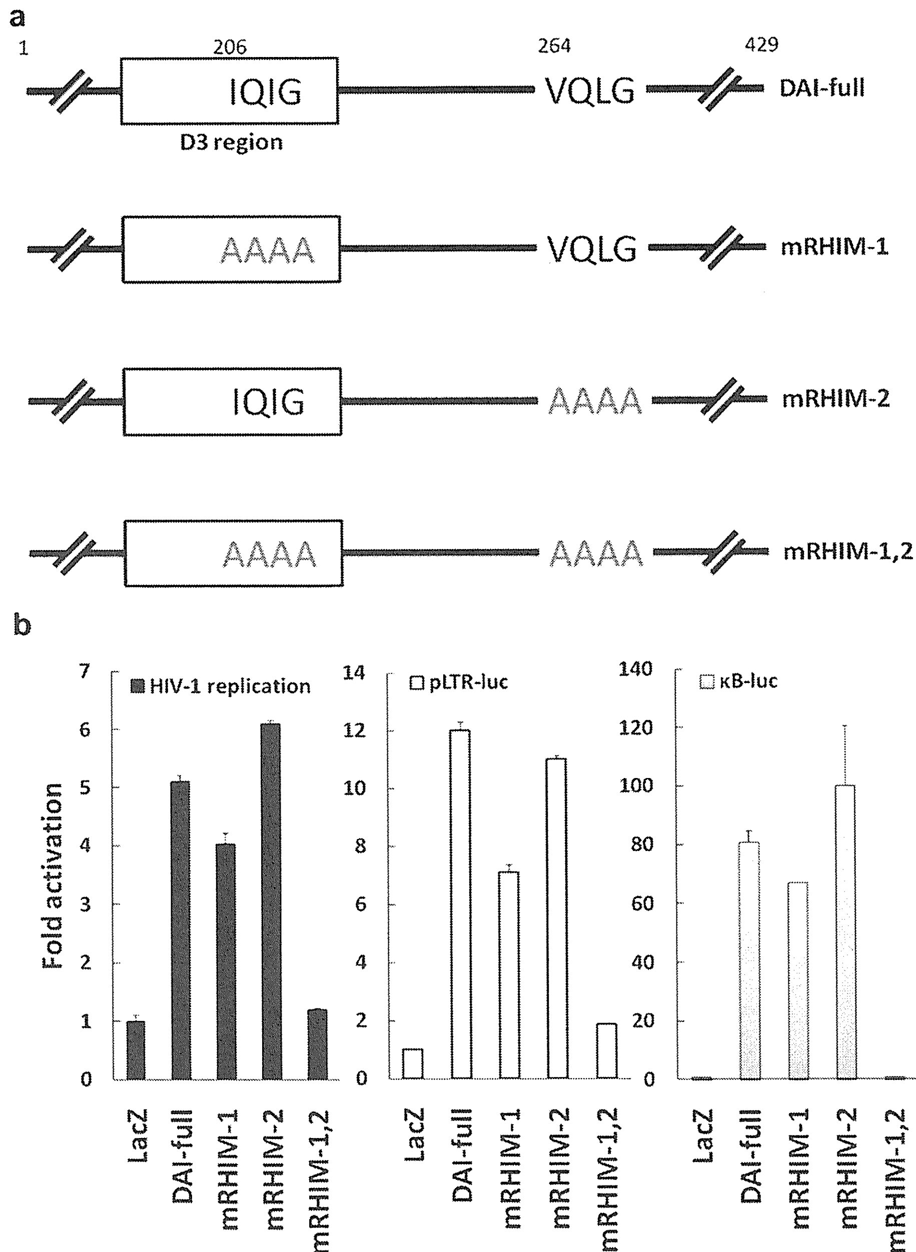

Fig. 5. Involvement of RHIMs in DAI promotion of HIV-1 replication. (a) In two RHIMs consisting of four conserved amino acids in the DAI expression vector, the first RHIM (IQIG), the second RHIM (VQLG), or both RHIMs were replaced with four alanine residues. (b) 293T cells were transfected with expression plasmids of LacZ, wild type DAI, or DAI mutants as-indicated, and infected with HIV-1/VSV-G pseudotype viruses (HIV-1 replication, left graph) after 2 days. HIV-1 LTR reporter assays (pLTR-luc, middle), or NF-κB reporter assay (κB-luc, right graph) were performed as-described in Fig. 2d, and the values of fold activation of luciferase activities relative to LacZ controls are shown. Data represent means ±S.D. of duplicate samples.

for both transcription factors, two κB sites in the U3 region of LTR, and an interferon-stimulated response element (ISRE)/ interferon-regulatory factor elements (IRF-E) sequence immediately downstream of the U5 region (Fig. 3a). To investigate whether these *cis*-elements were involved in the DAI-mediated enhancement of HIV-1 transcription, we constructed two mutant reporter plasmids that disrupted two κB sites (pLTRmκB-luc) or deleted ISRE/IRF-E (pLTRΔISRE-luc). Similar to the wild type LTR, transcription from pLTRΔISRE-luc was considerably increased by the expression of DAI (Fig. 3b). However, mutation of two κB sites in the LTR significantly impaired the enhancing effect of DAI (Fig. 3b). We also showed that co-expression of the super-repressor form of IκBα (SR-IκBα), NFκB activation-defective mutant, significantly impairing DAI-mediated enhancement of gene expression from HIV-1 LTR (Fig. 3c). This effect of SR-IκBα on the activity of DAI was specific for NF-κB since co-expression of DAI and SR-IκBα also impaired the transcription from κB-luc reporter plasmid, and it was comparable to control group without SR-IκBα when pLTRmκB-luc was used (Fig. 3c). Suppressive effect of SR-IκBα on the activity of DAI was reproduced in HIV-1 infection assay (Fig. 3d). These

results suggested that DAI promoted HIV-1 replication by enhancing transcription predominantly through NF-κB activation.

