IκBL and diseases

We have demonstrated that IκBL might regulate the immune system via modulating alternative splicing of immune-related genes, which coincides with the notion that the disturbance of alternative splicing in immune-related genes would link with autoimmune diseases[17–19]. However, functional evidence for that the pathogenesis of immune-related diseases is attributable to the deregulation of alternative splicing is still lacking. Even though splice variants of *CD45*, *CD72* and *CTLA4* have been suggested to regulate the function of B and T cells[25–27], further studies illustrating the causal relationship between the alternative splicing and diseases are required. Furthermore, IκBL appears to control a large variety of alternative splicing, but the mechanisms controlling the gene-specificity are waiting to be identified, and a comprehensive analysis of target genes is particularly essential. For this purpose, next generation sequencing could be applied for exploring the RNAs regulated by IκBL in cells involved in the immune regulation, appended with the information of exact interacting sites or motifs. These results will not only propose the characteristics of IκBL-interacting RNAs, but also provide an overview to which extent IκBL is involved in the alternative splicing of immune-related genes.

In order to investigate the role of IκBL in the autoimmune and inflammatory diseases, *IKBL*-knockout (KO) mice will undoubtedly be required. On the other hand, it was reported that *IKBL*-transgenic (Tg) mice show resistance to collagen-induced arthritis, an experimental model for RA[28]. It is worth trying to apply *IKBL*-KO or -Tg mice into other models of immune-related diseases such as myelin-induced experimental autoimmune encephalomyelitis, a model of MS. Besides, examining the alternative splicing of target genes in *IKBL*-KO or -Tg mice will be valuable for establishing the link between the alternative splicing and immune-related diseases.

IKBL also regulates the alternative splicing of influenza A virus *M* gene[15]. Given that inhibition of the synthesis of M2 variant accounts for decreased virus titer[29], *IKBL* provides us with an insight into the host-dependent control of viral replication. It also suggests that IκBL, as well as splicing factors, would be useful to prevent viral infection by modulating alternative splicing of viral genes. Beside of influenza A virus *M* gene, genes of other virus are known to undergo alternative splicing in infected cells, such as *tat*, *rev* genes of human immunodeficiency virus (HIV)[30]. Whether IκBL affects expressivity of HIV genes and lead to an impact on virus replication will be an attractive issue for investigation.

Conclusion remark

Acknowledging to genetic association studies, *IKBL* was identified to be a candidate gene involved in the immune regulation. Albeit several issues remain to be clarified, recent studies have suggested that IκBL modulates the alternative splicing in both human and viral genes. These observations led to further understanding about the function of HLA region in the immune system and in the pathogenesis of immune-related diseases. In the future, as an excellent achievement of biomedical research, we expect IκBL as a potential target of therapeutic strategy in clinical treatments.

Acknowledgments

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IκBL mapped within the HLA region is a novel regulator of alternative splicing involved in the pathogenesis of immune-related diseases

Jianbo An[1)], Akinori Kimura[1)]

[1)]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

HLA 領域には免疫にかかわる多数の遺伝子が存在するが，抗原提示において重要な役割を果たす HLA クラス I およびクラス II 遺伝子群以外の遺伝子も免疫制御に関わると考えられる。なかでも，HLA クラス III 領域内にマップされる IKBL（NFKBIL1）遺伝子は，その多型自己免疫疾患や慢性炎症疾患などの疾患感受性と関連することが知られている遺伝子であるが，その機能は不明であった。最近我々は，IKBL がコードする IκBL タンパクがヒト免疫関連遺伝子やインフルエンザウイルス遺伝子の選択的スプライシングを制御することを明らかにしたが，この知見は HLA 領域による免疫と感染の制御する機構として新たな視点をもたらすものである。本総説では，IKBL 研究に関する最近の動向を紹介する。

キーワード：NFKBIL1，CLK1，選択的スプライシング，疾患感受性，自己免疫疾患，インフルエンザウイルス

HLA-B*35:05 is a protective allele with a unique structure among HIV-1 CRF01_AE-infected Thais, in whom the B*57 frequency is low

Masahiko Mori, Nuanjun Wichukchinda, Reiko Miyahara, Archawin Rojanawiwat, Panita Pathipvanich, Tomoyuki Maekawa, Toshiyuki Miura, Philip Goulder, Michio Yasunami, Koya Ariyoshi and Pathom Sawanpanyalert

Objective: To identify protective human leukocyte antigen (HLA) alleles in an HIV-infected south-east Asian population, in whom HLA-B*57 prevalence is lower than other ethnic groups, and HIV-1 CRF01_AE is the dominant circulating subtype.

Design: Cross-sectional study of Thai patients with chronic HIV infection.

Methods: Five hundred and fifty-seven HIV-1 CRF01_AE-infected Thais were recruited. Their HLA type and viral load were determined to statistically analyze the association of each allele in viral control. In-silico molecular dynamics was also used to evaluate the effect of HLA structure variants on epitope binding.

