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H. 知的所有権の出願・取得状況（予定含む）
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Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

研究分担者 滝口雅文

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IV. 研究成果の刊行物・別刷

Adaptation of HIV-1 to human leukocyte antigen class I

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The rapid and extensive spread of the human immunodeficiency virus (HIV) epidemic provides a rare opportunity to witness host-pathogen co-evolution involving humans. A focal point is the interaction between genes encoding human leukocyte antigen (HLA) and those encoding HIV proteins. HLA molecules present fragments (epitopes) of HIV proteins on the surface of infected cells to enable immune recognition and killing by CD8⁺ T cells; particular HLA molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, are more likely to mediate successful control of HIV infection¹. Mutation within these epitopes can allow viral escape from CD8⁺ T-cell recognition. Here we analysed viral sequences and HLA alleles from >2,800 subjects, drawn from 9 distinct study cohorts spanning 5 continents. Initial analysis of the HLA-B*51-restricted epitope, TAFTIPSI (reverse transcriptase residues 128–135), showed a strong correlation between the frequency of the escape mutation I135X and HLA-B*51 prevalence in the 9 study cohorts ($P=0.0001$). Extending these analyses to incorporate other well-defined CD8⁺ T-cell epitopes, including those restricted by HLA-B*57 and HLA-B*27, showed that the frequency of these epitope variants ($n=14$) was consistently correlated with the prevalence of the restricting HLA allele in the different cohorts (together, $P<0.0001$), demonstrating strong evidence of HIV adaptation to HLA at a population level. This process of viral adaptation may dismantle the well-established HLA associations with control of HIV infection that are linked to the availability of key epitopes, and highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.

The extent to which HIV is evolving at the population level in response to immune selection pressure is under debate^{2–6}. Resolving the impact of HLA class I alleles on viral evolution is problematic because it can be obscured by other influences, such as founder effect⁶ (polymorphisms present within the early strains establishing the epidemic in a group). In addition, most HLA alleles do not drive significant selection pressure on HIV, a proportion of escape mutations revert to wild type after transmission, and different HLA alleles may drive the identical escape mutation⁷.

To test the hypothesis that the frequency of escape mutations in a given population is correlated with the prevalence of the relevant HLA allele in that population, we studied nine distinct cohorts from North America, the Caribbean, Europe, sub-Saharan Africa, Australia and Japan, in which we performed HLA typing, and defined the viral mutations arising within CD8⁺ T-cell epitopes. We focused initially on a well-characterized mutation, I135X, within the HLA-B*51-restricted epitope, TAFTIPSI (RT 128–135)⁸, because it arises in acute infection, non-HLA-B*51 alleles do not also select this mutation⁹, and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects⁹. Thus, if highly prevalent HLA alleles drive a high frequency of escape mutations in the population, this would be most obvious in relation to HLA-B*51 and the escape mutant I135X. We then considered an additional 13 well-defined escape mutations, including those known to reduce viral fitness and therefore liable to revert after transmission.

I135X was selected in 205 of 213 (96%) HLA-B*51-positive individuals analysed (Figs 1 and 2, and Supplementary Fig. 1). The I135X variants do not significantly affect viral replicative capacity *in vitro*, other than the rare I135V mutation. This was the only variant observed to revert to wild-type *in vivo* during a 3-year follow-up of 38 HLA-B*51-negative subjects identified during acute HIV infection who carried I135X mutant viruses at transmission (Fig. 1e). The I135X mutants substantially affect HLA binding, and therefore also recognition by CD8⁺ T cells (Fig. 1f–h). Thus, HIV transmission from HLA-B*51-positive subjects would probably involve transmission of I135X, which would persist in the new host. Newly infected HLA-B*51-positive subjects receiving an I135X mutant would be unable to generate an HLA-B*51-TAFTIPSI-specific response.

