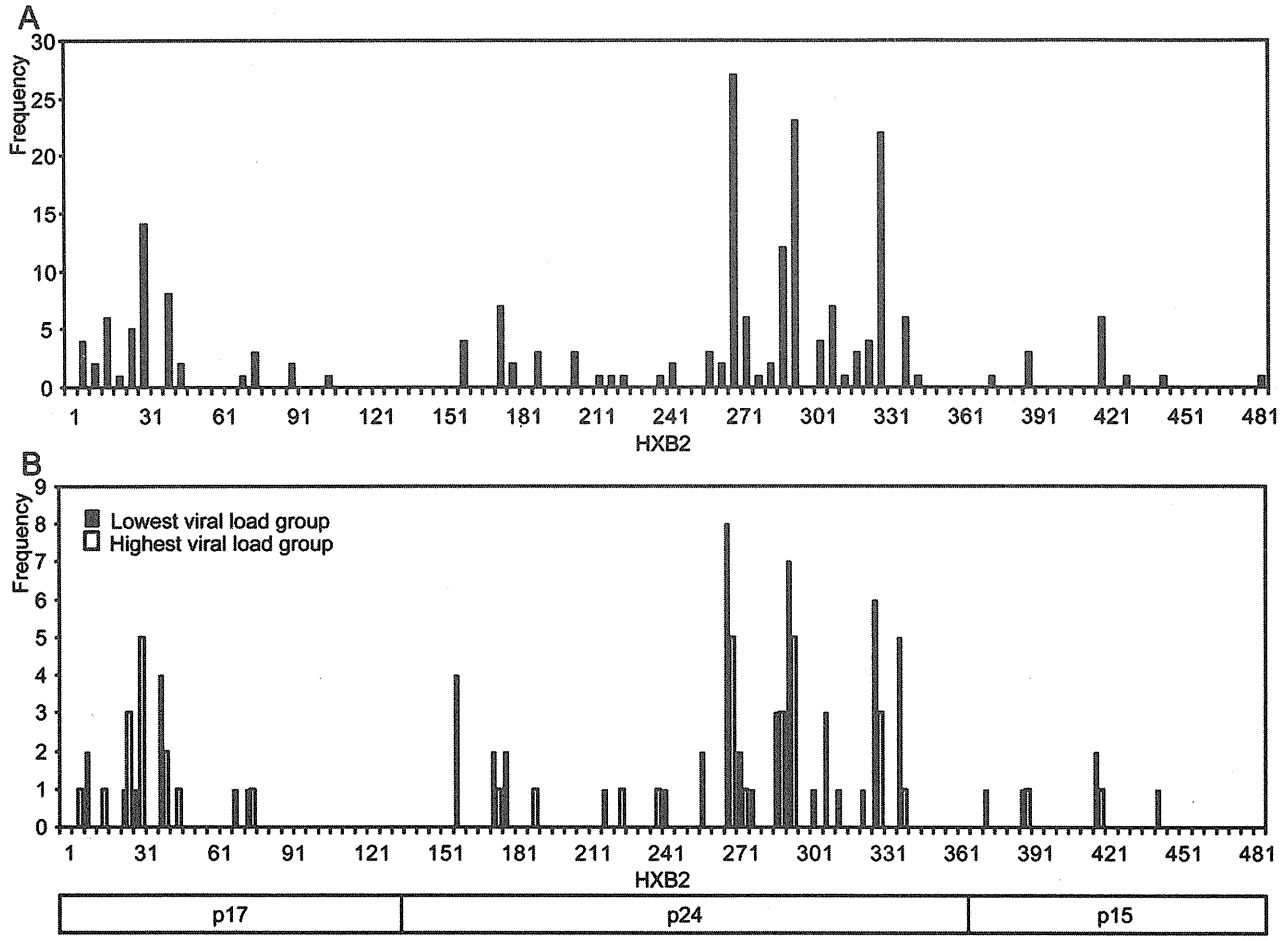

Figure 1. Pattern of CRF01_AE Gag CTL responses. Frequencies of overlapping peptide (OLP) responses in 112 individuals are shown (A); Frequencies of OLP responses in the lowest viral load group (lowest quartile, n = 34) and the highest viral load group (highest quartile, n = 34) were compared (B).
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243–1,057) in q1 and 429 cells/ul (range 204–856) in q4 ($p = 0.022$). Interestingly, individuals in q1 more frequently recognized p24 peptides than those in q4 (29/34 vs 18/34, respectively; $p = 0.0018$, Fisher's exact test), whereas individuals in q4 tended to recognize p17 peptides more frequently (9/34 vs 12/34, respectively; $p = 0.6$), although this difference was not significant.

ELISpot breadth, magnitude and clinical outcome

We next investigated the relationship between breadth and clinical outcome. The CD4+ T cell count was significantly higher in individuals with a greater breadth of response, with median CD4+ T cell count of 409 cells/ul (range 204–995), 455 cells/ul (range 243–793), 495 cells/ul (range 264–1,087) and 538 cells/ul (range 303–1,191) in individuals with 0, 1, 2 and ≥3 responses, respectively ($p = 0.018$ by Kruskal-Wallis test) (Figure 2A left). VL was significantly lower in individuals with a greater breadth of response, with median VL of 4.83 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.83), 4.26 log copies/ml (range 2.76–5.71) and 3.82 log copies/ml (range 2.60–5.04) in individuals with 0, 1, 2 and ≥3 responses, respectively ($p = 0.0015$) (Figure 2A right). In a site-specific