3.4. The region within DAI responsible for enhancement HIV-1 transcription

To determine the region in DAI that is responsible for the enhancement of HIV-1 transcription, a series of truncated DAI mutants was generated (Fig. 4a). The effect of each DAI truncation mutant on HIV-1 replication was evaluated (Fig. 4b). The enhancing effects of DAI on HIV-1 replication were moderately diminished by the deletion of first Z-DNA binding domain (ZBD) (Zα) and second ZBD (Zβ) (ΔZα and ΔZαβ). Disruption in the D3 region (ΔN199, ΔN234 and Zαβ) abolished the ability of DAI to activate the HIV-1 transcription. Involvement of the D3 region in DAI-mediated enhancement of viral transcription was confirmed by a D3 deletion mutant (ΔD3) which almost completely eliminated the enhancing effect on HIV-1 replication (Fig. 4b). Similar effects of each DAI mutant were reproduced when assayed in gene expression system using κB-luc reporter plasmid (Fig. 4b). Although slight differences of the levels of protein expression were observed in several DAI mutants, they seemed not to explain for the considerable decrease in their activities (Fig. 4a and b). Thus, the D3 region of DAI seemed to be the most critical region for enhancement of HIV-1 transcription although the Zα and Zβ regions also appeared to exert some influence.

3.5. Requirement of RHIM(s) in DAI for transcriptional enhancement of HIV-1

To further investigate the critical residues in D3, a series of five residues from 179 to 198 aa were alternately substituted by alanine residues (Fig. 4c) given that the mutant, DAIΔN199, lacked almost one-third (179–199 aa) of the D3 region and its activity was almost completely eliminated (Fig. 4b). However, none of these alanine substitutions resulted in a significant decrease in DAI activity (Fig. 4c). Finally, we looked for amino acid sequences within the D3 region, participating in the activation of NF-κB. A careful search based on the consensus motif for the activation of NF-κB, a typical motif known as RHIM was found in the D3 region, and another was found downstream of the D3 region [35]. Therefore, we constructed several DAI mutants with mutations in the RHIM(s) (Fig. 5a) and investigated the requirement of each in DAI-mediated enhancement of HIV-1 replication. Mutation at the first RHIM (mRHIM-1) but not the second RHIM (mRHIM-2) slightly impaired DAI-mediated transcriptional activation of HIV-1. However, the enhancing effects of DAI on HIV-1 replication were almost completely abolished when mutations were introduced into both RHIMs (mRHIM-1, 2) (Fig. 5b). Mutations at both RHIMs in DAI also abolished DAI-mediated enhancement of transcriptional activities driven by HIV-1 LTR and known κB sites (Fig. 5b). Thus, it appeared that although RHIM-1, lying at the center of the D3 region, might be more effective than RHIM-2, each RHIM could complementarily exert its function to enhance HIV-1 replication through NF-κB.

4. Discussion

In the present study, we demonstrated that DAI-mediated signals promoted HIV-1 transcription through activation of the NF-κB pathway, and determined the responsible regions in the HIV-1 LTR and DAI for enhancement of HIV-1 transcription. HIV-1 LTR contains four functional regions controlling HIV-1 transcription: the transactivation region (TAR) element; the core (basal) promoter; the core enhancer; and the modulatory region. The TAR lies in the R region, and the others in the U3 region of the LTR [37]. In these, the core promoter includes three Sp1 binding sites, and the core enhancer includes two NF-κB/NF-AT binding sites [37,38]. Sp1 and NF-κB, as well as Tat are especially essential for HIV-1 transcription [39–41]. In this study, DAI-mediated activation of HIV-1 transcription was largely due to the activation of NF-κB, as DAI hardly promoted the transcription from the HIV-1 LTR mutated at two κB sites or the Mo-MuLV LTR lacking intrinsic κB sites (Figs. 1 and 3b). In contrast, it might be unlikely that IRF3, another transcription factor known to be activated by DAI, was involved in DAI-mediated activation of transcription from HIV-1 LTR, as the deletion of ISRE from HIV-1 LTR barely affected the activity of DAI. This is consistent with a previous report which described only IRF1 but not IRF3 could activate the transcription of HIV-1 among the IRF family [42].

NF-κB plays critical roles in HIV-1 transcription in both acute and latent infections. In the early phases of HIV-1 replication, it has been shown that binding of the virions to CD4 molecules could activate NF-κB and promote HIV-1 transcription before Tat expression [43]. In the present study, HIV-1 transcription was significantly enhanced by DAI expression. In DAI-transfected cells, the early-onset of HIV-1 transcription was observed, reaching maximum levels 24 h following infection, much earlier than in the control LacZ-transfected cells (data not shown). These results might implicate that DAI promoted the initiation of HIV-1 transcription through NF-κB activation in the absence of Tat, and consequently enhanced Tat-dependent transcription. This is consistent with a previous report that PMA or TNF-α could promote transcription of a Tat-defective HIV-1, indicating the importance of NF-κB for Tat-independent HIV-1 transcription [44].

In latent HIV-1-infected cells, it has been suggested that continuous activation of NF-κB and subsequent Tat expression are required for sustaining the phosphorylation of RNA polymerase II C-terminal domain, and this induces efficient transcription and reactivation of HIV-1 [45]. Infection with opportunistic microorganisms, as a result of progressive immune dysfunction in HIV-1 infection, is an exogenous stimulus potentially triggering the host innate immune system and activating NF-κB. It has been described that superinfection with HSV-1 could activate HIV-1 in acute and latent HIV-1-infected cells through NF-κB activation [23,24,46,47]. PRRs such as DAI might be involved in such a vicious circle to

reactivate latent HIV-1 infection. Indeed, the potential cytosolic exposure of DNA has been reported in some viruses and bacteria detected by opportunistic infection [23–26]. The recognition of group B streptococcus, for instance, occurs in the cytoplasm and to some extent independently of TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs) or RLRs, and is supposedly mediated by cytosolic DNA sensor(s) [29].