To test the hypothesis that the population frequency of I135X is correlated with HLA-B*51 prevalence, HIV sequence and HLA data were collated from the nine study cohorts. One cohort comprised subjects with acute/early HIV infection; the remaining cohorts comprised chronically infected subjects. In all cohorts the odds ratio strongly favoured I135X in the HLA-B*51-positive subjects, even in the acute cohort where I135X was selected sufficiently early to be already over-represented in HLA-B*51-positive subjects (odds ratio 1.65, $P=0.07$, Fig. 2a). In Japan, where HLA-B*51 is highly

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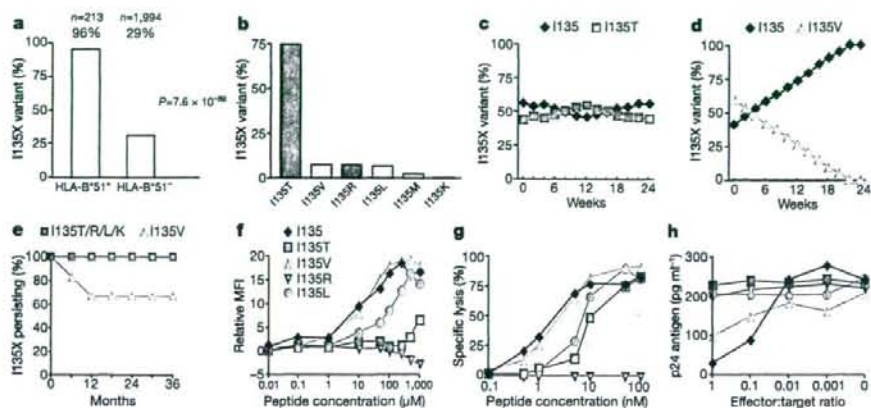


Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51-TAFTIPSI (RT 128–135)-specific CD8⁺ T cells. **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition assays between NL4-3 wild-type virus and I135X viral variants (I135T (**c**) and I135V (**d**)). I135R and I135L showed no fitness cost (not shown).

prevalent¹⁰ (21.9% of the study cohort), the frequency of I135X was >50%, and overall across all cohorts the I135X frequency was strongly correlated with HLA-B*51 prevalence ($P = 0.0001$, Fig. 2b). To control for the possibility that disproportionately more virus sequences from HLA-B*51-positive subjects were analysed, the same analysis comparing I135X frequency in HLA-B*51-negative subjects only was undertaken, with similar findings (Fig. 2c, $P = 0.0006$). These data suggest that HIV may be adapting to HLA-B*51 with respect to the HLA-B*51-TAFTIPSI response in localities where HLA-B*51 is at high prevalence.

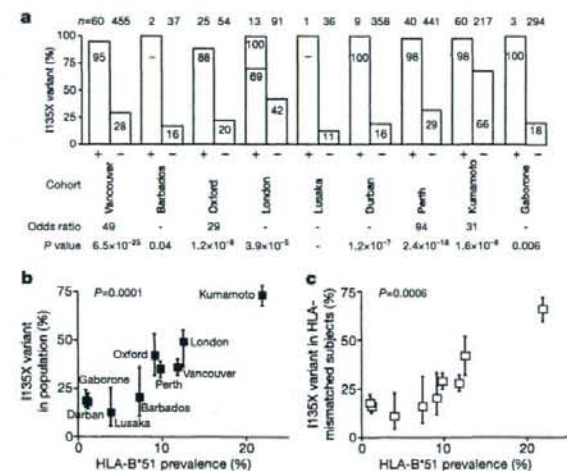


Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts. **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (-) subjects within nine study cohorts. In the acute cohort (London) 69% of HLA-B*51-positive subjects expressed I135X mutant at enrolment, 100% within 2 years of baseline (Supplementary Fig. 1). **b**, Correlation between frequency of I135X mutation and HLA-B*51 prevalence in the nine study populations. Logistic regression $P = 0.0001$ (Supplementary Table 1). **c**, Correlation between I135X frequency in HLA-B*51-negative subjects and HLA-B*51 prevalence in nine study populations. Error bars represent 95% confidence limits, obtained using a binomial error distribution.

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e, Persistence of I135X mutants in 38 HLA-B*51-negative subjects followed from acute infection. **f**, TAFTIPSI variant binding to HLA-B*51 (see Methods). MFI, mean fluorescence intensity. **g**, **h**, Recognition of peptide-depleted HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8⁺ T-cell clones.

Additional evidence that I135X is accumulating in Japan comes from the observation that only 3 of 14 (21%) HLA-B*51-negative Japanese haemophiliacs infected in 1983 carried I135X, compared with 30 of 43 (70%) HLA-B*51-negative subjects infected between 1997 and 2008 ($P = 0.002$). Furthermore, HLA-B*51 does not protect against disease progression in Japanese subjects infected between 1997 and 2008, whereas HLA-B*51-positive haemophiliacs infected in 1983 had lower viraemia levels and higher CD4 counts than HLA-B*51-negative haemophiliacs (Supplementary Fig. 2). These data are consistent with fewer HLA-B*51-positive subjects targeting TAFTIPSI during 1997–2008, owing to a population-level increase in the HLA-B*51 I135X escape mutation over this 14–25-year period.