Results: HLA-B*35:05 was identified as the most protective allele ($P=0.003$, $q=0.17$), along with HLA-B*57:01 ($P=0.044$, $q=0.31$). Structurally, HLA-B*35:05 belonged to the HLA-B*35-PY group of HLA-B*35 alleles; however, unlike the other HLA-B*35 alleles that carry Arg (R) at residue 97, it has unique sequences at T94, L95, and S97, located within the peptide-binding groove. Analysis of the three-dimensional HLA structure and molecular dynamics indicates that S97 in HLA-B*35:05 leads to less flexibility in the groove, and shorter distances between the α-helixes compared with the disease-susceptible HLA-B*35-PY allele, HLA-B*35:01.

Conclusion: These data indicate the existence of a protective effect of HLA-B*57 across ethnic groups and highlight HLA-B*35:05 as an allele uniquely protective in subtype CRF01_AE-infected Thais. The divergence of HLA-B*35:05 from conventional HLA-B*35-PY structural sequences at the peptide-binding groove is consistent with previous studies that have identified HLA residue 97 as strongly influential in shaping HLA impact on immune control of HIV, and that a more restricted peptide-binding motif may be associated with improved control.

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Keywords: HIV-1 CRF01_AE, HLA-B*35, PY, residue 97, south-east Asia

Introduction

Cytotoxic T lymphocytes (CTLs) play a critical role in the control of viral replication in HIV or simian immunodeficiency virus (SIV) infection [1,2]. The effectiveness of anti-HIV CTL activity is strongly influenced by the highly polymorphic host class I human leukocyte antigen (HLA) genes expressed, as well by

Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Sakamoto, Nagasaki City, Nagasaki, Japan.
Correspondence to Koya Ariyoshi, MD, PhD, Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, 1-12-4, Sakamoto, Nagasaki City, Nagasaki 852-8523, Japan.
Tel: +81 95 8197842; fax: +81 95 8197843; e-mail: kari@nagasaki-u.ac.jp
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differences in viral sequence. The HLA region is the most polymorphic loci in the human genome and is found on chromosome 6 [3]; as of July 2013, 6966 of class I HLA molecules (2244 of HLA-A, 2934 of HLA-B, and 1788 of HLA-C alleles) have been registered in the website database [4]. The HIV strains circulating in different geographical regions are also highly diverse; 13 prototype clades and 43 circulating recombinant forms (CRFs) have been reported [5]. Under such HLA polymorphism and HIV viral diversity environment, so far 1539 of CTL epitopes including 250 best-defined epitopes and their restricting HLA allele information have accumulated in the Los Alamos database (CTL/CD8+ Epitope Summary. http://www.hiv.lanl.gov/). However, most of these have been derived from subtype B-infected whites in Europe and North America, and from subtype C-infected Africans in sub-Saharan Africa. Studies remain sparse from south-east Asia, where 1.5 million patients are living with HIV, of whom approximately 90% are infected with the subtype CRF01_AE (Geography Search Interface, Los Alamos database. http://www.hiv.lanl.gov/).

The protective effect of HLA-B*57 alleles on HIV viral control has been consistently reported in both African and white populations [6–9]. However, the prevalence of HLA-B*57 alleles differs among ethnic groups, being expressed in 7–9% of Africans, in 5–7% of whites, but in less than 3% of Asians [1,10].

HLA-B*27 has also been identified as a protective allele in white populations [9,11] and HLA-B*58:02 as a disease-susceptible allele in African populations [6,8]. HLA alleles have also been classified into HLA supertypes according to the common structure of anchor positions [12,13], and the influence of these supertypes on clinical outcome has been described. For instance, the advantage of supertype B58s and B62s and disadvantage of B07s and B44s for viral control among African and white cohorts have been reported [14–16]. However, the impact of HLA supertypes for HIV viral control among Asians has not yet been investigated.

Recently, a European ancestry genome-wide association study (GWAS) reported the significant association between sequence variants at HLA residue 97 and HIV viral control [17,18]: Arg (R) at residue 97 affected peptide binding together with the surrounding Ser (S) at residue 116, allowing flexibility of R97 and subsequent binding of various peptides [19]. It is proposed that such flexibility at the HLA binding groove may increase self-peptide binding at the thymic level and in turn reduces the T-cell receptor (TCR) repertoire subsequently available for virus-specific T-cell responses [20], leading to a higher chance of viral escape mutations with higher viral set points [20–22]. The evaluation of single HLA amino acid variants on structural change or flexibility can also be analyzed by molecular dynamics modeling using three-dimensional crystal structure data, as developed for analysis of drug resistance and peptide–ligand interactions [23–25].

We here analyze the impact of HLA class I molecules on immune control of HIV in a study cohort in Thailand using a combination of statistical and molecular dynamics modeling approaches. Our objective was to identify protective HLA alleles in HIV-infected Asians, in whom HLA-B*57 prevalence is lower than other ethnic groups and HIV-1 CRF01_AE is the dominant circulating subtype of virus.