analysis, we did not find any significant association with CD4+ T cell count in any sites (Figure 2B). Interestingly, we found a significant association with VL only in p24 (4.57 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.80), 4.17 log copies/ml (range 2.60–5.23) and 3.37 log copies/ml (range 2.60–4.14) in individuals with 0, 1, 2 and ≥3 responses, respectively; $p = 0.00028$) but not in other sites (Figure 2C).

We also found that magnitude of ELISpot response was positively correlated with CD4+ T cell count ($p = 0.0032$ by Spearman's correlation test $y = 0.031x+453$ $R^2 = 0.080$) and inversely correlated with VL ($p = 0.0084$ $y = -0.0001x+4.41$ $R^2 = 0.055$) (Figure 3A). In a detailed site-specific analysis, magnitude in p24 had a significant correlation with clinical outcome both in CD4+ T cell count ($p = 0.048$ $y = 0.013x+493$ $R^2 = 0.010$) (Figure 3B) and VL ($p = 0.0018$ $y = -0.0001x+4.39$ $R^2 = 0.065$) (Figure 3C), but not in other sites.

We next investigated the effect of breadth on clinical progression using the initiation of antiretroviral therapy as the end-point. During the follow-up period, 66/137 (48.2%) individuals started antiretroviral therapy. Intriguingly, we found that individuals with a wider breadth of CTL response were less likely to start antiretroviral therapy than those with a narrower breadth of response (Figure 4A, $p = 0.001$ by log rank test): 18/25 (72.0%), 13/34 (38.2%), 30/57 (52.6%) and 5/21(23.8%) individuals with 0, 1, 2 and ≥3 responses, respectively, initiating antiretroviral therapy. These data imply that strong CTL responses delay

