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Abbreviations: **ART:** antiretroviral therapy · **HIV-1:** HIV type 1 · **IRS:** immune reconstitution syndrome · **Tcon:** conventional CD4$^+$ T cells

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Successful genotype-tailored treatment with small-dose efavirenz

King and Aberg [1] recently published an excellent review of the clinical implications of population differences and genomic variations in efavirenz (EFV) treatment. They elegantly summarized the relationship between EFV concentration under standard dosage (600 mg once daily) and the genotype of cytochrome p450 2B6 (CYP2B6), a primary liver enzyme in EFV metabolism. They also highlighted the importance of CYP2B6 516 G>T SNP as a marker of individuals at risk of high EFV concentration and potential development of central nervous system (CNS) side-effects. However, it is desirable to discuss possible personalization of treatment by EFV dose modification.

As we described in our recent clinical study [2], we reduced EFV dosage in 12 patients with CYP2B6 516G>T polymorphism who were found to have extremely high EFV concentrations when treated with the standard dosage. The dosage was reduced from 600 to 400 mg in five individuals and to 200 mg in seven, and their HIV-1 load was successfully suppressed below detection limit (50 copies/ml) at these dosages. Interestingly, nine of the 12 suffered from chronic CNS-related symptoms at the standard dosage, but these improved in all nine by EFV dose reduction. An example of these patients is a 71-year-old man who reported having nightmares almost every night since starting EFV-containing antiretroviral therapy at 600 mg 3 years ago (Fig. 1). Plasma EFV concentrations were extremely high

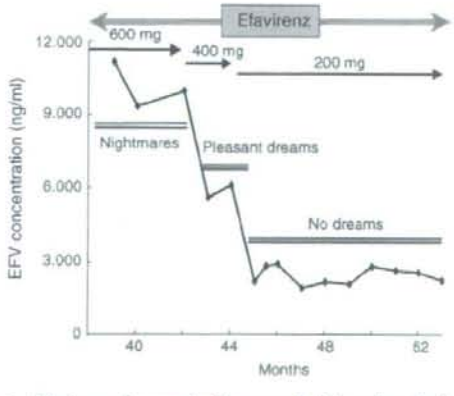

Fig. 1. Efavirenz dose reduction resulted in reduced efavirenz concentration and improved central nervous system related symptom. A CYP2B6 516T/T genotype holder reported having nightmares every night for 3 years, which disappeared after efavirenz (EFV) dose reduction.

and analysis of the 516G>T SNP showed CYP2B6 516 genotype T/T. The EFV dosage was reduced to 400 mg. This resulted in a dramatic change in dream contents from nightmares to pleasant dreams. These changes occurred although the EFV concentration remained high at 400 mg. Therefore, we further reduced the dose to 200 mg. The second reduction resulted in complete disappearance of dreams. Although he missed the dreams, the EFV concentration decreased to within the target range at 200 mg. The EFV dose has been at 200 mg for more than 2 years, and the HIV-1 load remains under detection limit.

Hasse *et al.* [3] also reported a patient with genotype CYP2B6 516T/T, who had chronic CNS symptoms and extremely high EFV concentration at 600 mg dose, but the symptoms resolved by reducing the EFV dose to 200 mg. Considered together, the above report and our study suggest that the quality of life of CYP2B6 516T/T genotype holders who suffer from CNS-related symptoms can be improved by reducing EFV dose from the standard to 400 or even 200 mg. In their review, King and Aberg [1] indicated that the cost remains an issue for identifying CYP2B6 516 genotype. However, one Japanese commercial laboratory has already developed a CYP2B6 516 genotype detection system based on the Invader assay [4], which costs only ¥8000 (~$75) per single test. Thus, the financial benefits of reducing EFV dosage should compensate for the cost of genotyping. Further large-scale studies are needed to discuss genotype-based tailored EFV treatment.

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Successful use of darunavir, etravirine, enfuvirtide and tenofovir/emtricitabine in pregnant woman with multiclass HIV resistance

A 38-year-old HIV-1-positive African woman was presented to clinic at 6 weeks of a twin pregnancy. In the past, she had received nucleoside reverse transcripase inhibitors (zidovudine, lamivudine, tenofovir and didanosine), nonnucleoside reverse transcriptase inhibitors (efavirenz and nevirapine) and protease inhibitors (nelfinavir and lopinavir). Her switches in antiretroviral therapy (ART) had been because of virological failure related to poor adherence. Her treatment was tenofovir/emtricitabine (TDF/FTC) and boosted atazanavir. Her viral load was 4660 copies/ml with CD4 cell count of 471 cells/μl.

Therapeutic drug monitoring showed levels in excess for atazanavir. Sequencing of HIV polymerase revealed resistance mutations – reverse transcriptase: D67N, V118I/V, M184V, Y188L, L210W and T215Y; protease inhibitor: L10I, I13V, G16E, K20I, M36I, M46I, I47V, F53L, I54V, D60E, D63T, H69K, I84V and L89M. A phenotypic assay confirmed high-level resistance to almost all licensed antiretroviral drugs. Thus, there was a risk of mother-to-child transmission (MTCT) of multiclass-resistant HIV [multidrug-resistant HIV (MDR-HIV)]. After discussion by a multidisciplinary HIV team, at 25 weeks gestation, she was prescribed darunavir 600 mg twice daily (b.i.d) with ritonavir 100 mg b.i.d, etravirine 200 mg b.i.d (via compassionate release program) and enfuvirtide (T20) 90 mg subcutaneously b.i.d given under direct observation and TDF/FTC (245 mg/200 mg) one tablet q.d. as optimized background therapy. Four weeks later, her viral load was fully suppressed (HIV RNA <50 copies/ml) with CD4 cell count of 356 cells/μl. A pharmacokinetic study of T20, etravirine and darunavir showed maternal plasma levels that were above the expected therapeutic ranges (Table 1).