The D3 region within DAI has been shown to be important in binding to dsDNA, as the IFN-β response to B-form DNA was significantly decreased in D3-deficient DAI [7]. Consistent with this, the mutant DAIs containing deletions extending into the D3 region abolished its ability to enhance HIV-1 transcription. This might be due to the disruption of intact structure of D3 region required for DAI function including interaction with RIP1/3 [35,36]. Although these mutants of DAI also have deletions and substitutions in regions other than D3 that decrease HIV-1 transcription to some extent, the deletion of the D3 region alone abolished all DAI effects. This suggests that the D3 region is essential for DAI activity promoting HIV-1 transcription, but full activity requires the entire intact structure of DAI.

Two recent reports have indicated the existence of two or three RHIMs in DAI [35,36]. Of these, RHIM-1 and RHIM-2 are highly homologous to RHIMs seen in RIP1, RIP3 and Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF), but the RHIM closest to the C-terminal of DAI does not possess the core consensus sequence, (V/I)Q(I/L)G. A previous study has reported that only RHIM-1 is required for interaction with RIP1 and RIP3, but both RHIM-1 and RHIM-2 can independently activate NF-κB. In the present study, however, DAI-mediated promotion of HIV-1 transcription was only slightly affected by mutation in RHIM-1 but not RHIM-2, and was almost completely eliminated by double mutations in RHIM-1 and RHIM-2. But, the reason for the difference in the contribution of RHIMs to DAI activity between the previous and present studies remains to be clarified.

Further subcellular fractioning experiments revealed that wild type DAI exhibited a strong tendency to localize a fraction containing the cytoskeleton and cytosol, whereas DAI with double mutations in RHIM-1 and RHIM-2 was diffusely detected in several cellular components including the cytoskeleton, membranes, cytosol and nucleus (data not shown). This is consistent with a report indicating that intracellular DNA stimulation induces accumulation of a portion of DAI into the area adjacent to the endoplasmic reticulum [2]. These observations suggest that each RHIM might have a role in forming a signalosome, containing DAI and RIP1/3, on the cytoskeleton to activate NF-κB, and/or transporting DAI along microtubules where the activation of NF-κB occurs.

We did not obtain any evidence supporting the notion that HIV-1 infection itself might activate DAI in 293T cells. This was presumably due to the low copy number of cytoplasmic HIV-1 dsDNA produced by reverse transcription, which differs from the intracellular amplification of viral DNA by DNA viruses. However, superinfection with DNA viruses or intracellular invading bacteria potentially stimulates DAI, possibly playing an important role in the reactivation of HIV-1 in latently infected cells.

In conclusion, the stimulated DAI induces NF-κB activation, which promotes the transcription of HIV-1.

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MINI-REVIEW

HIV-1 tropism

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Acquired immunodeficiency syndrome (AIDS) was reported in 1981 ((CDC), 1981). Human immunodeficiency virus type-1 (HIV-1), the virus causing AIDS, was isolated in 1983 (Barré-Sinoussi et al., 1983). Since AIDS patients were characterized by a profound decrease in CD4-positive T cell count (Gottlieb et al., 1981), it was a logical consequence that CD4 would be the most probable receptor for the virus. Actually, it was confirmed that CD4 is the primary receptor for HIV-1 in 1986 (Maddon et al., 1986). However, it was speculated that HIV-1 requires another factor to infect a cell, since CD4 alone could not support HIV-1 infection to nonhuman cells (Weiner et al., 1991). The co-receptor was an enigma for 10 years. A G protein-coupled 7 trans-membrane protein, originally called fusin and currently known as CXCR4, was isolated as a co-receptor for HIV-1 in 1996 using an assay system in which fusion between Env-expressing and CD4-expressing cells leads to activation of a reporter gene (*E. coli* lacZ) (Feng et al., 1996). Interestingly, CXCR4 acted as a preferential co-receptor for HIV-1 adapted to T cell lines *in vitro*, in comparison to its activity with primary HIV-1 isolates.

Long before the identification of CXCR4 as a co-receptor, it had been known that primary HIV-1 isolates show preferential growth in either CD4-positive T cell lines (T cell-line tropic or T-tropic strains) or monocyte-derived macrophages (macrophage tropic or M-tropic strains) while they replicate well in peripheral blood mononuclear cells (PBMC) (Asjö et al., 1986; Collman et al., 1989). It was speculated that T cell lines and monocytes/macrophages carry distinct co-receptors which discriminate for T-tropic and M-tropic strains (Schuitemaker et al., 1993). β-chemokines such as RANTES, MIP-1α and MIP-1β produced by CD8-positive T cells were proven to have HIV-1-suppressive activity (Cocchi et al., 1995). RANTES, MIP-1α and MIP-1β bind toCCR5, a member of CC-chemokine receptor family, as a common receptor. CCR5 has the characteristic structure of G protein-coupled 7 trans-membrane receptors. The discoveries of β-chemokines as HIV-1-suppressive agents and a molecular mimicry to CXCR4 led to a molecular identification of CCR5 as a co-receptor for M-tropic strains (Deng et al., 1996). SDF-1 was found to be a ligand of CXCR4 (Oberlin et al., 1996). A new classification for HIV-1 was proposed: R5 viruses for the isolates that use CCR5 but not CXCR4, X4 viruses for those that use CXCR4 but not CCR5 as a co-receptor (Berger et al., 1998). There are isolates that can use both CCR5 and CXCR4 and are called dualtropic.