To investigate HIV adaptation to other HLA alleles, we initially examined other escape mutations shown previously to persist stably after transmission^{5,7}. We selected the three non-reverting Gag polymorphisms that, from analysis of 673 study subjects in Durban, South Africa⁷, were most strongly associated with the relevant restricting allele ($P < 10^{-6}$ after phylogenetic correction), namely, S357X, D260X and D312X within epitopes restricted, respectively, by HLA-B*07 (GPSHKARVL, Gag 355–363), HLA-B*35 (PPIPVGDIY, Gag 254–262) and HLA-B*44 (AEQATQDVKNW, Gag, 306–316). In addition, we analysed a non-reverting I31V variant (LPPIVAKEL, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence⁵. These additional polymorphisms show a similar relationship to that between I135X and HLA-B*51, overall showing a strongly significant correlation between variant frequency and prevalence of the restricting HLA allele (Figs 3 and 4a, and Supplementary Fig. 3).

The spectrum of HLA-associated polymorphisms also includes mutations reducing viral fitness¹. These either revert to wild type after transmission, or persist in the presence of compensatory mutations. We extended these analyses to include epitopes restricted by HLA-B*27 and HLA-B*57, alleles strongly associated with successful immune control of HIV^{11,12}. The mutations analysed themselves are associated with precipitating loss of immune control^{13–16} and all inflict a documented viral fitness cost, either demonstrated by *in vitro* fitness studies and/or *in vivo* reversion^{7,14,17–21} (data not shown for V168I).

Again, a strong correlation between escape mutant frequency and prevalence of the restricting HLA allele was observed (Figs 3c–f and 4b, and Supplementary Fig. 3; overall, for these nine variants affecting viral fitness, $r = 0.69$, $P < 0.0001$). Unexpectedly, this correlation

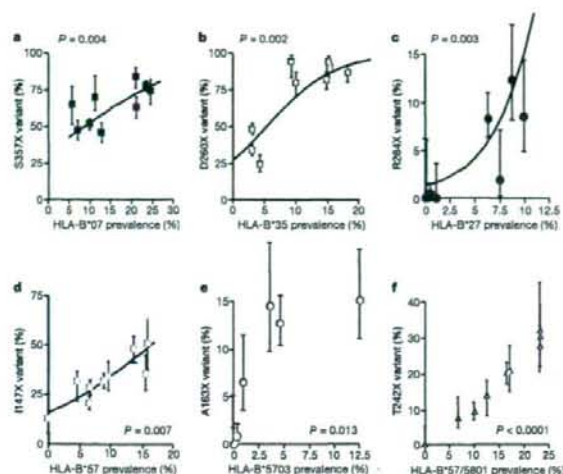


Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b**, Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDII (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIIIGLNK (Gag 263–272). **d**, Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e**, Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f**, Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting^{18,20} mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan¹⁰, 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific CD8⁺ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted CD8⁺ T-cell responses. Non-reverting variants^{5,7}, as well as those previously shown to arise at a fitness cost^{7,14,16–21}, were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms^{7,20}. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those

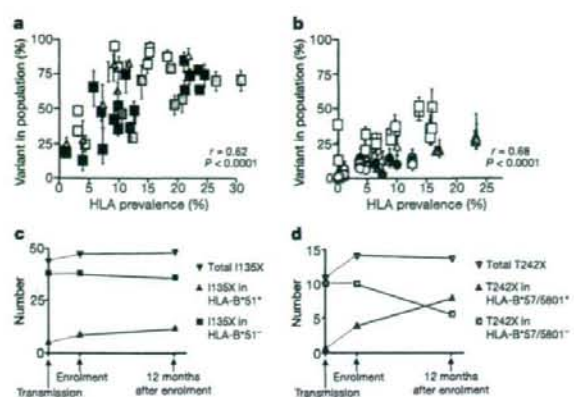


Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. **a**, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares, A146X; sky-blue squares, V168I; yellow circles, I247X). **c**, **d**, Data from acute London cohort. **c**, Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d**, The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

believed to contribute significantly to containment of HIV^{4,7,13–19}. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms⁶. Phylogenetic correction of sequence data excludes founder effect as a confounder^{6,7,9}, and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain CD8⁺ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV

transmission rate, and HLA allele prevalence. Models would need to include factors such as the selection of compensatory mutations to slow reversion rates, and antiretroviral therapy access that would slow transmission rates.