At 32 weeks gestation, she developed premature contractions and received oxytocin receptor antogonist (atosiban). After spontaneous rupture of membranes at 34 weeks gestation, a caesarean section was performed 3 h after onset of labour. She delivered a healthy baby boy and girl weighing 1.810 and 1.860 kg, respectively. Neither zidovudine nor nevirapine were administered during labour, but she received an extra dose of her current antiretroviral drugs 2.5 h before the caesarean section. At delivery, viral load remained undetectable, and her CD4 cell count was 451 cells/μl. The babies received a postnatal prophylactic antiretroviral drug regimen comprising T20 for 2 days, nevirapine for 1 week and didanosine for 2 weeks. Analysis of cord blood samples from both placentas showed undetectable levels of T20, whereas significant levels of darunavir, ritonavir and etravirine were found (Table 1). At 4 months of age, four HIV-1 DNA polymerase chain reaction tests performed on blood samples from each twin have been negative and no laboratory abnormalities noted.

Adverse events experienced by the mother were mild and included T20 injection site reactions, high fasting triglycerides and anaemia. She developed liver dysfunction at week 4 which peaked at week 8 of therapy (29 and 33 weeks gestation, respectively). All adverse events resolved spontaneously. Serological markers showed that she was hepatitis B immune with negative hepatitis B virus (HBV) DNA and negative for hepatitis A, C, cytomegalovirus (CMV), parvovirus, Q fever and rubella infections. Liver ultrasound was normal.

Use of ART in pregnancy significantly reduces MTCT of HIV [1]. This goal is more challenging in pregnant women with MDR-HIV. Newer antiretroviral drugs lack

Table 1. Levels of T20, ritonavir, darunavir and etravirine at week 4 (29 weeks of pregnancy) and at delivery (week 34) in cord blood samples.

					Cords sample (delivery)	
Twenty-nine weeks of pregnancy	Predose	1 h postdose	3 h postdose	6 h postdose	Twin 1	Twin 2
T20 (ng/ml)	3188	3766	4183	4313	Undetectable	Undetectable
Ritonavir (ng/ml)	199	365	188	212	25.7	123
Darunavir (ng/ml)	1960	2820	3940	3320	577	1020
Etravirine (ng/ml)	896	939	1110	1210	414	345

Levels of drugs were assayed by liquid chromatography-mass spectrometry/mass chromatography. Mean trough for wild-type virus in adults: T20: ranged from 2600 to 3400 ng/ml [12], darunavir: 3578 ng/ml (after 600/100 mg twice daily) [13] and etravirine: 297 ng/ml [14]. Ritonavir was used as booster.

safety, tolerance and efficacy data in pregnancy. Neonatal MDR-HIV infection poses an even greater problem to treat, as there is no pharmacokinetic data for the newer drugs on infants.

Recent case reports highlighted the efficacy of T20 in preventing MTCT [2–4]. The lack of transplacental crossing renders this drug attractive for use in MDR-HIV-experienced pregnant women with anticipated little or no foetal toxicity [5]. Nevertheless, in one case report, T20-based regimen failed to prevent MTCT, despite an undetectable plasma viral load at delivery suggesting lack of genital tract penetration of such ART [6].

Unlike T20, placental crossing of darunavir and etravirine is not known. To our knowledge, ours is the first report to show transplacental transfer of darunavir and etravirine in human pregnancy. While raising concerns regarding possible foeto-toxicity, this finding also offers the prospect that these drugs could have prevented HIV infection in babies. Pregnant women have been excluded from all the major clinical trials conducted to date involving the three drugs [7–10]. In animal studies, however, no maternal foetal toxicity was observed for either darunavir or etravirine [11]. The extra dose of antiretroviral drugs given before the delivery may have influenced the cord blood levels, but caused no apparent neonatal toxicity. Clinicians should, where possible, give babies the same ART as the mother's regimen for prophylaxis, and we would advocate caesarean section as preferred mode of delivery in such cases.

In conclusion, we report successful prevention of MTCT in a case of MDR-HIV using a darunavir/etravirine-based regimen with evidence of placental transfer of both drugs.

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OR, 6.2; 95% CI, 2.5–15.4). There was no increase in the risk of *T. vaginalis* infection among women who were infected with *T. vaginalis* during the immediately preceding interval (4.4%), compared with women who were not (3.9%). However, 13 (62%) of 21 new infections occurred in women who had been previously infected with *T. vaginalis*, and 11 (85%) of 13 had negative test results during the immediately preceding interval (figure 1).

Some of the women might have acquired infections during sexual contact that they did not report, and some might have had infections that were not detected at the baseline visit. However, many women were treated for infection, had negative test results, and then had positive test results again, which suggests that *T. vaginalis* was undetected by testing but still present for months after treatment. The possibility of long-term asymptomatic carriage is consistent with the age distribution of infected women; *T. vaginalis* is found more often in older women [8, 9]. This pattern is different from the pattern for bacterial sexually transmitted diseases but similar to that for incurable viral infections, such as herpes simplex virus type 2 [10]. Trials have suggested cure rates of >90%, but most have tested women once within a few weeks after treatment [11]. When women were tested again a few months after treatment, some of the previously cured women had infection detected again [11], and none of the studies continued testing women beyond a few months. Cultures might not detect infections if the concentration of *T. vaginalis* is low, which would be expected in asymptomatic infections [6, 12, 13]. Nucleic acid amplification tests may be better, but reports are inconsistent and the tests are not commercially available in the United States [14]. Similarly, self-obtained vaginal swab specimens occasionally miss infections, but the sensitivity of tests performed with self-obtained specimens has compared favorably with that of tests performed with clinician-obtained specimens [15].