The envelope glycoprotein of HIV-1 is synthesized as a precursor form, gp160. Trimeric gp160 molecules are transported to the Golgi apparatus, where they are cleaved by a cellular protease generating mature envelope glycoproteins: gp120, the exterior glycoprotein and gp41, the trans-membrane glycoprotein (Wyatt and Sodroski, 1998). The viral determinant of the co-receptor usage had been mapped to the third variable (V3) domain of the envelope glycoprotein gp120 (O'Brien et al., 1990; Hwang et al., 1991; Shioda et al., 1991). V3 amino acids in X4 strains have a significantly higher positive charge than R5 isolates (Fouchier et al., 1992). The presence of a positively charged amino acid at 11th or 25th positions of the V3 loop is a predictive marker for the X4 phenotype (Resch et al., 2001). The high-affinity co-receptor binding site is hidden from the surface of gp120 and sequestered from the host immune surveillance (Wyatt and Sodroski, 1998). Binding to CD4 molecules induces large conformational changes in gp120 (Wu et al., 1996). The V3 loop after CD4 binding has three structural regions: a conserved base, which forms an integral portion of the gp120 core; a flexible stem, which extends away from the core; a β-hairpin tip (Huang et al., 2005). The tropism determining positions 11 and 25 are within the variable stem. This stem-β-hairpin tip structure may allow V3 to

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function as a molecular hook for the co-receptor. Further conformational change in gp120 induced after co-receptor binding triggers the fusion process between viral envelope and the plasma membrane of the host cell.

It has been known that the co-receptor usage is highly related to the clinical course of HIV-1 infection. During the early stage of the infection, R5 viruses predominate. Data from cross-sectional studies show that 70%–80% of patients with early-stage disease harbor only R5 virus (Japour et al., 1995). During the natural course of the infection, viral variants that use CXCR4 appear in roughly 50% of the patients (de Roda Husman et al., 1997). It is still an enigma why R5 viruses have an advantage to replicate in the early phase of the infection. Appearance of X4 viruses is correlated often with the rapid clinical progression (Tersmette et al., 1989), which is relevant to the differential expression of CCR5 and CXCR4 according to the T cell development (Bleul et al., 1997).

Small molecular inhibitors for CCR5 and CXCR4 are available (Kuritzkes, 2009). CCR5 antagonists have been named with a suffix, "viral receptor occupancy (-viroc)." CCR5 inhibitor, maraviroc, has been approved for clinical use and vicriviroc is on the clinical trial in the resource rich countries. AMD3100 and related compounds AMD11070 have been developed as CXCR4-specific inhibitors; however, none of them have been approved for clinical use. Determinations of the co-receptor usage of patients' viruses are a prerequisite to choose maraviroc for treatment.

There are several ways to determine the HIV-1 co-receptor usage: one is the phenotype-based method and other is genotype-based (Table 1) (Koot et al., 1992; Trouplin et al., 2001; Braun and Wiesmann, 2007; Van Baelen et al., 2007). Before the co-receptors were molecularly cloned, a method to classify HIV-1 isolates according to the ability to induce syncytium formation in MT-2 cell line had been published (Koot et al., 1992). MT-2 cells express CXCR4 but not CCR5. Therefore, MT-2 assay was actually a phenotypic assay for the co-receptor usage. Virtually speaking, T-tropic viruses were syncytium-inducing (SI) and M-tropic viruses were non-syncytium inducing (NSI) viruses. More sophisticated phenotypic assays are available using reporter cell lines expressing CD4 and a co-receptor, either CCR5 or CXCR4, on the cell surface. They have been used to characterize HIV-1 present in the patients' plasma. Trofile™ (Monogram Biosciences, South San Francisco, California, USA) is one of them. HIV-1 gp160 gene in a patient's plasma is amplified, cloned into an expression vector and co-transfected with an HIV-1 genomic vector carrying a reporter luciferase gene, to generate pseudotype virions. HIV-1 tropism is judged according to the luciferase activity in the reporter cells after infection (Whitcomb et al., 2007).

Since the Trofile assay has been a single globally approved phenotypic assay for the clinical use of maraviroc, the effort to speculate the co-receptor usage from sequencing data has been continued. For example, Web PSSM (http://indra.mullins.microbiol.washington.edu/webpssm/) (Jensen et al., 2006), Geno2Pheno [coreceptor] (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) (Sing et al., 2007) and WetCat (http://genomiac2.ucsd.edu:8080/wetcat/v3.html) (Pillai et al., 2003) are algorithms to predict HIV-1 co-receptor tropism based on the V3 sequences. The V3 sequence prediction may fail to correlate with the phenotype test, since Env region other than V3 may influence the co-receptor usage (Huang et al., 2007, 2008). The sensitivity and specificity of these assays are under debate, but evidence is accumulating that the genotypic assay is useful in clinical decision-making.

HIV-1 tropism has been characterized almost exclusively on subtype B HIV-1, which is predominant in the resource rich countries. Analysis of the HIV-1 tropism in the strains predominating in Africa and Asia awaits future research. A simpler phenotypic assay system would be valuable to characterize these strains.

Table 1 Methods currently used to determine HIV-1 coreceptor tropism

assay		Env flagment	type	detection	reference
trofile	phenotype	gp160	single cycle	Luc from the viruses	Whitcomb et al., 2007
phenoscript	phenotype	V1-V3	multiple cycle	β-Galactosiase from the cells	Trouplin et al., 2001
MT-2	phenotype	NA	multiple cycle	Syncytia formation, p24	Koot et al., 1992
tropism-testing platform	genotype and phenotype	C1-V4	multiple cycle	GFP from the viruses	Van Baelen et al., 2007
Xtrack^{c}/PhenX-R	genotype and phenotype	V1-V3	multiple cycle	β-Galactosiase from the cells	Braun et al., 2007
Web PSSM	genotype	V3	NA	sequencing	Jensen et al., 2006
Geno2 Pheno [corecep-tor]	genotype	gp120	NA	sequencing	Sing et al., 2007
WetCat	genotype	V3	NA	sequencing	Pillai et al., 2003

NA, not available

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

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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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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′-AAATCTCTAG-CAGTGGCGCCCGAACAG-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′-TCTCGACGCAGGACTCGGCTT-GCT-3′, and antisense inner primer, Gag-R2 5′-CCTCCAATT-CCCCCTATCATTTTTGG-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