HLA adaptation to certain CD8⁺ T-cell responses may also alter currently established HLA associations with slow disease progression. Data here suggest that, whereas 25 years ago HLA-B*51 was protective in Japan^{11,12}, this is no longer the case (Supplementary Fig. 2). The apparent increase in I135X frequency in Japan over this time supports the notion that HLA-B*51 protection against HIV disease progression hinges on availability of the HLA-B*51-restricted TAFTIPSI response. However, whether this is the case remains unknown.

For HLA-B*27 and HLA-B*57, there is more clear-cut evidence that their association with HIV control depends on the Gag-specific epitopes presented and analysed here^{4,7,13–15,18,19}. For each of the HLA-B*27- and HLA-B*57-associated Gag mutations studied, an *in vitro* fitness cost or *in vivo* reversion has been observed. A strong correlation between variant frequency and HLA prevalence even for rapidly reverting variants can be explained, either by mutant acquisition exceeding reversion rate (Fig. 4D), or by selection of compensatory mutations slowing or halting reversion altogether. The clearest example of the latter is the HLA-B*27-associated R264K mutation, 'corrected' by S173A¹⁹. Compensatory mutations are also well described for the HLA-B*57-associated Gag mutations^{14,18}. These data suggest that the escape mutations in these HLA-B*27- and HLA-B*57-restricted epitopes are accumulating over time. Several studies have now demonstrated that transmission of viruses encoding escape mutants in the critical Gag epitopes to individuals expressing the relevant MHC class results in failure to control viraemia^{21,22}. The accumulation at the population level of these escape mutations in HLA-B*27 and HLA-B*57 Gag epitopes is therefore likely to reduce the facility of these alleles to slow HIV disease progression.

The longer-term consequences of this process for immune control of HIV are unknown. Loss of currently immunodominant epitopes would promote subdominant CD8⁺ T-cell responses, which can be more effective^{23,24}. Also, the adapted virus provides new epitopes that can be presented, potentially with beneficial effects. In hepatitis C virus, for example, HLA-A*0301 holds a particular advantage, but only against the specific strain of virus responsible for the Irish outbreak²⁵. In HIV, HLA-B*1801 is associated with high viraemia in C clade but not in B clade infection^{10,11,26}; the opposite applies to HLA-B*5301.

Thus, the data presented here, showing evidence that the virus is adapting to CD8⁺ T-cell responses, some of which may mediate the well-established associations (HLA-B*57, HLA-B*27 and HLA-B*51) with immune control of HIV, highlight the dynamic nature of the challenge for an HIV vaccine. Important questions to be addressed include the speed and extent of sequence change, particularly in Gag, the most effective target for CD8⁺ T-cell responses^{1,7,13,21}. The induction of broad Gag-specific CD8⁺ T-cell responses may be a successful vaccine strategy, but such a vaccine will be most effective if tailored to the viral sequences prevailing, and thus may need to be modified periodically to keep pace with the evolving virus. Moreover, the strong associations between certain HLA class molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, and slow disease progression may decline as the epidemic continues, particularly where these HLA alleles are highly prevalent, and where HIV transmission rates are high.

METHODS SUMMARY

Overall 2,875 subjects were studied, from 9 previously established study cohorts. These cohorts comprised subjects from North America, the Caribbean, Europe, sub-Saharan Africa, Australasia and Asia. All subjects were antiretroviral-therapy-naïve. Apart from the London acute cohort ($n = 142$), all cohorts comprised chronically infected subjects. The 14 variants studied are well-defined escape mutations within well-characterized CD8⁺ T-cell epitopes, and included those

persisting after transmission and likely to have little effect on viral fitness ($n = 5$), as well as those shown previously to reduce viral fitness ($n = 9$). Autologous HIV-1 sequences, and HLA class I types, were determined for all study subjects. The replicative capacity of I135X variants selected within the HLA-B*51-restricted epitope TAFTIPSI (RT 128–135) was assessed via *in vitro* competition assays and also via longitudinal follow-up of HLA-B*51-negative subjects infected acutely with I135X variants. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model taking into account the different numbers of study subjects in each cohort. Demonstration of an HLA allele driving escape at Gag 146 in the Japanese cohort was undertaken first by identification of an association between HLA-B*4801 and A146P, subsequent definition of an HLA-B*4801-restricted CD8⁺ T-cell response to a novel epitope Gag 138–147 (LI10), and finally demonstration that A146P reduced viral recognition by LI10-specific CD8⁺ T cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Accession numbers for newly determined viral sequences are included in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.G. (philip.goulder@paediatrics.ox.ac.uk).