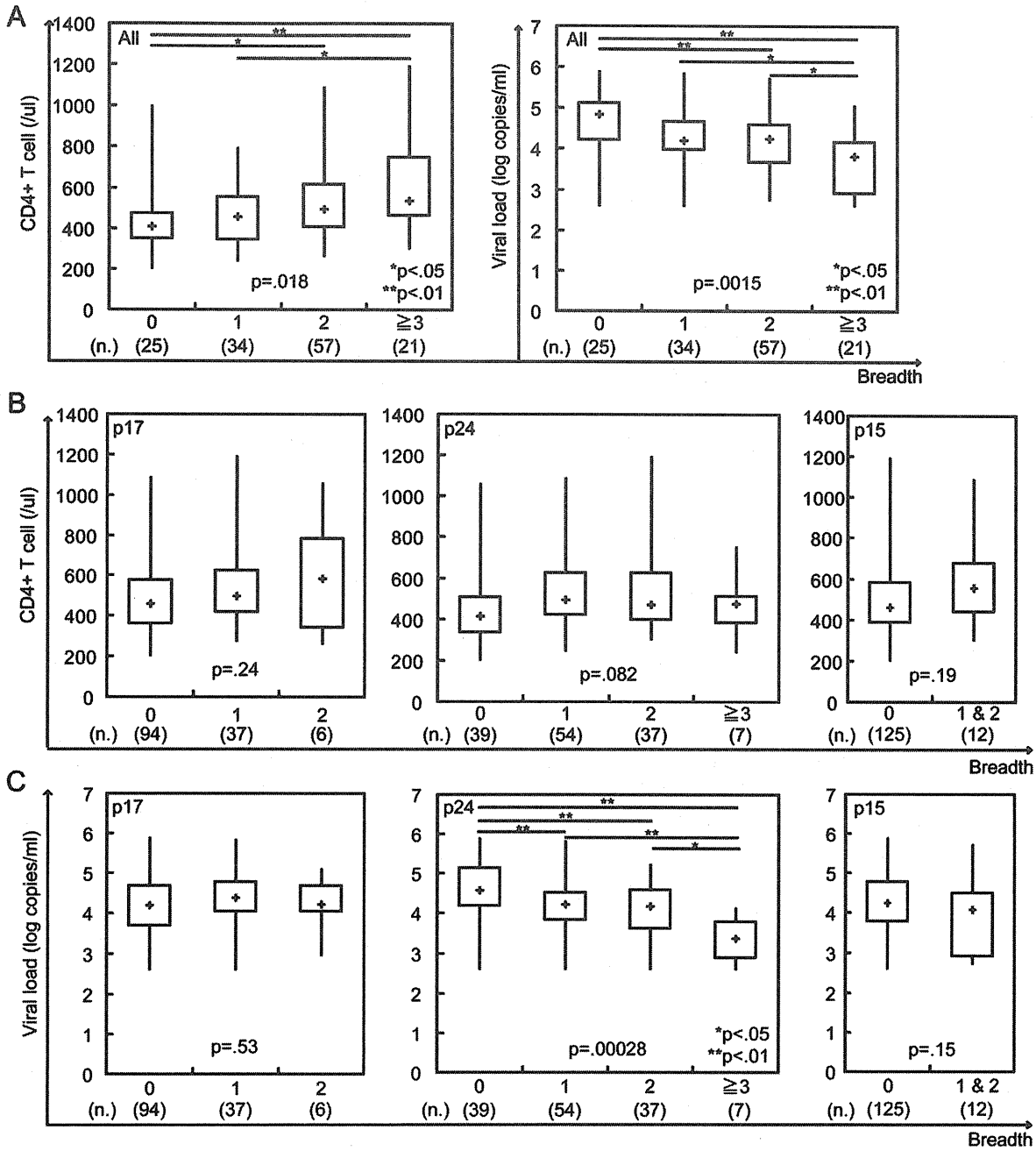

Figure 2. ELISpot breadth is associated with CD4+ T cell count and viral load. The associations between ELISpot breadth (the number of reacting OLP) and CD4+ T cell count or viral load were analyzed using the Kruskal-Wallis test (A). The p17, p24 or p15 site-specific ELISpot breadth was also compared with CD4+ T cell count (B) and viral load (C); * and ** showed a significant difference of $p<0.05$(*) and $p<0.01$(**) by Mann-Whitney u-test.

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clinical progression by slowing the decline in CD4+ T cell count. In a detailed site-specific analysis, individuals with a p24 response, but not other responses, were significantly less likely to start antiretroviral therapy than individuals without a p24 response ($p = 0.001$). However, the breadth of p24 response did not seem to correlate with clinical progression (Figure 4B).