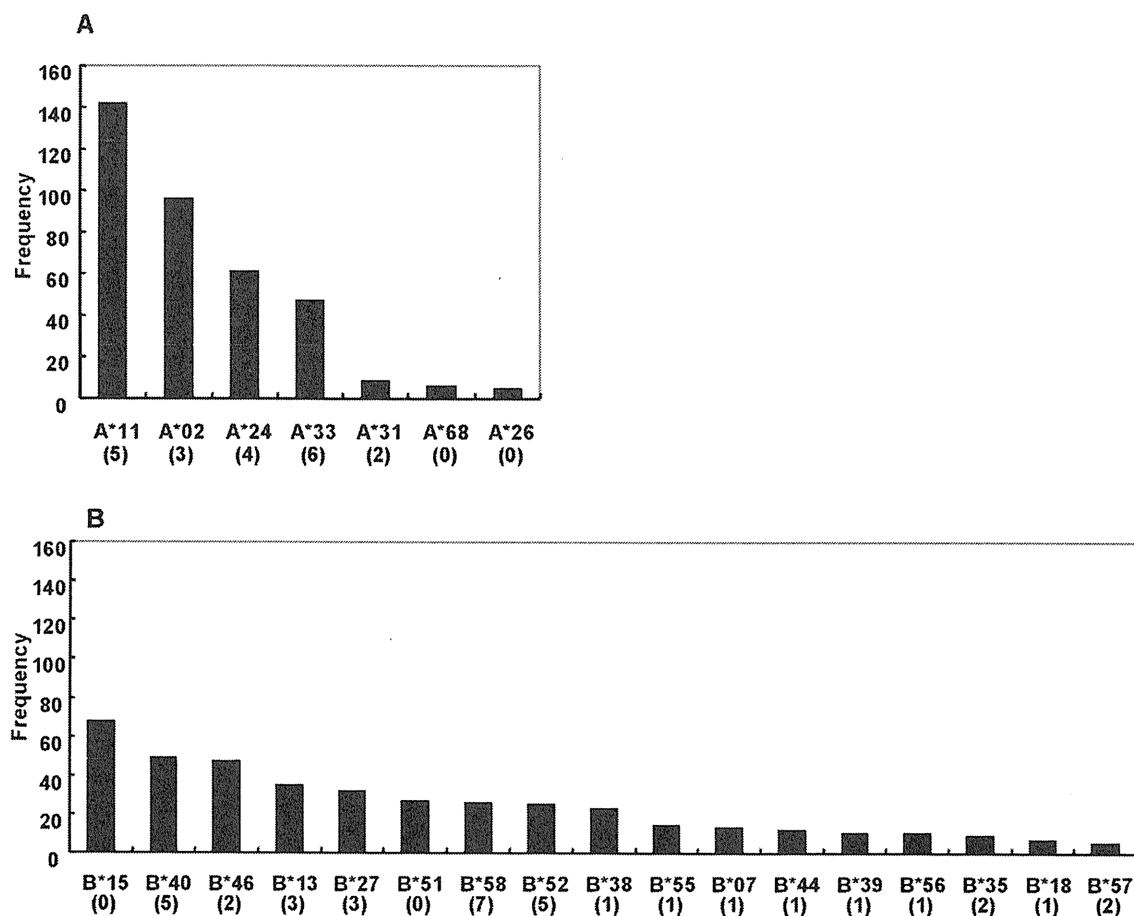

Figure 1. HLA_A and HLA_B allele distributions among patients. HLA allele distributions: the frequencies of HLA_A and _B alleles occurring in at least five or more individuals are shown in (A) and (B), respectively. The number of HLA-associated amino acid variations is indicated in brackets under each 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: KELYPLTSL
	(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: KELYPLTSL
	(P482X)			−	7	102		11.25		
	HXB2 485X	Purify	B*40	+	7	28	3.64	1.18	0.008631568	B*4001: KELYPLTSL
	(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