METHODS

Study subjects. The study cohorts have been described more fully elsewhere^{17,9,11,14,18,20,21,27}. All comprise chronically infected and highly active anti-retroviral therapy (HAART)-naive study subjects, with the exception of the London acute cohort ($n = 142$), who were enrolled immediately after seroconversion between 1999 and 2004, and 54 subjects enrolled during acute infection in Japan between 1997 and 2008. Viral sequences in all 2,679 chronically infected study subjects (all of whom were HAART-naive) were determined from time points after 2000, with the exception of 9 study subjects in the Japanese chronic cohort (1998–99) and all of the British Columbia cohort (1996–99). Sequencing data were obtained from 566 study subjects in the British Columbia cohort, 53 study subjects in the Barbados cohort, 106 in the Oxford cohort, 673 in the Durban cohort, 226 in the Lusaka cohort (chronically infected subjects enrolled between 2005–08), 481 study subjects in the Perth cohort, 277 chronically infected subjects in the Kumamoto cohort, 297 in the Gaborone cohort, and 142 subjects in the acute London cohort. An additional cohort in Japan comprised 117 haemophiliacs who were infected before 1985, the majority of which were believed to have been infected in 1983, and who were enrolled and followed up in out-patient clinics since 1997. These haemophiliacs are all now on HAART except for 4 HAART-naive subjects.

HLA-associated HIV amino acid polymorphisms studied. Variants studied that were shown to reduce viral fitness comprised polymorphisms within the HLA-B*27-restricted Gag epitope KRWILGLNK (Gag 263–272; R264X and L268X) and mutations in three HLA-B*57-restricted Gag epitopes: ISPTLNLAW (ISW9, Gag 147–155), KAFSPVEIPMF (KF11, Gag 162–172) and TSTLQEQIAW (TW10, Gag 240–249). T242X is strongly selected by HLA-B*5801 in addition to HLA-B*57 subtypes^{7,14,17}. The HLA-B*57-associated polymorphisms at residues Gag 146, 147 and 248 are selected by all HLA-B*57 subtypes, whereas Gag 163, 165, 166 and 247 are only selected by the HLA-B*5703 subtype (refs 7, 18 and H.C., unpublished data).

Statistics. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model. To take account of the different numbers of study subjects in each cohort, appropriate confidence limits for the mutation frequencies were calculated, using the Adjusted-Wald method for binomial variables²⁸. Logistic regression was calculated by GLMStat (<http://www.glmstat.com>) using a binomial error distribution and a logit link function. In addition, the Spearman's rank correlation coefficient was calculated in the context of a linear regression model (data shown in Supplementary Tables 1 and 2).

HLA class I typing. Because HLA typing was not undertaken consistently to four-digit resolution in all cohorts, two-digit HLA types only were used for these analyses, with the exception of the HLA-B*5703-associated polymorphisms (the Barbados and Oxford cohorts being excluded from these latter analyses as HLA-B*57 subtyping data were not available). Genomic DNA samples were initially typed to an oligo-allelic (two-digit) level using Dynal RELITM reverse SSO kits for the HLA-A, HLA-B and HLA-C loci (Dynal Biotech). Refining the genotype to the allele level was performed using Dynal Biotech sequence-specific priming (SSP) kits in conjunction with the previous SSO type. HLA phenotypic frequencies were determined from the HIV-infected study cohorts themselves.

Sequencing of viral RNA and proviral DNA. Viral sequencing of gag and pol from plasma RNA and proviral DNA was undertaken, using primers as previously described²⁹. PCR products were sequenced directly or they were cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a Big Dye terminator v1.1. cycle sequencing kit (Applied Biosystems) and analysed by an ABI PRISM 310 genetic analyser.