Multivariate analysis of the relationship between CTL response and initiation of antiretroviral therapy, using Cox proportional hazards model, showed that the association between breadth of CTL response and initiation of HAART was independent of the baseline CD4+ T cell count (>350 cells/ul or not) and VL (<4.0 log copies/ml, 4.0–4.9 log copies/ml and ≥5.0 log copies/ml): adjusted Hazard Ratio (aHR) for individuals making ≥3 OLP responses was 0.23 ($p = 0.005$ with 95% CI of 0.08–0.64).

Detection of reactive OLP-HLA association

Associations between OLP responses and HLA were statistically analyzed. In total, 14 peptides (4 in p17, 9 in p24 and 1 in p15) with 31 OLP-HLA associations were identified (Table S2). 13 associations were found both with HLA-B and Cw alleles each and

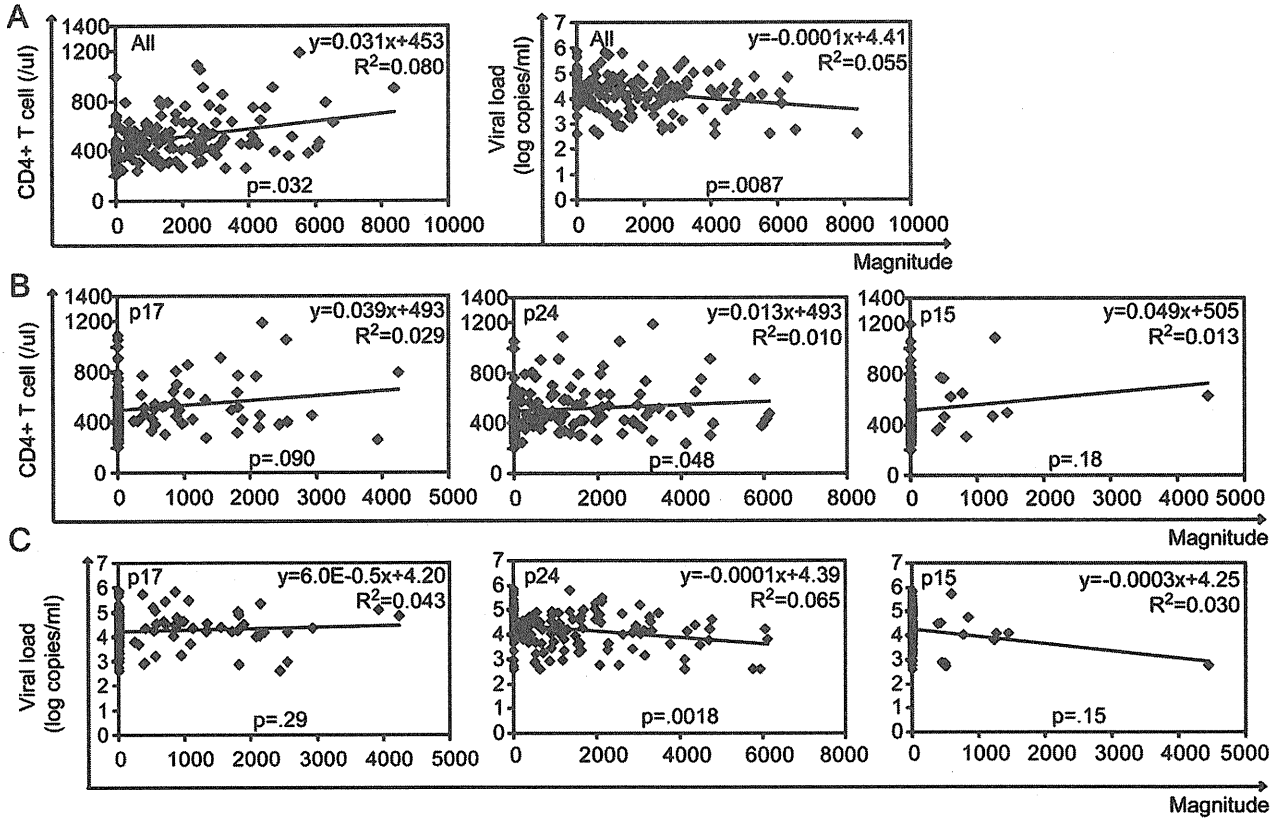

Figure 3. ELISpot magnitude is associated with CD4+ T cell count and viral load. The associations between ELISpot magnitude (total SFU per 1.0 M PBMC) and CD4+ T cell count or viral load were analyzed by Spearman's correlation (A). The p17, p24 or p15 site-specific response was also compared with CD4+ T cell count (B) and viral load (C).
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5 were found with HLA-A alleles. Two adjacent OLPs shared the same responsible HLA allele: HLA_A*0207, B*4601 and Cw*0102 in OLP 54–55, and B*4601 in OLP 58–59, suggesting that CTL epitopes reside in the overlapping region of these peptides. Some of the OLP-HLA associations may not reflect genuine CTL epitopes. 10 OLP responses were associated with two or more responsible HLA alleles. Of these, 9 OLP responses were associated with a pair of HLA alleles in linkage disequilibrium (LD), which were identified using the Los Alamos database (HLA Linkage Disequilibrium. Los Alamos National Lab. http://www.hiv.lanl.gov/). Among the 10 OLP responses, 7 included reported epitopes in either one of the HLA alleles. OLP 54, 55 and 59 responses were also associated with HLA alleles that have haplotype associations: HLA_A*0207-B*4601-Cw*0102. In total, 11 OLP-HLA associations were compatible with previously reported CTL epitopes: 4 epitopes were already reported as cross-clade epitopes including CRF01_AE or subtype A and the remaining 7 epitopes were reported in other subtypes but neither in subtype A nor CRF01_AE. Consequently, we identified at least 17 OLP-HLA associations in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these are likely to contain unique CRF01_AE Gag CTL epitopes.