Treatment failure could explain many of our findings, because 13 women had a documented preceding infection. However, our results were not simply attributable to treatment failure. Most of the women ($n = 11$) had an intervening negative test result before having a positive result during an interval when they reported not having sex. This suggests that, after treatment, *T. vaginalis* infection can become nondetectable for months and then reappear. Because these findings were unexpected and obtained with a small number of participants, additional studies are needed to confirm or refute these observations.

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Detection of HIV Type 1 Load by the Roche Cobas TaqMan Assay in Patients with Viral Loads Previously Undetectable by the Roche Cobas Amplicor Monitor

To the Editor—In March 2008, the Roche Cobas TaqMan assay replaced the Roche Cobas Amplicor Monitor, version 1.5, for measuring plasma HIV type 1 (HIV-1) load in Japan. This has resulted

in the detection of an HIV-1 load >50 copies/mL in some of the patients whose HIV-1 load had been undetectable (<50 copies/mL) by the Amplicor Monitor over several years and for whom antiretroviral treatment regimens had not been changed [1, 2].

A total of 1387 HIV-1–infected patients visited our outpatient clinic from March through June 2008, and their HIV-1 load was measured by the TaqMan assay. Among these patients, 876 regularly visited the clinic (once every 1–3 months) and had an undetectable HIV-1 load by the Amplicor Monitor at the last visit. Surprisingly, the TaqMan assay detected an HIV-1 load >50 copies/mL in 253 (28.9%) of the 876 patients, although antiretroviral treatment had not been modified for these patients. Furthermore, another 22 patients (2.5%) were found to have an HIV-1 load >40 copies/mL with use of the TaqMan assay. The same assay also detected HIV-1 RNA at levels lower than the linear range of the assay (<40 copies/mL) in 128 (14.6%) of the 876 patients.

We analyzed the relationship between TaqMan detectability and time during which the HIV-1 load was undetectable by the Amplicor Monitor. This time was defined as the period from the first HIV-1 load undetectable by the Amplicor Monitor to the viral load first measured by the TaqMan assay, without any HIV-1 load rebound or blip detected during the period. Interestingly, among the patients who had a viral load undetectable by the Amplicor Monitor for <1 year, 43.7% had an HIV-1 load >50 copies/mL detected by the TaqMan assay; among the patients who had a viral load undetectable by the Amplicor Monitor for >4 years, 18.5% had an HIV-1 load >50 copies/mL detected by the TaqMan assay (figure 1). Conversely, 37.3% of patients who had a viral load undetectable by the Amplicor Monitor for <1 year had HIV-1 RNA undetectable by the TaqMan assay, and 70.2% of patients who had a viral load undetectable by Amplicor Monitor for >4 years had an HIV-1 load undetectable by the TaqMan assay. Thus, the proportion of patients who had an HIV-1 load >50 copies/mL was inversely correlated with the duration that the viral load was undetectable ($R^2 = 0.895$), and the proportion of patients with undetectable viral load was positively correlated with the duration that the viral load was undetectable ($R^2 = 0.979$). These findings indicate that the longer the effective treatment, the greater the number of patients with HIV-1 RNA undetectable by the TaqMan assay.

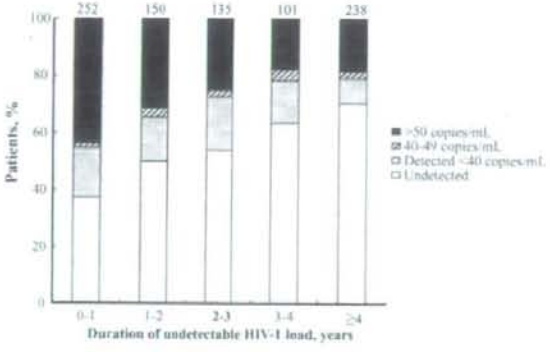

Figure 1. Results of the TaqMan assay and duration of undetectable HIV-1 load in 876 patients whose HIV-1 load was undetectable (<50 copies/mL) when the last Amplicor Monitor was performed. The number of patients is shown above each bar.

We observed significant discrepancy of HIV-1 detectability between the TaqMan assay and the Amplicor Monitor [3–5]. The TaqMan assay detected HIV-1 RNA in a significant percentage of treated patients with HIV-1 loads previously undetectable by the Amplicor Monitor; this is confusing to clinicians and patients and may be a critical problem in ongoing clinical trials of antiretroviral treatment. To determine the permissible range of detectable HIV-1 load during successful antiretroviral treatment, year-long clinical follow-up of treated patients is necessary. Our observation revealed that the detection rate of HIV-1 RNA with use of the TaqMan assay was inversely correlated with the previous duration of undetectable HIV-1 load, suggesting that long-term antiretroviral treatment can further suppress HIV-1 load even after it has decreased to below the detection limit of the Amplicor Monitor.

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LETTERS

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 infection[1]. 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 effect[6] (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 mutation[7].