Methods

Ethical statement

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

Participants

This study was conducted as a part of Lampang cohort study, which has been described in detail elsewhere [26,27]. Briefly, this is a hospital-based cohort study of 557 HIV-1 CRF01_AE-infected participants, comprising individuals attending a government referral hospital in northern Thailand. Recruitment was undertaken between July 2000 and October 2002. All study participants were antiretroviral treatment naive and tested for viral load at enrollment. Ethylenediaminetetraacetic acid (EDTA)-treated buffy coat samples were used in this study.

Class I human leukocyte antigen and typing

Genomic DNA was extracted from the buffy coat using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) and four digits class I HLA typing for A, B, and C allele was performed 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 against 557 participants. For gene polymorphism associations with viral load, a Kruskal–Wallis test was performed for alleles expressed in four or more persons, that is, at population frequency of 0.7% or greater. To determine whether individual alleles were associated with significantly high or low viral load, Mann–Whitney *U*-test with false discovery rate analysis ($q < 0.2$ as significance) was performed comparing viral load in participants with and without the allele as previously reported [28–31].

The same analyses, seeking associations with high or low viral load, were performed among HLA supertype comprising alleles. Linkage disequilibrium among HLA alleles was determined by Fisher's exact test using the Los Alamos database program (HLA Linkage Disequilibrium. http://www.hiv.lanl.gov/).

Human leukocyte antigen sequence, three-dimensional crystal structure, and in-silico molecular dynamics analysis among HLA-B*35 alleles

For the comparison of HLA sequence and structure among HLA-B*35 alleles, we obtained HLA sequence information from the IGMT/HLA database [4], and HLA crystal structure information from the RCSB Protein Data Bank (PDB; http://www.rcsb.org/). For this study, we compared the HLA sequence variants among HLA-B*35 alleles identified in this cohort (HLA-B*35:01, B*35:03, and B*35:05). The structural model of HLA-B*35:01 was constructed from coordinates derived from PDB with the accession code 2CIK. Using 2CIK as a template, structural model for HLA-B*35:05 was made by homology modeling method with commercial software Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Canada). The effect of residue 97 variants on the HLA structure was also analyzed by the in-silico molecular dynamics program NAMD [32], measuring the distance between α-helixes of residues 74 Tyr (Y) and 147 Trp (W), which are adjacent with residue 97. Distance was measured in each 0.5 ps (pico second), with repeats up to 1000 ps of movement and 2000 times in total. The distance differences between two alleles were analyzed by a *t*-test.

Sequence difference analysis at HLA-B*35:01-restricted Gag $_{253-262}$ NPPIPVGDIY (NY10) between HLA-B*35:01 and HLA-B*35:05

Recently, Gag $_{253-262}$ NPPIPVGDIY (NY10) was reported as a HLA-B*35:01-restricted epitope, with a D260E escape mutation causing HLA-epitope binding instability and consequent increase in viral load [33]. We analyzed sequence differences at this site and its flanking residue of Asn (N) 252 between HLA-B*35:01 positives and HLA-B*35:05 positives, among 216 CRF01_AE-infected Thais using our previously published data wherein the sequence data were obtained by direct sequencing of PCR products [27], and updated four digit HLA information.

Results

Characteristic of study population and human leukocyte antigen allele expression

Of 557 HIV-1 CRF01_AE-infected individuals recruited, 300 were women and 257 were men. The median age was 32 years (range 15–63 years), median baseline $CD4^+$ T-cell 86 cells/μl (range 0–1191/μl), and median baseline viral load 5.26 log copies/ml (range 2.60–6.72 log copies/ml). With respect to HLA type, 111 alleles (27 HLA-A, 58 HLA-B, and 26 HLA-C alleles) were detected (Supplemental Table 1, http://links.lww.com/QAD/A479). The most prevalent HLA-A allele was HLA-A*11:01 (58.7% in population frequency), followed by HLA-A*24:02 (25.0%) and HLA-A*02:03 (24.4%). Of the HLA-B alleles, HLA-B*46:01 was the most prevalent (26.6%), followed by HLA-B*40:01 (20.1%) and HLA-B*13:01 (17.8%). HLA-B*57:01 was detected in only 1.4% of the study cohort. Of the HLA-C alleles, HLA-C*01:02 was the most prevalent (31.6%), followed by HLA-C*07:02 (30.3%) and HLA-C*08:01 (18.3%).