Discussion

This is the first study to investigate Gag CTL epitopes and their effect on clinical outcome in a systematic way in a CRF01_AE-infected Asian cohort. In this study, which tested optimal OLPs in a well-described cohort, we succeeded in predicting a number of unique CRF01_AE Gag epitope and novel cross-clade epitope candidates. Although one third of CTL epitope candidates in CRF01_AE infection were not compatible with previously reported CTL epitopes in other subtypes, both cross-sectional and longitudinal analysis showed the pattern of protective CTL responses was similar to previous studies; specifically, that a Gag CTL response, particularly against p24, was associated with better control of viral replication and slower clinical progression [7,11–15]. These findings are also compatible with our previous study in which an association with clinical outcome was found only for the number of HLA-associated mutations in p24 but not in other sites [16]. Both studies imply that immune pressure on p24 Gag influences the clinical outcome in CRF01_AE infected Asian individuals. Several papers have discussed the advantages of CTL immune pressure against p24 for viral control , which include selection of escape mutations that lead to viral fitness cost [17,18], sequence stability compared with other viral particles [4,19,20], the abundance of Gag protein in incoming virions [21], and more rapid antigen presentation of Gag epitopes following viral infection [18].

While our findings showed the clear-cut relationship between ELISpot breadth and clinical parameters, the slopes of the trend lines between ELISpot magnitude and clinical parameters were rather shallow. Furthermore ELISpot magnitude did not correlate with onset of HAART initiation. These findings are consistent with a recently published study that breadth of the CTL response rather than magnitude associated best with clinical outcome [22].