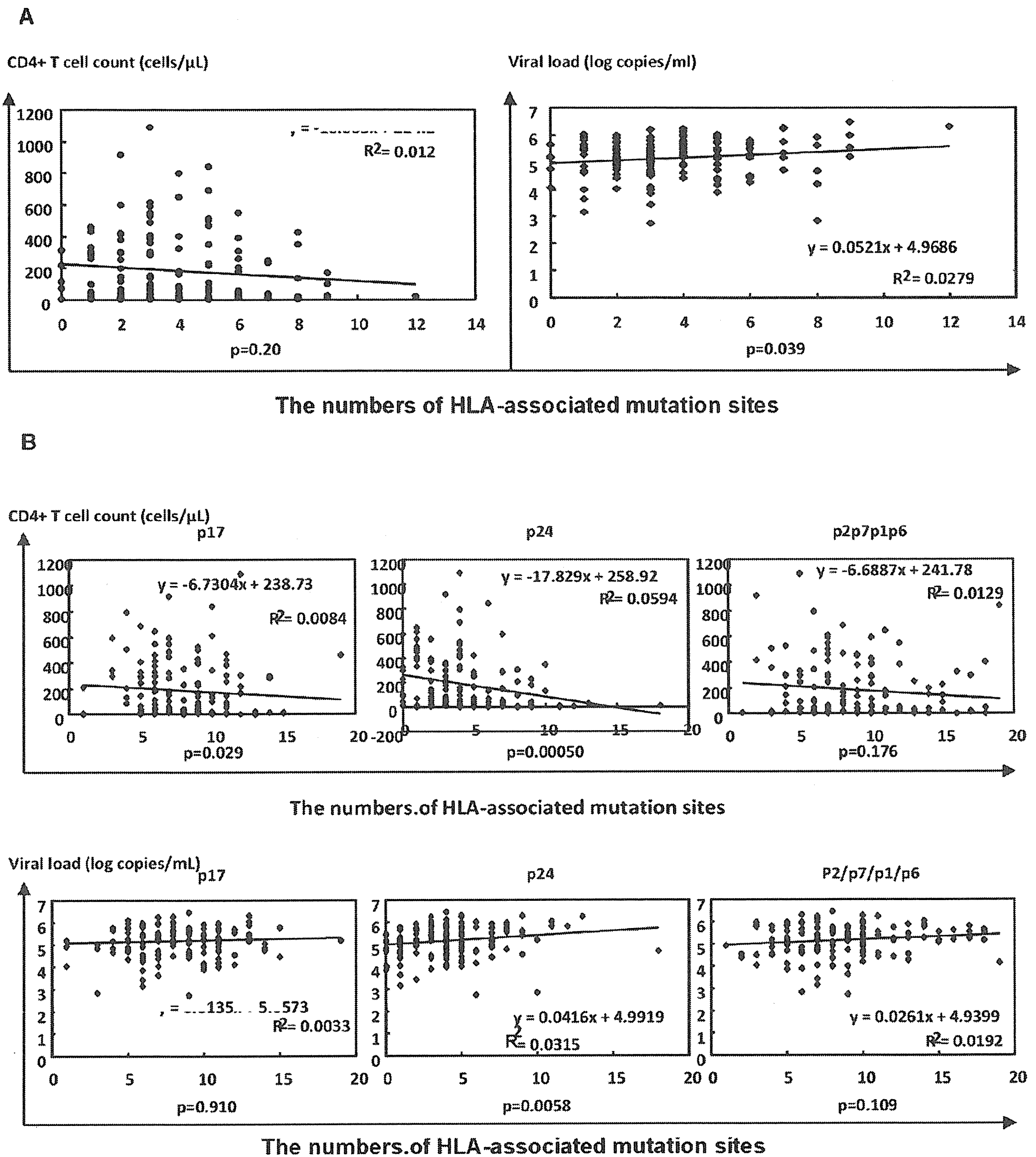

Figure 2. Associations between numbers of HLA-associated mutation sites and CD4 cell count or viral load. A regression line is drawn in each graph: (A) shows the relationships with HLA-associated amino acid variations in the whole Gag sequence, and (B) shows the relationships with amino acid variations in p17, p24, and p2/p7/p1/p6.
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reversion rate of these putative CTL escape mutations upon transmission to HLA-unmatched recipients varies considerably, suggesting that the rate of accumulation of CTL escape mutations in a given population differs substantially between mutations. Our data also suggest that the transmission of CTL-selected attenuated viruses is likely to confer a survival benefit on HLA-unmatched recipients, at least during the early days of the infection.

These associations between HLA and viral sequences can be explained in several ways. The majority of associations are probably attributable to specific mechanisms by which the virus escapes from HLA-restricted, HIV-specific CTLs, such as loss of

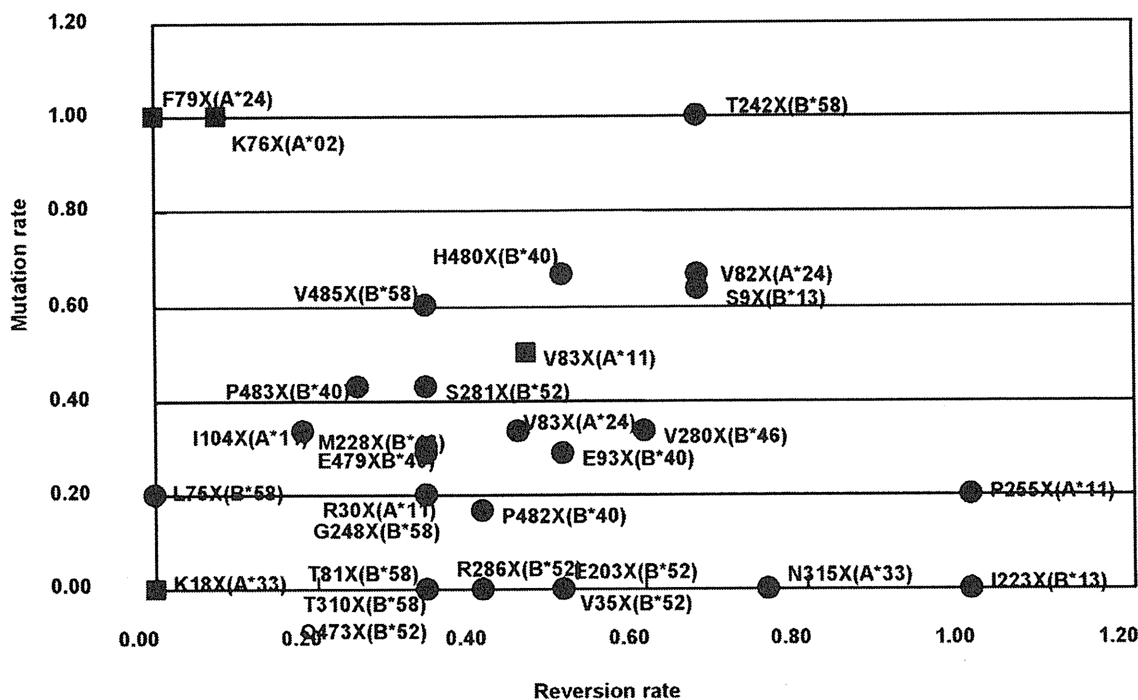

Figure 3. Rates of reversion and mutation for each HLA-associated amino acid variant. HLA-associated mutation and reversion rates after viral transmission to contact spouses with distinct HLA profiles were calculated and plotted. Amino acid variations selected by non-dominant amino acid (●) and dominant amino acid (■) are shown.
doi:10.1371/journal.pone.0011179.g003