Unique protective human leukocyte antigen allele associated with HIV viral load in an Asian population

Figure 1 shows the association of class I HLA alleles with viral load. This analysis included the 71 alleles that were expressed by more than three individuals and showed that viral load varied significantly among them ($P=0.034$, Kruskal–Wallis test; Fig. 1a). Viral load varied more as a result of differences between HLA-B alleles ($P=0.068$), than between HLA-A alleles ($P=0.077$) or HLA-C alleles ($P=0.29$; Fig. 1b). Three individual alleles were significantly associated with low viral load, namely, HLA-B*35:05 ($P=0.003$), HLA-B*57:01 ($P=0.044$), and HLA-A*24:07 ($P=0.025$). Four individual alleles were significantly associated with high viral load, namely, HLA-B*15:04 ($P=0.038$), HLA-B*56:01 ($P=0.011$), HLA-A*11:02 ($P=0.015$), and HLA-C*07:02 ($P=0.018$). In each case, these associations were statistically significant ($P<0.05$, Mann–Whitney *U*-test) without correction for multiple tests. Only in the case of HLA-B*35:05 was this association significant after correcting for multiple tests, using $q<0.2$ ($q=0.17$, Supplemental Table 1, http://links.lww.com/QAD/A479). Significant viral control by HLA-B*35:05 was also identified among 209 participants with $CD4^+$ T-cell counts more than 200 cells/μl (median 3.9 log copies/ml of viral load, $P=0.010$ and $q=0.16$) (Supplemental Table 2, http://links.lww.com/QAD/A480).

We next investigated whether linkage disequilibrium might explain some of these associations (Supplemental Figure 1, http://links.lww.com/QAD/A481). Among these alleles, we observed linkage disequilibrium between HLA-B*57:01 and HLA-C*06:02, and among HLA-A*24:07, HLA-B*35:05, and HLA-C*04:01. All HLA-B*57:01-positive persons also carried HLA-C*06:02 and no significant difference in viral load was observed in HLA-C*06:02 persons not expressing HLA-B*57:01 ($P=0.58$) (Supplemental Figure 1A, http://links.lww.com/QAD/A481). In the case of the HLA-A*24:07-B*35:05-C*04:01, a significantly lower viral load was