In this study, we could not detect any OLP-HLA associations in HLA_B*57, which is well-known as one of the most protective

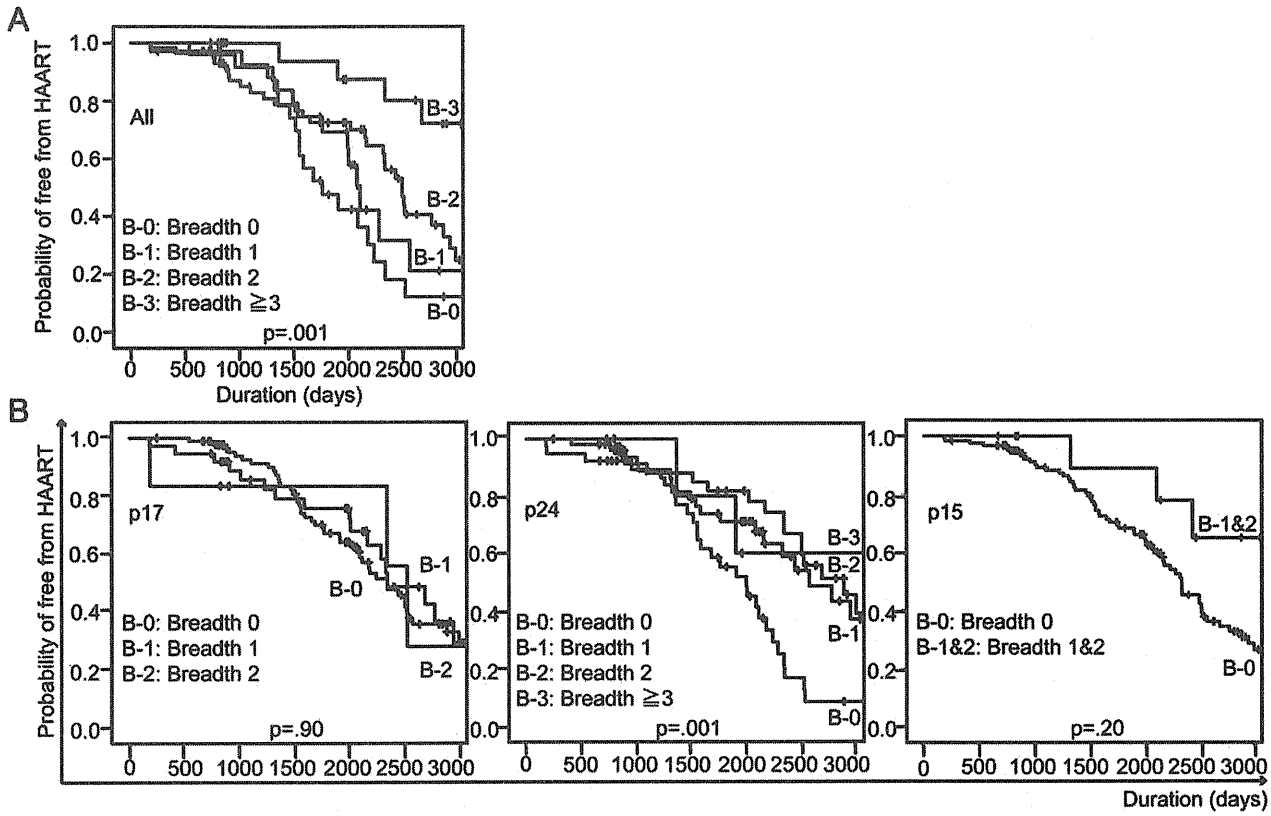

Figure 4. ELISpot breadth is related to delayed initiation of antiretroviral therapy. The impact of ELISpot breadth on antiretroviral therapy initiation was evaluated by Kaplan-Meier analysis, using the log rank test (A). The effect of p17, p24 or p15 site-specific ELISpot breadth was also analyzed (B).
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alleles for viral control [2,3,23]. Three individuals expressed B*5701; however, none had any response to OLP 47, which contains the TW10 (TSTLQEQIGW) epitope [24]. We have previously found in our cohort that all B*57 patients had the T242N escape mutation [16]. This suggests that the virus circulating in B*57 individuals lacks the wild-type TW10 sequence *in vivo* and no longer stimulates TW10 CTL cells [25].