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 mutation[7,9], and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects[8]. 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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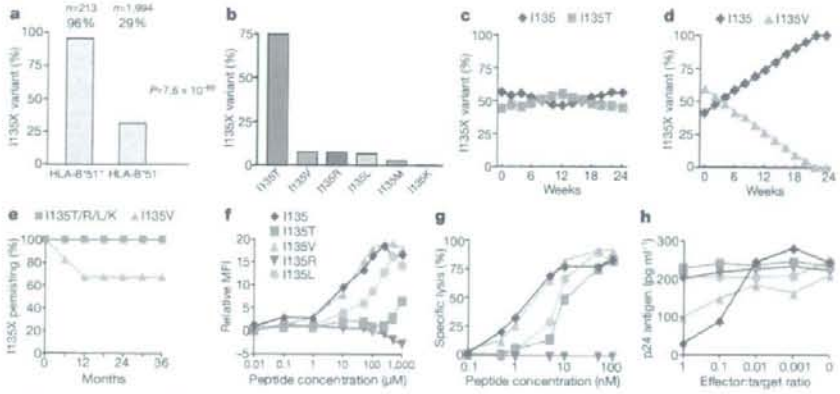

Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51–TAFTIPSI (RT 128–135)-specific CD8$^+$ T cells. **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition 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). **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-pulsed HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8$^+$ T-cell clones.

prevalent[10] (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.

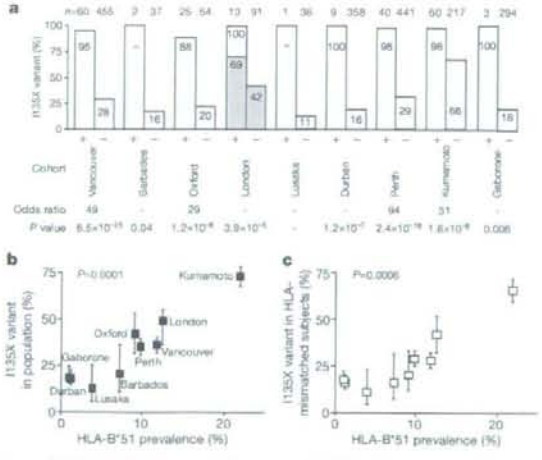

Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts. **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (−) subjects within nine study cohorts. In the acute cohort (London) 69% of 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.

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 Africa[7], 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 (LPPIVAKEI, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence[5]. 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 fitness[1]. 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

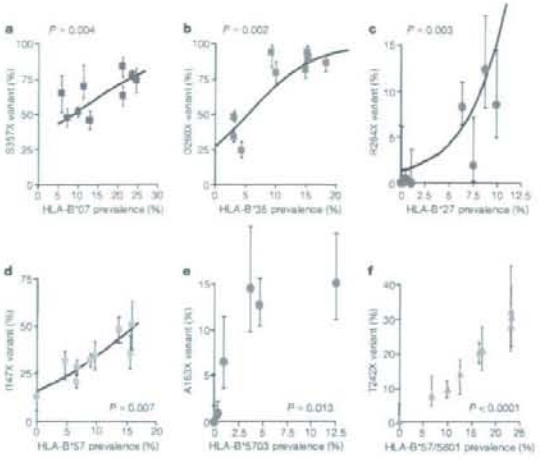

Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a**, Frequency of 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 PPIPVGDIY (Gag 254–262). **c**, Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (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.

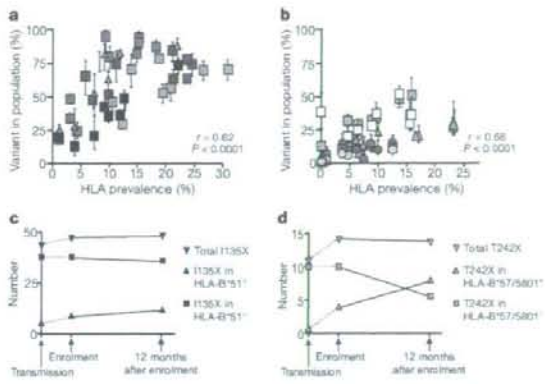

Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. a, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b**, Eight variants demonstrated 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.

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 Japan[10], 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[7,14,18–21], 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 believed to contribute significantly to containment of HIV[5,7,13–19]. 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 polymorphisms[6]. 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 S173A[16]. 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[2,21,22]. 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 outbreak[25]. 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-naive. 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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Supplementary Information is linked to 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).

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METHODS

Study subjects. The study cohorts have been described more fully elsewhere[3,7,9,13,14,16,20,21,27]. All comprise chronically infected and highly active antiretroviral 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 KRWIILGLNK (Gag 263–272; R264X and L268X) and mutations in three HLA-B*57-restricted Gag epitopes: ISPRTLNAW (ISW9, Gag 147–155), KAFSPEVIPMF (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 variables[28]. 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 described[7,9]. 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 previously[29]. 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 cells[8].

Assays to determine recognition of peptide-pulsed or virus-infected targets. C1R and .221 cells expressing HLA-B*5101 or HLA-B*4801 were generated as previously described[30]. 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 C1R-B*5101 cells pre-pulsed with peptide measured by the standard ^{51}Cr release assay was as previously described[8]. .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 mutation[4] 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 described[8]. 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 N11 (NLQGQMVHQAI) peptide and then culturing them for 2 weeks[8]. Cytotoxicity of CD8$^+$ T cells for target cells pre-pulsed with peptide measured by the standard ^{51}Cr release assay was as previously described[8].

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 described[30].

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 described[30].