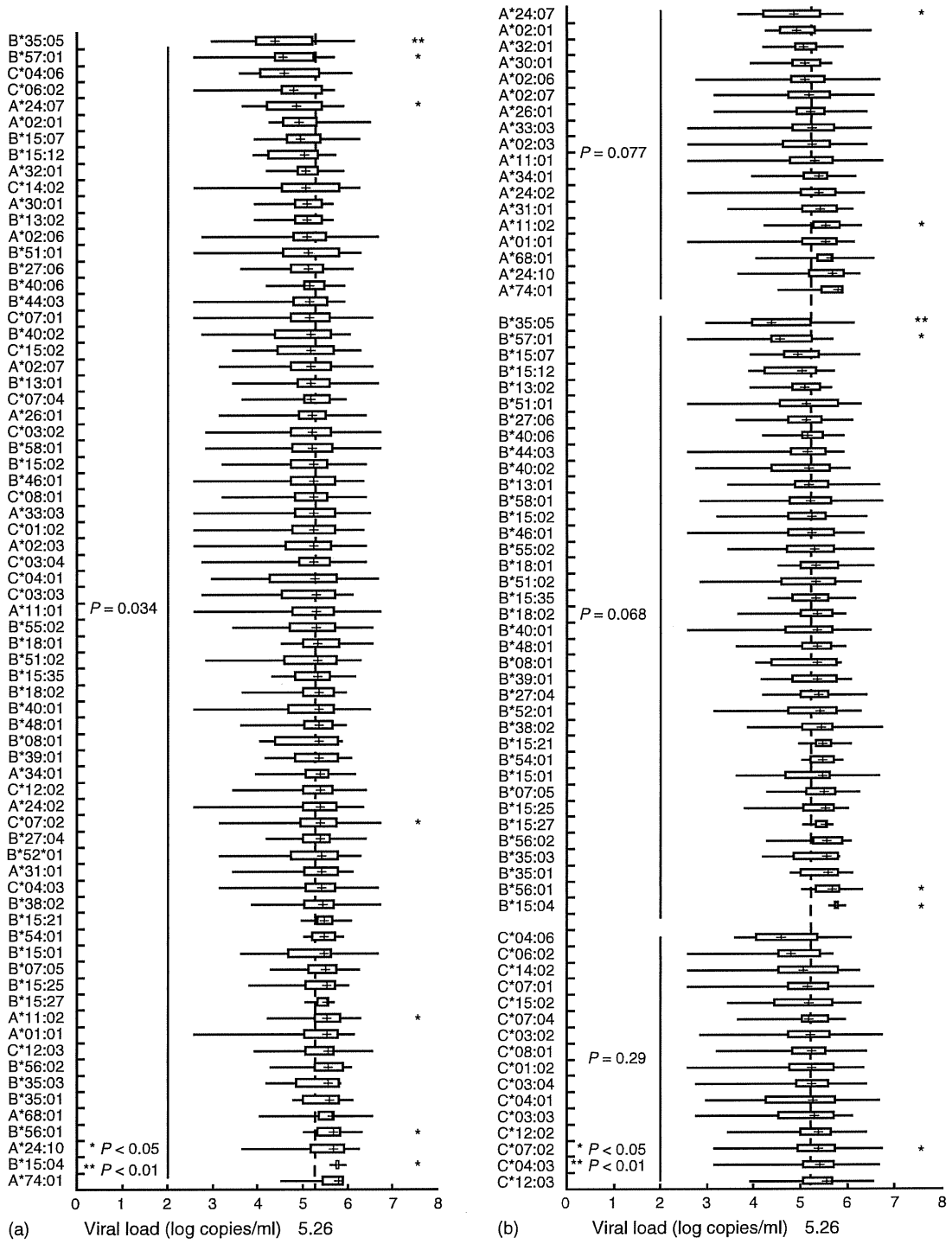


Fig. 1. Class I human leukocyte antigen allele expression and viral load association. (a) The associations between the 71 class human leukocyte antigen (HLA) alleles expressed by more than three individuals and viral load were analyzed using the Kruskal–Wallis test. A Mann–Whitney *U*-test was performed to compare viral load in individuals with and without the alleles; * and ** show a significant difference of $P < 0.05$ (*) and $P < 0.01$ (**). (b) Difference in viral load was analyzed for A, B, and C alleles.

found in HLA-B*35:05-positive/HLA-A*24:07-negative individuals, whereas no such effect was found in HLA-A*24:07-positive/HLA-B*35:05-negative individuals (Supplemental Figure 1B, http://links.lww.com/QAD/A481). There was no linkage disequilibrium association to explain the association with disease susceptibility of HLA-A*11:02, HLA-B*15:04, HLA-B*56:01, or HLA-C*07:02.

Significant viral load difference among B07s comprising human leukocyte antigen alleles

We next analyzed viral load differences among HLA supertypes, and here we did not find any significant viral load difference among A supertypes ($P=0.17$ by Kruskal–Wallis test) nor B supertypes ($P=0.61$). This result was not unexpected, given the coexistence of HLA alleles associated with low viral load and high viral load within the same supertype. For example, B07s comprised eight alleles, including the protective HLA-B*35:05 and the susceptible HLA-B*56:01 ($P=0.027$ by Kruskal–Wallis test, Fig. 2). However, there were no significant viral load differences among comprising alleles in other supertypes. Viral load discrepancy among supertype comprising HLA alleles was previously reported in B58s among subtype C-infected African, including protective HLA-B*57:03 and HLA-B*58:01, then susceptible HLA-B*58:02, suggesting the difficulty of CTL-induced vaccine development based on HLA's structural similarity, that is, supertope [34].

Impact of human leukocyte antigen sequence variation among HLA-B*35 alleles

HLA-B*35:05 has Asp (D) at residue 114 and Ser (S) at 116, as does the disease-susceptible HLA-B*35:01 (Fig. 3a). However, distinct from other HLA-B*35 alleles with Ile (I) at residues 94 and 95, and Arg (R) at residue 97, HLA-B*35:05 has Thr (T) at residue 94, Leu (L) at 95, and Ser (S) at 97, located at the peptide-binding groove (Fig. 3b, 3c, and 3d).

We compared the effect of sequence variation at residue 97 on the structure of the peptide-binding site of HLA-B*35:01 and HLA-B*35:05. Supplemental Figure 2, http://links.lww.com/QAD/A482 shows the difference in the flexibility at residue 97 between the two alleles by a space-filling method. Arg (R) 97 in HLA-B*35:01 protrudes further into the peptide-binding groove than

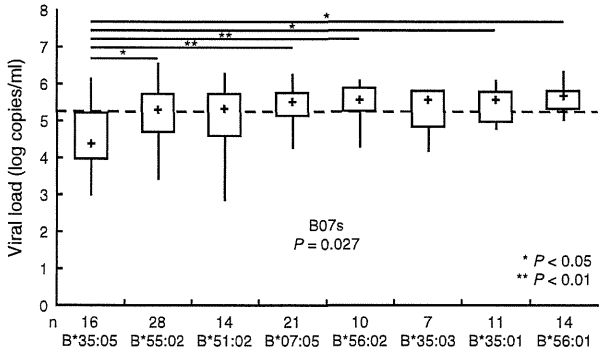


Fig. 2. Viral load difference among human leukocyte antigen supertype comprising alleles. The viral load difference among supertype comprising alleles was analyzed in B07s using the Kruskal–Wallis test; * and ** show a significant difference of $P<0.05$ (*) and $P<0.01$ (**) by Mann–Whitney U-test between individuals positive for the alleles.