Competitive HIV-1 replication assay. Freshly prepared H9 cells (3×10^5) were exposed to the mixtures of paired virus preparations (300 blue cell-forming

units) each of NL-432 versus mutant virus (I135T, I135V, I135R and I135L), to be examined for their replication ability for 2 h, washed twice with PBS, and cultured as described previously³⁰. On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and the proviral HIV-1 reverse transcriptase gene was sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells. The cells harvested at the end of every other passage (that is, at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 weeks) were subjected to direct DNA sequencing of the HIV-1 reverse transcriptase gene, and the viral population change was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

HLA-B*5101 stabilization assay. Binding of HIV-1-derived peptides to HLA-B*5101 was measured as previously described by using RMA-S-B*5101 cells⁸.

Assays to determine recognition of peptide-pulsed or virus-infected targets. CIR and .221 cells expressing HLA-B*5101 or HLA-B*4801 were generated as previously described³⁰. All cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg ml⁻¹ hygromycin B. Cytotoxicity of CD8⁺ T cells for CIR-B*5101 cells pre-pulsed with peptide measured by the standard ⁵¹Cr release assay was as previously described⁸. .221-B*4801 and .221 cells infected with NL4-3 or NL4-3 A146P mutant virus were used as target cells for intracellular cytokine staining assay.

Generation of the NL4-3 A146P mutant virus. The p82-2 plasmid containing the A146P mutation⁴ was digested with BssHII and ApaI. The BssHII–ApaI 1.3-kb fragment was purified and then ligated into the same site of BssHII–ApaI-digested pNL-432 plasmid. To obtain pNL-432 including the A146P mutant (pNL-432 A146P), 293T cells were transfected with pNL-432 A146P using Lipofectamine 2000 (Invitrogen). Supernatants from transfected 293T cell cultures were stored at –80 °C.

Generation of CD8⁺ T-cell clones and peptide-specific CD8⁺ T-cell lines. Cytotoxic T lymphocyte (CTL) clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution as previously described⁸. Peptide-specific CD8⁺ T-cell lines were generated by stimulating peripheral blood mononuclear cells (PBMCs) from the HLA-B*4801-positive HIV-1-seropositive individual KI-092 with the NI11 (NLQGMVHQAI) peptide and then culturing them for 2 weeks⁸. Cytotoxicity of CD8⁺ T cells for target cells pre-pulsed with peptide measured by the standard ⁵¹Cr release assay was as previously described⁸.

Suppression assay of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described³⁰.

Intracellular cytokine staining assays. PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12–14 days. These cultured PBMCs were assessed for IFN-γ-producing activity as previously described³⁰.

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Original article

Escape mutation selected by Gag28-36-specific cytotoxic T cells in HLA-A*2402-positive HIV-1-infected donors

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Abstract

Gag-specific CTLs are known to have stronger ability to control HIV-1 replication than others that are protein-specific. Therefore, the analysis of Gag escape mutants is expected to clarify the mechanisms of immune control in HIV-1-infected donors. However, only a limited number of Gag escape mutants have been identified so far. A previous study suggested the possibility that Gag28-3R (KW9-3R) is an escape mutant from HLA-A*2402-restricted KW9-specific CTLs but did not show any evidence of it. Here we sought to demonstrate that KW9-3R is selected as escape mutant by KW9-specific CTLs. KW9-specific CTLs showed a remarkable reduction in recognition of target cells infected with the KW9-3R mutant. The sequence analysis of HIV-1 from 58 HIV-1-infected individuals showed that the frequency of the KW9-3R mutant was significantly higher in HLA-A*2402⁺ individuals than in HLA-A*2402⁻ individuals. Longitudinal analysis of an HLA-A*2402⁺ individual with HIV-1 early infection showed that this escape mutant was selected over an approximately 2-year period. These results together indicate that Gag28-3R is an escape mutant selected by HLA-A*2402-restricted KW9-specific CTLs. Further analysis of this epitope will clarify the role of HIV-1-specific CTLs in the control of HIV-1 among the Japanese population, since 70% of them carry this allele.