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SUPPLEMENTARY INFORMATION

Subject	Timepoint	TAFTIPSI
Subject-1	Baseline	-------T
	12 months	-------T
Subject-2	Baseline	-------T
Subject-3	Baseline	-------R
	12 months	-------R
Subject-4	Baseline	--------
	12 months	--------
	18 months	--------
	24 months	-------T
Subject-5	Baseline	-------R
	Baseline	-------R
	Baseline	-------R
Subject-6	Baseline	-------R
	12 months	-------R
	24 months	-------R
	36 months	-------R
Subject-7	Baseline	-------T
	10 months	-------T
	12 months	-------T
	24 months	-------T
	36 months	-------T
Subject-8	Baseline	--------
	12 months	-------R
	24 months	-------T
Subject-9	Baseline	-------T
	7 months	-------T
	12 months	-------T
Subject-10	Baseline	--------
	12 months	-------T
	24 months	-------T
Subject-11	Baseline	-------T
	12 months	-------T
	24 months	-------T
Subject-12	Baseline	-------T
Subject-13	Baseline	--------
	6 months	-------T
	12 months	-------T

Supplementary Figure 1. Deduced amino acid sequences encoded by plasma viral RNA sequences determined from 13 HLA-B*51-positive study subjects enrolled in London during acute infection or shortly following seroconversion.

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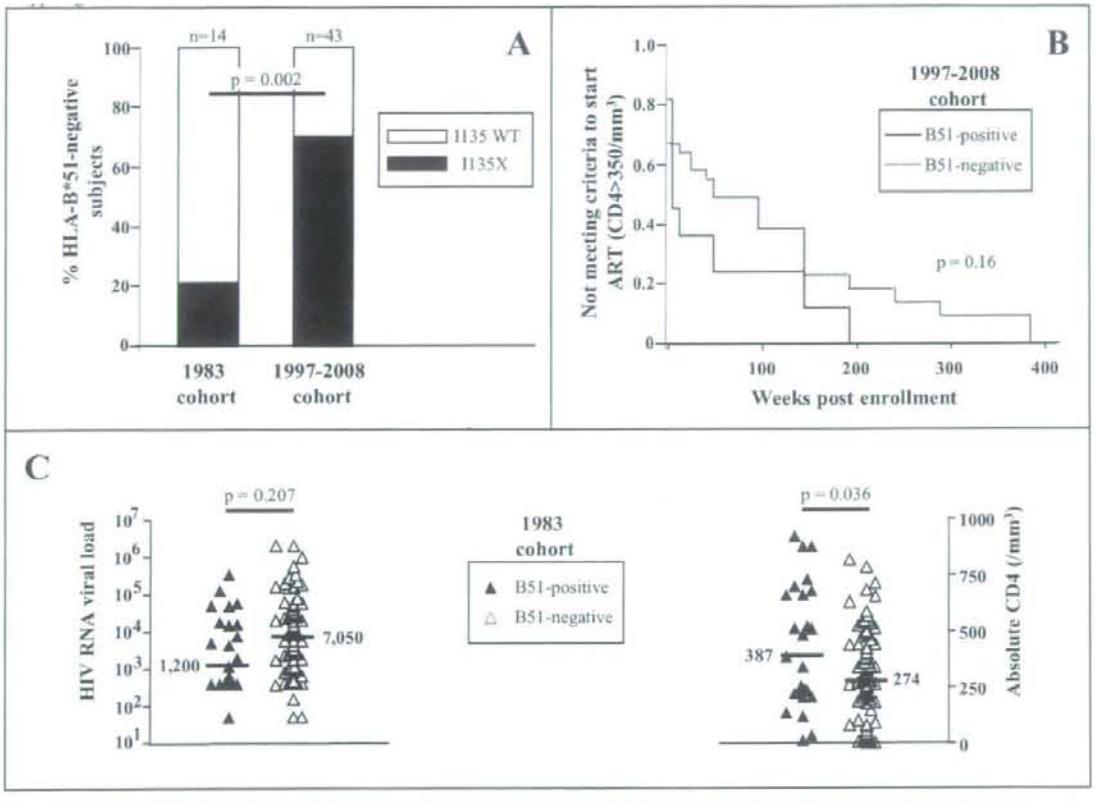

Supplementary Figure 2. A. Frequency of I135X variant in 14 HLA-B*51-negative haemophiliacs infected in 1983 and in 43 HLA-B*51-negative subjects infected between 1997 and 2008 via sexual transmission. **B.** Kaplan Meier analysis showing time to meeting criteria to start HAART in the acute cohort enrolled 1997-2008, criteria being a CD4<350/mm3 and/or severe clinical symptoms. 2-sided p value obtained from the logrank (Mantel-Cox) test. **C.** HIV viral and absolute CD4 count differences in haemophiliacs infected in 1983 when enrolled in 1997.

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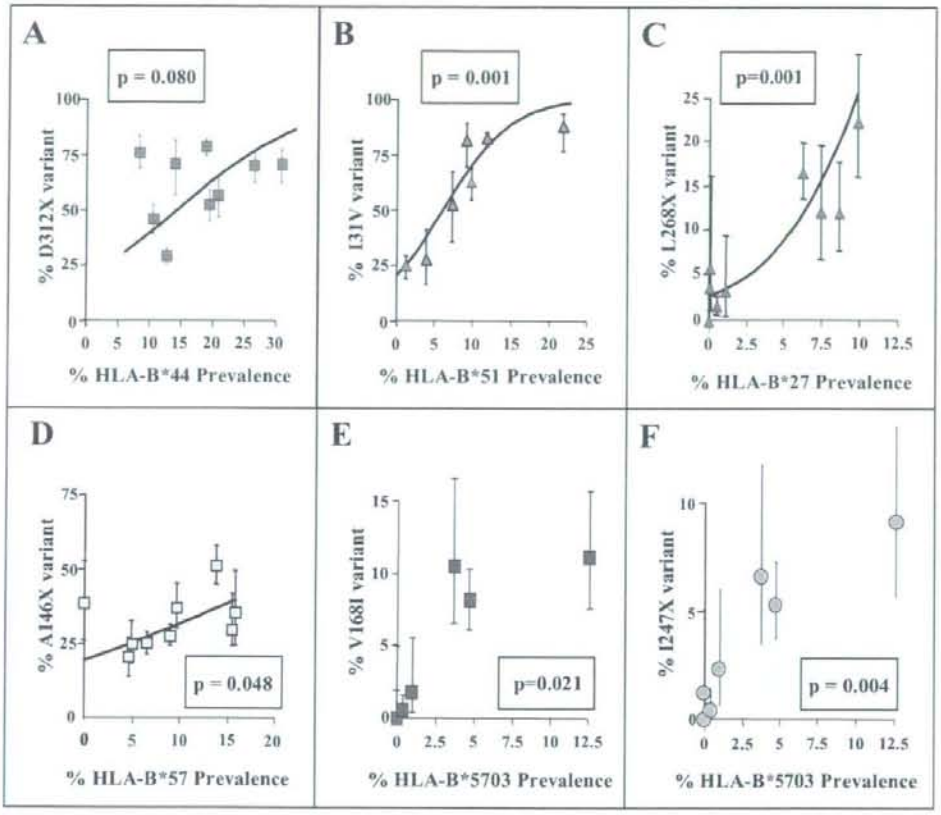