Ser (S) 97 in HLA-B*35:05, with its short side chain. Analysis of the interaction of residue 97 with its neighboring residues showed that Arg (R) 97 in HLA-B*35:01 forms a salt bridge with Asp (D) 114 (Fig. 4a), whereas Ser (S) 97 in HLA-B*35:05 forms a predominant hydrogen bond of <2.5Å (2.3Å) with the α-helix-comprising residue of Tyr (Y) 74 (Fig. 4b). In the distance calculation between α-helixes Tyr (Y) 74 and Trp (W) 147, which lie adjacent to residue 97 (Fig. 4c), a significantly shorter distance was identified in HLA-B*35:05 compared with HLA-B*35:01 (7.1 ± 0.6 in HLA-B*35:05 vs. 7.8 ± 0.8 of mean ± SD Å in HLA-B*35:01, respectively, $P<0.0001$ by t-test; Fig. 4d). Because of these structural differences conferred by residue 97 variants, these two alleles will have a difference in peptide-binding capacity at the F pocket side, with a greater chance of peptide binding in HLA-B*35:01, and lesser one in HLA-B*35:05.

Significant D260E escape mutations at HLA-B*35:01-restricted Gag $_{253-262}$ NPPIPVGDIY (NY10) in HLA-B*35:01, but not in HLA-B*35:05

Lastly, to examine differences in viral adaptation between HLA-B*35:01 and HLA-B*35:05, we analyzed the sequence difference at the Gag $_{253-262}$ NPPIPVGDIY (NY10) epitope, which was recently identified as an HLA-B*35:01-restricted epitope with a D260E escape mutation causing epitope-HLA binding instability [33]. In 216 individuals of CRF01_AE-infected Thais, significant D260E mutation was identified in HLA-B*35:01 positives [3/5 in HLA-B*35:01+ vs. 3/211 in HLA-B*35:01−, $P=0.0001$ by Fisher's exact test, odds ratio (OR) 104, 95% confidence interval (CI) 12–868], but not in HLA-B*35:05 positives (0/4 in HLA-B*35:05+ vs. 6/212 in HLA-B*35:05−, $P=1$; Table 1). In other sites, significantly higher mutations at I261X (2/5 vs. 2/209, $P=0.0025$, OR 70, 95% CI 7.2–672) and the flanking N252X (5/5 vs. 94/209, $P=0.019$) were also identified in HLA-B*35:01, but not in HLA-B*35:05 (0/4 vs. 6/212, $P=1$ in I261X, and 2/4 vs. 97/212, $P=1$, OR 1.2, 95% CI 0.2–8.6 in N252X).

Discussion

This is the first study to investigate the effect of HLA alleles on clinical outcome in a systematic way in an HIV-1 CRF01_AE-infected Asian cohort. In this study, we identified unique protective HLA alleles associated with viral control. As reported from other ethnic groups, and in HIV clade B and C infection [6–9], HLA-B*57 was also one of the most protective alleles in this Asian cohort. In addition, we identified a novel protective allele in HLA-B*35:05. This HLA-B*35:05 is identified among south-east Asian (2–5% in population frequency), but rare in African and whites. HLA-B*35 alleles have been classified into two groups, namely

(a)

HLA	Residue				
	94	95	97	114	116
B*35:01 (PY)	I	I	R	D	S
B*35:03 (Px)	-	-	-	N	F
B*35:05 (PY)	T	L	S	-	-

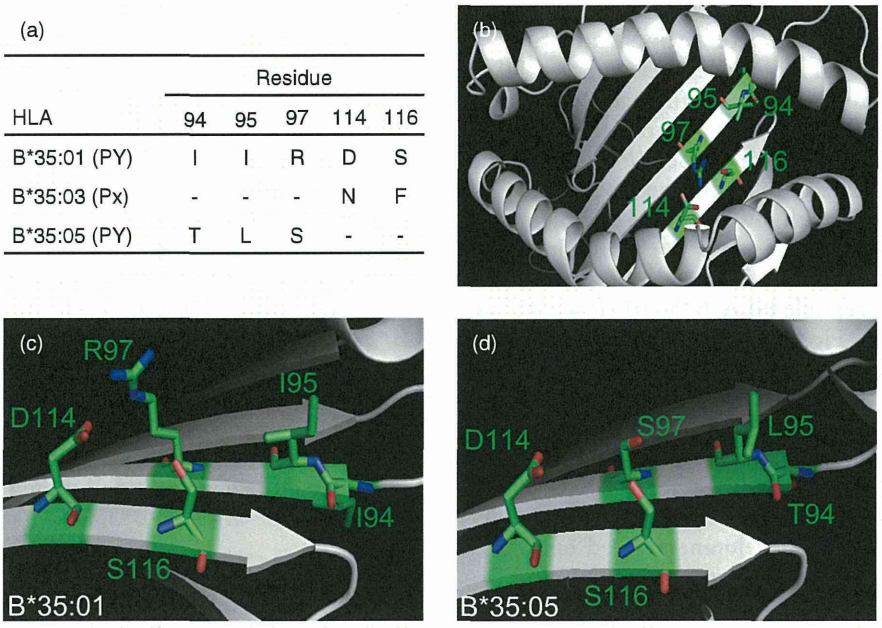
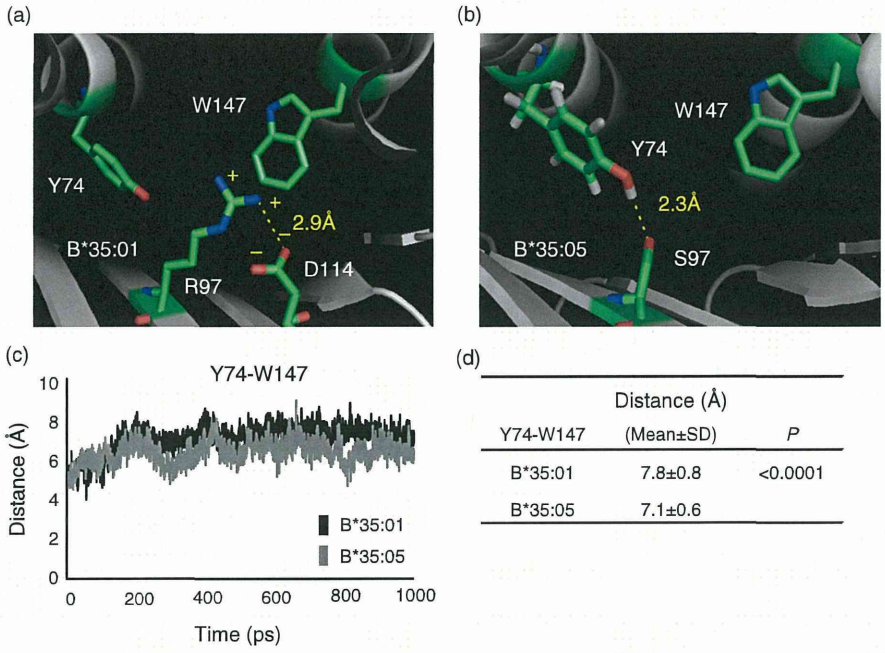