peptide binding, loss of T-cell receptor recognition, and/or changes in peptide processing and presentation [1]. In fact, we found that two thirds of the HLA-associated variations were located within known CTL epitopes or their flanking regions. However, most previously reported CTL epitopes were identified in studies of other clades, indicating that these CTLs are probably cross-clade CTLs. Because studies of CTL epitopes in CRF01_AE infections are limited, we believe that several other mutations may occur in clade-specific CTL epitopes that have not yet been reported. Our current work on CTL epitope mapping using overlapping CRF01_AE Gag peptides has identified several new CTL epitopes, and at least two amino acid mutations have been found within the newly identified CRF01_AE Gag CTL epitopes (data are in preparation). Linkage disequilibrium can also explain the associations between HLA and viral sequences. There is a strong association between HLA_A*33 and _B*58 [22,23]. The A*33-associated T242X and G248X mutations are widely known to be selected by HLA_B*57/*5801 [26], so they are likely to be reflected in the linkage disequilibrium with B*58. Some HLA-associated mutations may also be part of the structural and functional compensatory mechanism underlying the development of primary CTL escape mutations, which can arise at sites considerably remote from the relevant epitopes [30].

This study has relatively low statistical power compared with previous studies. However, one of its strong aspects is that we identified a substantial number of HLA-associated amino acid variations that appear to be unique to a CRF01_AE-infected Thai population. Many of these variations do not appear in the list of HLA-associated mutations identified in subtype-B infections in over 1,500 subjects [15], and this cannot be explained by the relatively low statistical power of the present study. For instance, mutations such as S9X, V280X, and P453X had convincingly strong associations with HLA_B*13, _B*46, and _B*55, respectively ($p<0.001$; Table 1). However, none of these associations have been noted in subtype B, suggesting that there are a number of unidentified CTL pressures on Gag in the context of CRF01_AE infections in the Asian population. This indicates that an extensive search for CTL epitopes in various clades is warranted to facilitate the development of globally effective CTL vaccines.

Several publications have suggested that the Gag CTL response, as measured by interferon γ ELISPOT, has the most profound effect on the clinical outcome in subtype B and subtype C infections. Responses to the capsid protein are also likely to be most crucial in the containment of viral replication *in vivo* [31]. In this study, we have shown that variations in the capsid protein (p24) had the strongest association with viral load and CD4 cell count, indicating that regardless of the HIV clade, the p24 capsid is the most preferred target of HIV-1-specific CTLs. Therefore, p24 may be one of the important targets for effective CTL-based vaccines.

Reversion rates and mutation rates

When the virus is transmitted into a new host with a different HLA environment, the virus, which had already adapted to the previous host, must adapt again to the new HLA environment by reversion of the previous mutations and/or the creation of new mutations. One of the most interesting results of the present study is the variability in the reversion and mutation rates for each HLA-associated variant. We found that these rates varied considerably, depending upon the amino acid position, and that the reversion rate tended to correlate inversely with the mutation rate. It is plausible that an amino acid change with a low reversion rate tends to accumulate in the population, and that the rate of accumulation is higher if the mutation rate is higher and especially if its HLA allele is dominant. In fact, we found that all the

Table 2. Clinical status and amino acid variations around TW10 and compensatory mutations (H219, I23, and M228) for T242N in eight couples with B*57/B*58 index cases.

Sex	AIDS	CD4 (/µl)	VL (log copies/ mL)	HLA_A1	HLA_A2	HLA_B1	HLA_B2	H219	I223	M228	D235	I236	A237	G238	T239	T240	S241	T242	L243	Q244	E245	Q246	I247	G248	W249	M250	T251	N252	N253	P254
M	No	11	5.35	A*11	A*33	B*40	B*57	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	G	-	-
F	No	311	5.37	A*02	A*11	B*15	B*15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
M	No	13	5.21	A*24	A*33	B*52	B*58	-	-	-	-	-	-	-	S	-	-	N	-	-	-	-	-	-	-	-	-	S	-	-
F	No	196	5.41	A*11	A*34	B*15	B*56	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	S	-	-
M	Yes	3	5.92	A*02	A*33	B*40	B*58	Q	-	I	-	-	-	-	-	-	-	N	-	-	-	-	-	T	-	-	-	-	-	-
F	No	627	3.71	A*02	A*11	B*13	B*38	-	-	I	-	-	-	-	-	-	-	N	-	-	-	-	-	A	-	-	-	-	-	-
M	Yes	230	6.28	A*11	A*11	B*58	B*58	Q	-	I	-	-	-	-	-	-	-	N	-	-	-	-	-	A	-	-	-	-	-	-
F	No	284	5.63	A*11	A*11	B*40	B*51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
M	Yes	9	5.72	A*11	A*33	B*15	B*58	Q	-	I	-	-	-	-	-	-	-	N	-	-	-	-	-	A	-	-	-	-	-	-
F	No	195	5.76	A*11	A*24	B*40	B*51	Q	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	G	-	-
M	No	204	5.65	A*11	A*33	B*38	B*58	-	-	I	-	-	-	-	-	-	-	-	-	P	-	-	-	A	-	I	-	-	-	-
F	No	11	5.31	A*02	A*11	B*35	B*40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
M	No	688	4.35	A*24	A*33	B*15	B*58	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
F	No	576	3.91	A*11	A*24	B*15	B*52	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
M	Yes	19	6.32	A*11	A*33	B*56	B*58	Q	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
F	No	441	4.98	A*24	A*24	B*18	B*27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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variations selected by dominant amino acid were associated with common HLA alleles and rarely changed from the consensus sequence, even after they were transmitted to HLA-unmatched recipients. These findings suggest that the viruses circulating within the population had already adapted to the common HLA alleles, resulting in best-fit sequences that could escape from those alleles. The results of our study will increase our understanding of the influence of immune pressure on HIV and on the future direction of virus evolution. Conversely, it is also plausible that an amino acid change with a high reversion rate, presumably with a functional or structural constraint at that position, is unlikely to accumulate rapidly in the population. It will be important for future CTL vaccine development to consider whether an escape mutant will accumulate or not. Therefore, further studies of this kind are required to provide valuable insights for future vaccine design.