Supplementary Figure 3. Correlation between variants associated with HLA alleles and prevalence of the respective HLA allele. A. Frequency of the D312X mutation within the HLA-B*44-restricted epitope AEQATQDVKNW (Gag 306-316). **B.** Frequency of I31V mutation within the HLA-B*51-restricted epitope LPPIVAKEI (Int 28-36; Integrase was not sequenced and therefore data unavailable for the (acute) London cohort or the Gaborone cohort). **C.** Frequency of the L268X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263-272). **D.** Frequency of the processing mutation4 A146X associated with the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147-155). **E.** Frequency of the V168X mutation within the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162-172). **F.** Frequency of the I247X mutation within the same HLA-B*57/5801-restricted epitope TSTLQEQIAW (Gag 240-249).

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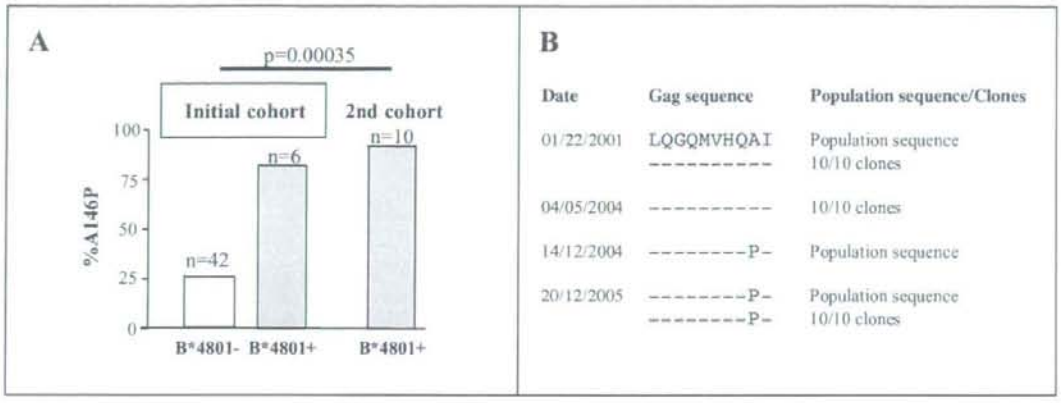