Fig. 3. Human leukocyte antigen sequence differences at residues 94, 95, 97, 114, and 116 among HLA-B*35 alleles, and three-dimensional crystal structure difference between HLA-B*35:01 and HLA-B*35:05. A hyphen (-) indicates the concordant sequence with HLA-B*35:01 at each residue (a). HLA sequence difference sites listed in (a) are shown. View from above (b), side of HLA-B*35:01 (c), and HLA-B*35:05 (d) are shown.

HLA-B*35-PY with a binding motif of Pro (P) at B pocket of HLA and Tyr (Y) at F pocket, including HLA-B*35:01 and HLA-B*35:08, and HLA-B*35-Px, with a binding motif of Pro (P) at B pocket, but not Tyr (Y) at F pocket, including HLA-B*35:02, HLA-B*35:03, HLA-B*35:04, and HLA-B*53:01 [35]. The disadvantage of Px for viral control was originally reported from clinical outcome studies among Africans and whites [35]. The mechanism underlying the difference in HIV disease outcome resulting from the two HLA-B*35 groups has



(d)

Y74-W147	Distance (Å) (Mean±SD)	P
B*35:01	7.8±0.8	<0.0001
B*35:05	7.1±0.6	

Fig. 4. Sequence interactions with residue 97, and distance analysis between α-helix structures by in-silico program NAMD. Salt bridge between R97 and D114 in HLA-B*35:01 (a), and hydrogen bond between Y74 and S97 in HLA-B*35:05 with hydrogen figure in Y74 (b) are shown. Distance between α-helix structures of Y74 and W147 was analyzed by in-silico molecular dynamics program of NAMD (c), with significant shorter distance in HLA-B*35:05 (d).

Table 1. Sequence difference at the epitope Gag p24 $_{253-262}$ NPPIPVGDIY (NY10) between HLA-B*35:01 positives and B*35:05 positives.

ID	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	N	N	P	P	I	P	V	G	D	I	Y
P206	A*02:03	A*11:02	B*18:02	B*35:01	C*04:01	C*07:04	D	–	–	–	V	–	–	–	E	–	–
P381	A*11:01	A*33:03	B*15:02	B*35:01	C*04:01	C*08:01	S	–	–	–	–	–	–	–	–	L	–
P453	A*24:02	A*24:02	B*15:25	B*35:01	C*07:02	C*12:03	S	–	–	–	–	–	–	–	–	–	–
P1349	A*02:03	A*11:01	B*35:01	B*38:02	C*03:03	C*07:02	S	–	–	–	–	–	–	–	E	F	–
P2097	A*24:02	A*31:01	B*35:01	B*46:01	C*01:02	C*04:01	S	–	–	–	–	–	–	–	E	–	–
ID	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C	N	N	P	P	I	P	V	G	D	I	Y
P115	A*02:06	A*11:01	B*35:05	B*46:01	C*01:02	C*04:01	–	–	–	–	–	–	–	–	–	–	–
P1013	A*02:03	A*11:01	B*35:05	B*52:01	C*04:01	C*07:01	S	–	–	–	–	–	–	–	–	–	–
P1497	A*02:01	A*11:01	B*13:01	B*35:05	C*04:01	C*04:06	S	–	–	–	–	–	–	–	–	–	–
P1651	A*02:06	A*11:01	B*35:05	B*40:06	C*04:01	C*08:01	–	–	–	–	–	–	–	–	–	–	–