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) have an important role in the control of HIV-1 replication during acute and chronic phases of an HIV-1 infection [1–3]. However, CTLs cannot completely eradicate HIV-1, because HIV-1 can escape from the host immune system by various mechanisms, including mutations of its immunodominant CTL epitope. The substitution of just a single amino acid within a CTL epitope is crucial for the failure of binding to HLA class I molecules or of the interaction between the T cell receptors (TCRs) of specific CTLs and the

peptide-HLA class I complex. Both mechanisms result in the loss of CTL activities against target cells infected with mutant HIV-1 and contribute to the selection of a virus that can escape from CTLs [4–10]. There are many studies demonstrating that CTL-mediated immune pressure selects CTL escape variants during both acute and chronic HIV-1 and simian immunodeficiency virus (SIV) infection [11,12] and that the appearance of the escape mutants could result in the loss of immune control and disease progression [4,13]. The escape of HIV-1 from CTL responses has been proposed to be an important obstacle for HIV-1 vaccine development [14,15].

Gag-specific CTLs are known to have a stronger ability to control HIV-1 replication than others that are protein-specific [16]. Therefore, it is assumed that the appearance of Gag escape mutant results in the failure of HIV-1 control by Gag-specific CTLs. In fact, several such cases have been reported [4,7,17,18]. Analysis

Abbreviations: HIV-1, human immunodeficiency virus type 1; CTL, cytotoxic T-lymphocytes; HLA, human leukocyte antigens.

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of Gag escape mutants is very important to clarify the mechanisms of AIDS pathogenesis and AIDS vaccine development.

Approximately 70% of the Japanese population expresses HLA-A*2402 [19]. Gag28-36 (KW9) is the only immunodominant epitope of 3 epitopes presented by this allele [20,21]. A previous study showed that Gag28-3R (KW9-3R) was detected in 2 of 3 Japanese HIV-1-infected HLA-A*2402⁺ individuals and that HLA-A*2402-restricted KW9-specific CTLs did not recognize the KW9-3R peptide [22]. However, it was still unclear whether the appearance of the mutant was associated with HLA-A*2402 and if the HLA-A*2402-restricted KW9-specific CTLs selected the mutants.

Here, we sought to clarify that KW9-3R is an escape mutant from KW9-specific CTLs by demonstrating that specific CTLs showed markedly reduced killing activity towards target cells infected with KW9-3R mutant virus. In addition, we investigated whether this mutant is more highly detectable in HLA-A*2402⁺ donors than in HLA-A*2402⁻ donors in order to clarify whether this mutant is selected at the population level. Furthermore, we performed longitudinal analysis of this mutant in a case of early HIV-1-infection.

2. Materials and methods

2.1. Patient samples

Informed consent was obtained from all subjects according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA type was determined by standard sequence-based genotyping.

2.2. Sequence of autologous virus

Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute Virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H-Reverse Transcriptase and random primer (Invitrogen). The Gag region was amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were then agarose gel-purified and sequenced directly or cloned by use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer.

2.3. Cells

C1R cells expressing HLA-A*2402 (C1R-A*2402) were previously generated [23] and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B. 721.221-CD4-A*2402 cells were generated by transfection of the CD4 gene and the HLA-A*2402 gene into 721.221 cells, and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

2.4. Generation of CTL clones

Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 µl of cloning mixture (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMC from a healthy donor, and 1×10^5 irradiated C1R-A*2402 cells prepulsed with a 1 µM concentration of the corresponding peptide, KW9 [KYKLIKHIWV]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by the standard ⁵¹Cr release assay. All CTL clones were cultured in RPMI 1640–10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

2.5. HIV-1 clones

NL-432gag^{SF2} was created by replacing the NL-432 gag gene with the SF2 gag gene. For NL-432gag^{SF2} KW9-3R, the mutation was introduced by site-directed mutagenesis (Invitrogen) based on overlap extension.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxicity activity was measured by the standard ⁵¹Cr release assay, as previously described [24]. Target cells (2×10^5) were incubated for 60 min with 100 µCi Na₂⁵¹CrO₄ in saline and then washed 3 times with RPMI 1640 medium containing 10% newborn calf serum (NCS). Labeled target cells (2×10^3 /well) were added to 96-well round-bottom microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After a 1 h incubation, effector cells were added, and the mixtures were then incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ⁵¹Cr release was determined by measuring the counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ⁵¹Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as $(\text{cpm exp} - \text{cpm spn}) / (\text{cpm max} - \text{cpm spn}) \times 100$, where "cpm exp" is the counts per minute in the supernatant in the wells containing both the target and effector cells.

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4-A*2402 cells were exposed to the virus for several days. The cells were used as target cells for a CTL assay when infection of approximately 80% cells was confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ⁵¹Cr as described above. Labeled target cells were added along with effector cells into

round-bottom microtiter plates (Nunc), and the mixtures were incubated for 6 h at 37 °C.