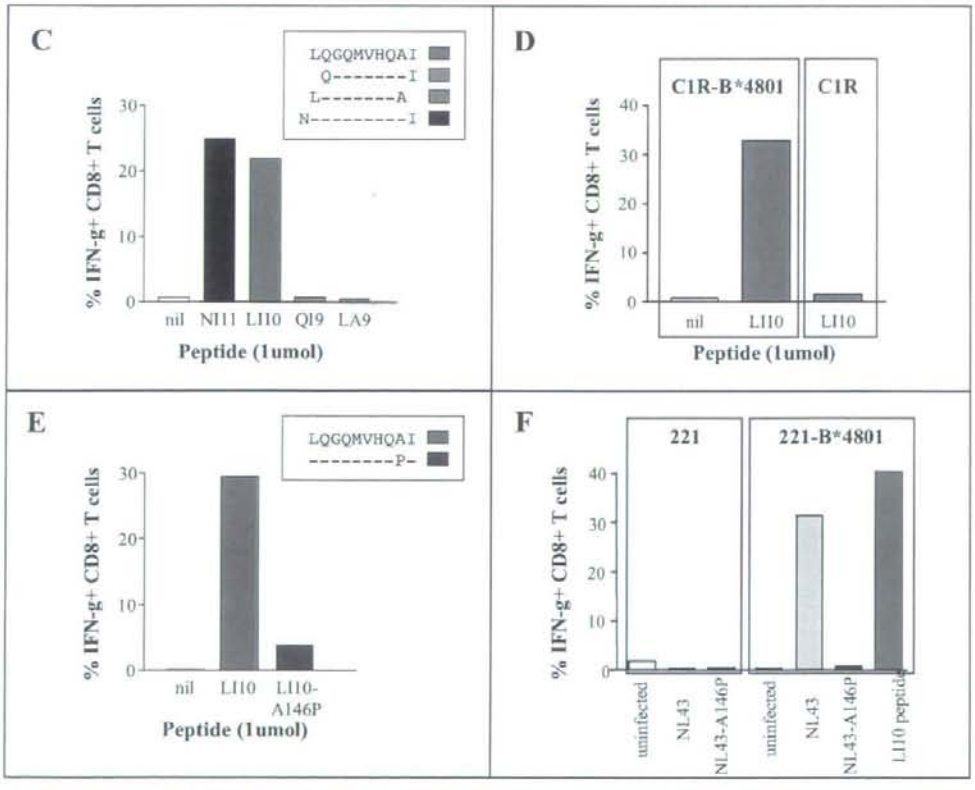

Supplementary Figure 4. Selection of the A146P variant within Gag in B*4801-positive subjects, definition of the HLA-B*4801-restricted LQGQMVHQAI (Gag 138-147)-specific epitope and recognition by LQGQMVHQAI-specific CD8+ T-cells. A. Association between A146P and HLA-B*4801, initially in a first cohort, subsequently demonstrated in a second cohort. **B.** Viral sequencing from an individual B*4801-positive study subject, KI-092. Population sequencing: direct sequencing of the region encoding Gag 138-147 from PCR amplified gag; sequencing of 10 clones per timepoint also shown. **C.** Recognition of peptide LQGQMVHQAI ('LI10') truncations. **D.** B*4801-restriction of LI10-specific CD8+ T-cells. **E.** A146P LI10 variant peptide-pulsed targets are poorly recognised. **F.** Wildtype virus-infected 221-CD4-transfected cells are not recognized by LI10-specific CD8+ T-cells, nor is the A146P viral variant. 221-CD4-transfectants, also transfected with B*4801, infected with wildtype virus are seen, whereas the same targets infected with A146P mutant virus are not.

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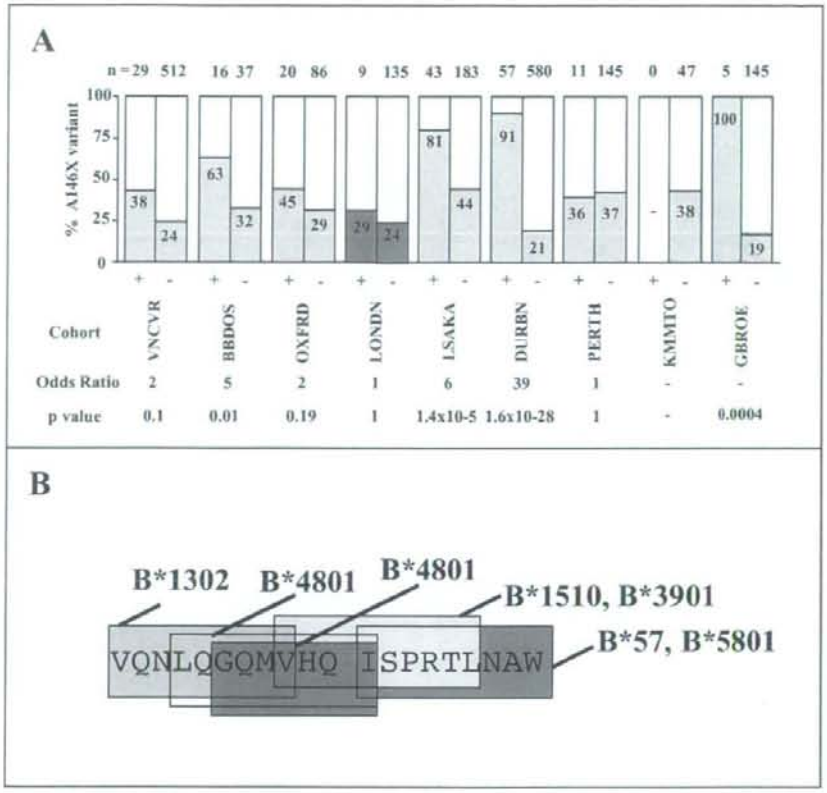

Supplementary Figure 5. Correlation between A146P frequency and HLA-B*57 prevalence is affected by selection of A146P by other alleles, such as HLA-B*4801. A. Frequency of A146X variant in 9 study populations, in HLA-B*57-positive subjects (+) and HLA-B*57-negative subjects (-). Numbers of study subjects, odds ratios and p values as shown for each study cohort. **B.** Clustering of epitopes in the region Gag 135-155 selected by 6 distinct HLA alleles, each of which selects the same A146P mutation. All epitopes are published (http://www.hiv.lanl.gov/content/immunology/) except the B*3901 epitope, predicted on the basis of peptide-binding motif.

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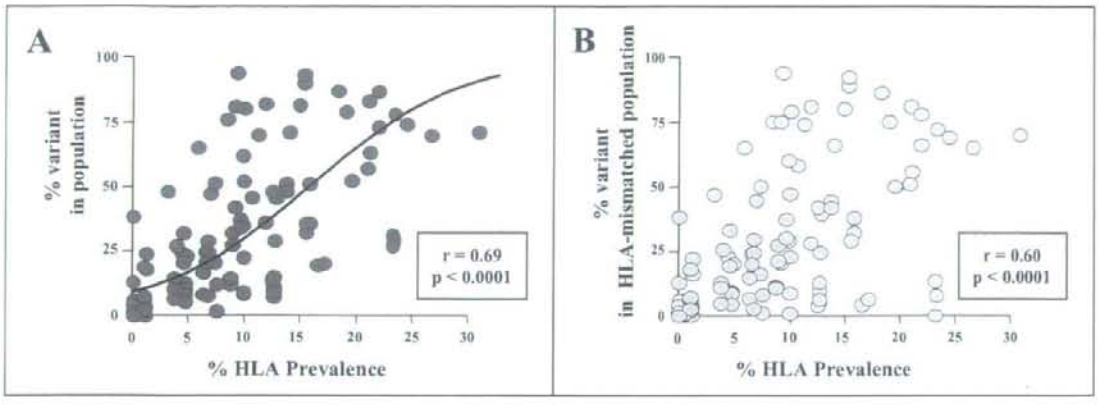

Supplementary Figure 6. Correlation between frequency of variant in population and HLA prevalence for the 14 studied variants. A. Correlation of variant in the population versus HLA prevalence, r value and p value shown from linear regression analysis; from logistic regression analysis, p<0.0001. **B.** Correlation of variant in the HLA-mismatched population versus HLA prevalence, r value and p value shown from linear regression analysis; from logistic regression analysis, p<0.0001.