Sequence difference at the epitope Gag p24 $_{253-262}$ NPPIPVGDIY (NY10) with its flanking N252 between HLA-B*35:01 positives and B*35:05 positives among 216 patients are shown.

not been determined, but may relate to the presence of Gag-specific CTLs capable of reducing viral load that have been detected in individuals expressing HLA-B*35:01 [35,36]. In addition, greater binding affinity to immunoglobulin-like transcript 4 (ILT4) has been described in individuals expressing HLA-B*35:03 compared to individuals expressing HLA-B*35:01, which causes dendritic cell dysfunction [37]. Furthermore, the existence of CTLs, which were not reactive against the original sequence epitope but only against variant sequence epitopes (heteroclitic reaction) have been described in individuals expressing HLA-B*35-Px alleles [38].

HLA-B*35:05, identified as one of the most protective alleles in this cohort, structurally belongs to the PY group [39], in common with HLA-B*35:01 carrying Asp (D) at HLA amino acid residue 114 and Ser (S) at residue 116 (D114-S116), compared to Asn (N) at 114 and Tyr (Y) or Phe (F) at 116 in HLA-B*35:03 (non-D114-S116). However, HLA-B*35:01 was associated with a significantly higher viral load than HLA-B*35:05 (Fig. 2, median 5.58 log copies/ml in HLA-B*35:01-positives, and 4.38 log copies/ml in HLA-B*35:05-positives, respectively, $P = 0.010$, Mann–Whitney *U*-test). Susceptibility of HLA-B*35:01 to high viral loads is consistent with two recent reports from studies of subtype B-infected whites and African–Americans, respectively [7,17]. In addition, HLA-B*35:01 is strongly associated with high viral load in clade B-infected cohorts in Japan and in Mexico [33]. These studies suggest that there may be distinct mechanisms underlying the association of low viral load in this Thai cohort with HLA-B*35:05. Of note, distinct from other HLA-B*35 alleles including those in the PY group, which share Ile (I) as HLA residues 94 and 95, and Arg (R) at residue 97, HLA-B*35:05 has Thr (T) at 94, Leu (L) at 95, and Ser (S) at 97 [4] (Fig. 3). Among these three residues, an association between sequence variants at residue 97 and HIV viral control has been previously reported and summarized in a European ancestry GWAS [17,18]. In the present study, we showed that the Arg (R) at residue 97 in HLA-B*35:01 protrudes into the peptide-binding groove, and conferred longer distances between α-helixes compared to Ser (S) at residue 97 in HLA-B*35:05. We propose that these results suggest Arg (R) has a dual role of an adhesive for epitope binding and a prop for a wider α-helix structure. Related with structure difference in peptide-binding groove of HLA and epitope variants, the difference between HLA-B*44:02 and HLA-B*44:03, with single amino acid difference of Asp (D) at residue 156 in HLA-B*44:02, and Leu (L) at residue 156 in HLA-B*44:03, was previously reported [40]. Although HLA-B*44:02 and HLA-B*44:03 shared more than 95% of their peptide repertoire in the examination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDITOF MS), HLA-B*44:03 with wider groove also presented more unique peptides compared to HLA-B*44:02. Indeed, HLA-B*35:01 has binding motif of Tyr (Y), Phe (F), Met (M), Leu (L), Ile (I), or no specificity at F pocket, whereas HLA-B*35:05 has binding motif of Phe (F) only, according to the Los Alamos database (HIV HLA Anchor Residue Motifs. http://www.hiv.lanl.gov/). These data are consistent with the hypothesis proposed by Kosmrlj *et al.* [20] that HLA molecules with a more restricted peptide-binding repertoire, such as HLA-B*27 and HLA-B*57, and in this case, HLA-B*35:05, are more likely to be protective. In support of our hypothesis, we observed selection of D260E escape mutations within the Gag $_{253-262}$ NPPIPVGDIY (NY10) HLA-B*35:01-positive individuals and not in HLA-B*35:05-positive individuals.

In contrast to the consistent association between HLA-B*57 alleles and low viral load in this Thai cohort, as well as in clade B-infected and C-infected cohorts previously reported [6,8,9], the closely related allele HLA-B*58:01 was not protective in this Thai cohort. This contrasted with clade C-infected Native Africans and B-infected whites [6,8,9], but was consistent with subtype B-infected African–Americans [7]. HLA-B*58:01 was the fourth most prevalent HLA-B allele in this cohort, 17.6% by population frequency, higher than in Africans (6%) and whites (less than 2%) [7]. It is plausible, therefore, that the dominant virus in this population has already adapted to the immune pressure imposed on it by this allele [41]. Indeed, in our previous report, 15 of 20 (75%) of HLA-B*58:01-positive patients