In this study, OLP-HLA associations were predicted by statistical analysis. Thus these associations are not necessarily a reflection of new CTL epitopes with responsible HLA alleles. We excluded LD associations, including haplotypes and adjacent OLP responses with the same HLA allele association, in which CTL epitopes presumably reside in the overlapping region of these peptides. The most immunodominant OLP, number 54 (NKIVRMYSPVSILDI), was associated with three HLA alleles: A*0207, B*4601 and Cw*0102. "RMYSPVSIL" was previously identified as an A*0207-restricted CTL epitope [26]. All three responsible HLA alleles were found to be in LD. However, the association with B*4601 and Cw*0102 was much stronger than for A*0207 (odds ratio 29.4 in B*4601 and 104 in Cw*0102 vs 5.5 in A*0207) and further analysis including by ^{51}Cr release assay is warranted.

From this study, we have substantially increased information about CTL epitopes in CRF01_AE infection, reporting at least 6 unique CRF01_AE CTL epitope and 7 novel cross-clade epitope candidates. CRF01_AE is a recombinant HIV-1 with Gag derived from subtype A [4], from which CTL epitope information is limited, compared to subtypes B or C. We anticipate that if a more detailed epitope mapping study were to be conducted in subtype A-infected populations, there would be a large number of epitopes cross-recognized between CRF01_AE and subtype A.

Although details of OLP-HLA associations are substantially different between subtypes, interestingly we found a similarity in the immunodominant regions between subtypes. Our data showed that the second half of p24 was the most immunodominant regions, followed by the first half of p17 regions. This finding is consistent with previous reports [13,15,27]. We were concerned that the compatibility between OLP sequences and circulating Gag sequences may vary depending on the conservativeness and influence on the pattern of Gag CTL responses. However, the proportion of gag clones that were completely matched to the amino-acid sequence of OLPs was not associated with the frequency of OLP responses (data not shown).

Cross-clade CTL responses are said to be influenced by the viral sequence variability between subtypes, especially the sequence at anchor positions of the HLA binding motif [4,28–31]. Among the 7 newly identified cross-clade epitope candidates, 6 shared the same sequences with reported epitopes at both the B and F pockets. We also compared sequence compatibility at the anchor positions of the best-defined 12 epitopes, not identified in our study. 11 out of 12 also had compatible sequences at anchor positions, implying that sequence compatibility at anchor positions per se does not predict cross-clade reactivity. Other factors should be considered, such as sequences at flanking regions affecting peptide cleavage by the proteasome [32,33] and epitope-HLA complex recognition by T cell receptors (TCRs) [34,35].

This study has a number of limitations. First, we focused on Gag CTL immune responses and did not investigate whole viral proteins. However, since this type of analysis requires a large number of cells, and the volume of blood that we were able to take was rather limited, we decided to focus on Gag responses, as Gag is known to be the most important viral target. Instead of testing a large number of OLPs individually, we undertook experiments in triplicate, using a matrix system, to improve reliability. However, it would have been ideal if we had obtained enough volume of blood to confirm all responses using the individual peptides. Second, we detected OLP-HLA associations by a statistical method and not by the standard HLA-restriction analysis. This approach is easily influenced by sample size and the impact of LD. Thus our study does not provide direct evidence. Third, we have not yet confirmed these OLP responses with CTL using the ^{51}Cr release assay. However, ELISpot assays are now widely accepted as a technique for mapping CTL epitopes [36]. Fourth, these data are based on single cytokine release of gIFN; we did not evaluate multi-functionality of CTL with other cytokines such as IL2 or TNFα [37].

However, our data indicate the existence of a substantial number of unique CTL epitopes in CRF01_AE infection; it is therefore worth conducting a systematic analysis of CTL epitopes when vaccine trials are undertaken in different populations infected with different subtypes.

Supporting Information

Table S1 HLA allele frequencies in the study population.
(XLS)

Table S2 Gag overlapping peptide responses and their HLA allele associations.
(XLS)

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Author Contributions

Conceived and designed the experiments: BS PS KA. Performed the experiments: NW CB MM. Analyzed the data: MM NT. Contributed reagents/materials/analysis tools: BS PP KA. Wrote the paper: MM KA. Clinical evaluation and patient recruitment: PP. Critical review: TM.

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