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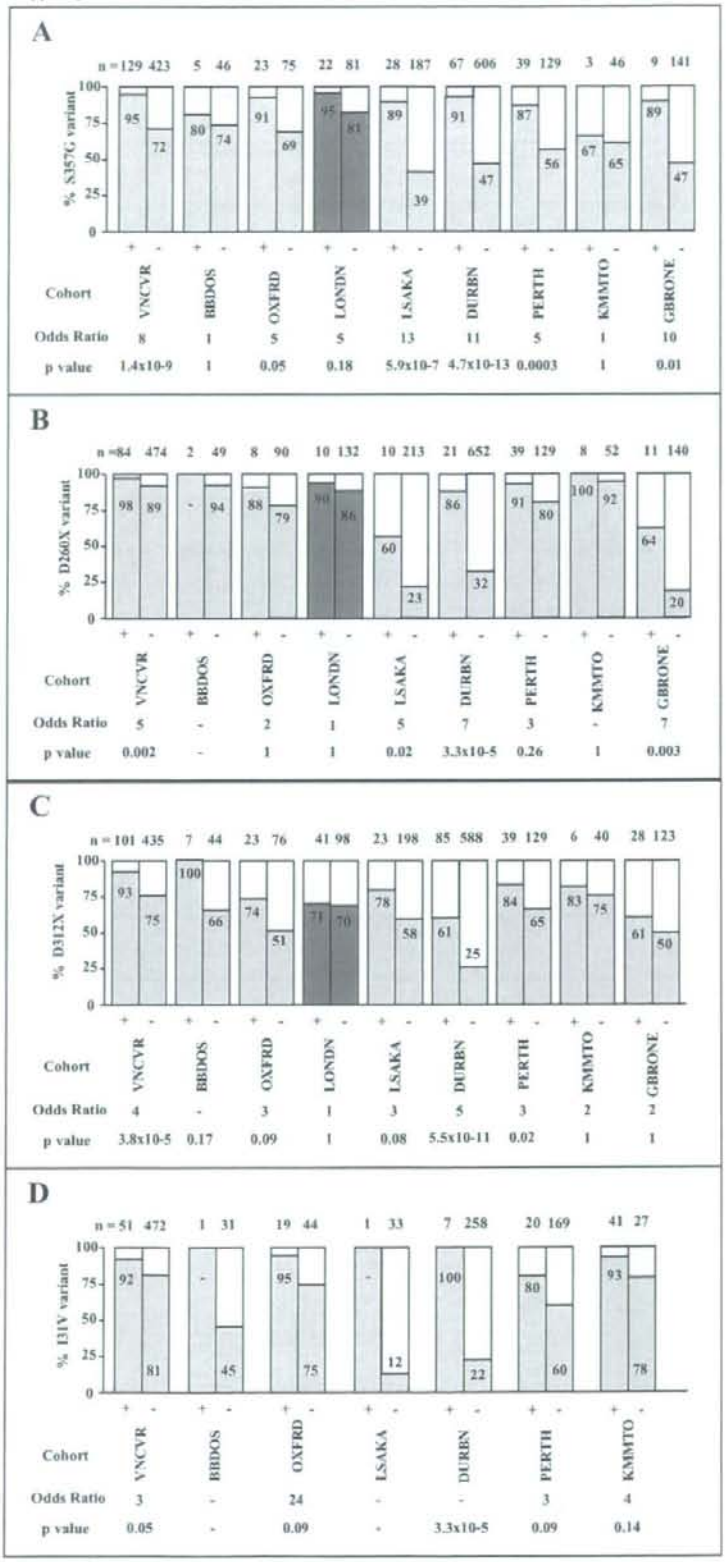
Suppl Fig 7



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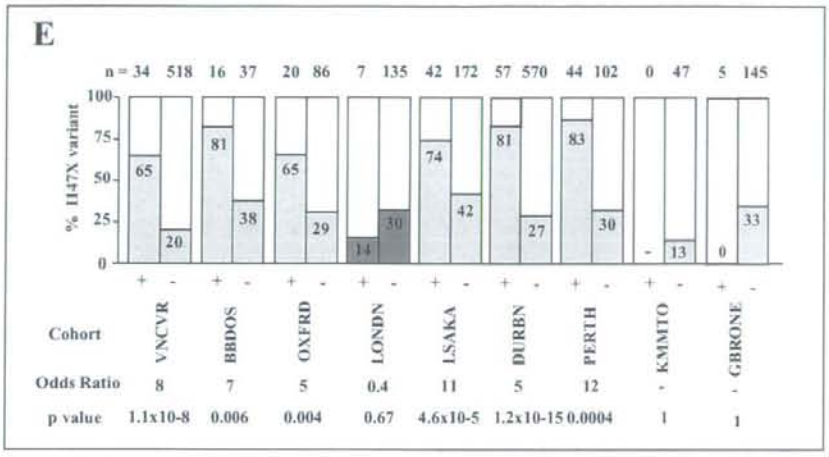
Suppl Fig 7



Supplementary Figure 7. Data as shown in Figure 3 panels A-D, and panel F, but showing numbers of study subjects, percentage expressing the respective mutations, odds ratios and p values, p value not calculated where numbers of subjects expressing the respective HLA allele was <5.