T242N issues

This is the first report indicating that the p24 T242N escape mutation from the B*57/*58 CTL has a significant impact on the HIV-1 viral load in CRF01_AE infections and that the mutations, H219 and M228, compensate for the crippling effect of T242N. Although this is a rather predictable result because the TW10 site is conserved throughout the HIV-1 clades, it increases our insight. Two other dominant CTL epitopes within the capsid protein are restricted by B*57 (IW9: ISPRTLNAW; KF11: KAFSPEVIPMF). However, the founder virus of CRF01_AE has a well-described 'peptide-processing mutation' at IW9 that affects the epitope presentation on HLA class I molecules, and a well-described CTL escape mutation within KF11 [33]. In fact, 100% of the CRF01_AE sequences in our cohort carried these amino acid substitutions (data not shown; see the Methods, for the GenBank accession number), indicating that these two CTL epitopes are less likely recognized in CRF01_AE infections. Several lines of evidence indicate that TW10 is the most important target determining the viral load set point: TW10 is the earliest target during primary infection [32]; and among all the described HLA-associated mutations, T242N is the earliest escape mutation that emerges during acute infections [13]. In light of these previously reported data and the nature of the CRF01_AE sequence, it would be interesting to determine whether TW10 is sufficient to protect B*57/*5801-positive subjects from disease progression. Unfortunately, we saw no clear protective effect of B*57/*5801 in the cohort reported here. However, as shown by the low median $CD4^{+}$ T-cell count, our cohort was substantially advanced in terms of disease progression. Therefore, the accumulated compensatory mutations might have masked the true protective effect of B*57/*5801. Supporting this explanation, we observed substantially lower plasma viral loads in the B*57/*5801-positive subjects with T242N but without the compensatory mutations ($p = 0.012$ by ANOVA; Figure 4).

We have also shown that the transmission of virus crippled by T242N might be associated with lower plasma viral loads in HLA-unmatched recipients, supporting a previous study of clade C infections [34]. However, the long-term effects of the transmission of virus attenuated by CTL escape mutations remain unknown and these contact cases should be examined longitudinally to determine whether virological escape accompanies the reversion of the T242N escape mutation.

Limitations

One of the limitations of this study is that we had no information regarding the timing of HIV transmission within the couples, although the rate of mutation is known to depend on the time from transmission [35]. However, perhaps because the duration of marriage in our discordant couples was quite long (median of seven years), we detected negligible amino acid changes between the first and second samplings. Therefore, we believe that most of the associations between amino acid mutations and HLA alleles observed in this study occurred in chronic infections. Another limitation is that this type of analysis depends heavily on statistical power. Therefore, it is difficult to identify HLA-associated variations if the allele frequencies and rates of mutation are low. Moreover, we did not use multiple testing corrections because of the small sample size, so we must admit there would have been a substantial number of false positive results. However, the high odds ratios of some of the novel HLA-associated mutations in the context of CRF01_AE infections in this Asian population strongly encourage us to obtain more information about CTL epitopes in different geographical regions where distinct HIV clades circulating, to develop globally effective CTL-based vaccines.

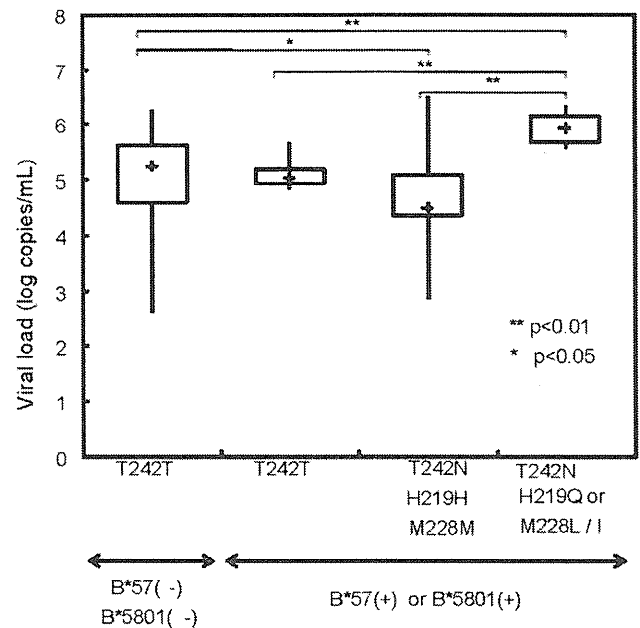

Figure 4. Lower plasma viral loads in B*57/*5801-positive subjects with T242N. Plasma viral loads of patients grouped by the presence or absence of B*57/*5801 with T242N with or without the compensatory H219 and M228 mutations.
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Our contact cases were not incident cases. Inevitably, there is also concern that the estimated direction of viral transmission was not true. However, because the HIV epidemic in Thailand started with commercial sex workers transmitting to their male clients and then from husbands to their wives [16], and partly because our study was conducted in a hospital close to a rural community, our interviews clearly indicated which spouse displayed risk behavior for HIV infection in most couples.

In conclusion, our data suggest two points: (a) HLA-associated amino acid mutations and CTL selection pressure on the p24 antigen appear to have the most significant impact on HIV replication in this CRF01_AE infection in an Asian population; and (b) the rates of accumulation of CTL escape mutations at the population level differ substantially between escape mutations, because the reversion rates varied considerably among the HLA-associated mutations.

Supporting Information

Figure S1 The inserted box magnifies the phylogenetic tree to show how the couples were identified.
Found at: doi:10.1371/journal.pone.0011179.s001 (0.48 MB TIF)

Table S1 The codon-based analysis demonstrated selection sites in the Gag protein.
Found at: doi:10.1371/journal.pone.0011179.s002 (0.28 MB XLS)

Table S2 Sequence mutation or reversion list.
Found at: doi:10.1371/journal.pone.0011179.s003 (0.04 MB XLS)

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Author Contributions

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

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