2.8. Intracellular cytokine (ICC) production assay

PBMCs from HLA-A*2402-positive HIV-1-infected patients were stimulated with KW9 peptide (1 μ M) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN- γ production activity by using a FACSCalibur. Briefly, bulk cultures were stimulated with C1R-A*2402 cells pulsed with KW9 peptide (1 μ M) for 2 h at 37 °C. Brefeldin A (10 μ g/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (mAb; Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were permeabilized in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4 °C for 10 min and stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN- γ mAb (PharMingen, San Diego, CA). After a thorough washing with the permeabilization buffer, the cells were analyzed by using the FACSCalibur.

2.9. HLA-peptide tetrameric complexes

The tetrameric complexes were synthesized as previously described [25]. Briefly, an ectodomain of HLA class I proteins and human β_2 microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4 °C. The resultant 45-kDa complex was purified by size-exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England) and anion-exchange (MonoQ column; Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England) chromatographies. The purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE- or allophycocyanin (APC)-conjugated avidin (Molecular Probes) at a molar ratio of 4:1.

2.10. Tetramer analysis

Cryopreserved PBMC of HIV-1-infected donors were stained with PE- or APC-conjugated tetramers at 37 °C for 30 min. Following 2 washes with RPMI 1640–10% FCS, the cells were stained with FITC-conjugated anti-CD8 mAb at 4 °C for 30 min and subsequently analyzed using flow cytometry (FACSCalibur).

3. Results

3.1. Immunodominancy of HLA-A*2402-restricted Gag28-36 (KW9) epitope

A previous study using a cytolytic assay showed that KW9-specific T cells are detected in 4 of 12 chronically

HIV-1-infected individuals [20]. We first re-evaluated whether KW9-specific CD8⁺ T cells could be frequently detected in chronically HIV-1-infected Japanese donors carrying HLA-A*2402. PBMC from these donors and HIV-1-seronegative HLA-A*2402⁺ donors as negative control were stimulated with the epitope peptides and cultured for 2 weeks. The specific CD8⁺ T cells in these cultures were measured by performing an ICC production assay using KW9 epitope peptides (Fig. 1A). KW9-specific CD8⁺ T cells were detected in 8 of the 12 individuals (Fig. 1B), confirming that KW9 is recognized as the immunodominant epitope in HIV-1-infected HLA-A*2402⁺ Japanese donors.

3.2. Association of an HLA-A*2402 allele with KW9 mutations

To clarify whether KW9-specific CTLs select the escape mutants at the population level, we analyzed the sequences of the KW9 epitope and the flanking regions of this epitope of HIV-1 from 32 HLA-A*2402⁺ and 26 HLA-A*2402⁻ donors. Several mutations were found, occurring at positions 1, 3, 7, and 8 in the KW9 epitope (Fig. 2). The frequency of the 3R mutation was significantly higher in the HLA-A*2402⁺

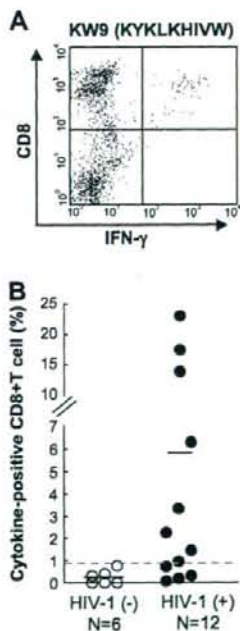


Fig. 1. HLA-A*2402-restricted KW9-specific CD8⁺ T cells in chronically HIV-1-infected HLA-A*2402⁺ individuals. (A) After PBMC from an HLA-A*2402⁺ HIV-1-infected individuals had been stimulated with KW9 peptides for 2 weeks, KW9-specific CD8⁺ T cells were detected by measuring IFN- γ -producing CD8⁺ T cells in the culture stimulated with KW9 peptide. A representative result is shown in "A". (B) Summary of ICC assays for HLA-A*2402⁺ HIV-1-infected and HIV-1-uninfected individuals. The average + 2 SD of IFN- γ -producing CD8⁺ T cells in HIV-1-uninfected individuals was defined as a positive value (>0.87%). Dotted bars indicate the average + 2 SD; and horizon bars, the average in HIV-1-infected individuals.