Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703–positive individuals and their transmission recipients

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HLA-B*57 is the class I allele most consistently associated with control of human immunodeficiency virus (HIV) replication, which may be linked to the specific HIV peptides that this allele presents to cytotoxic T lymphocytes (CTLs), and the resulting efficacy of these cellular immune responses. In two HIV C clade–infected populations in South Africa and Zambia, we sought to elucidate the role of HLA-B*5703 in HIV disease outcome. HLA-B*5703–restricted CTL responses select for escape mutations in three Gag p24 epitopes, in a predictable order. We show that the accumulation of these mutations sequentially reduces viral replicative capacity in vitro. Despite this, in vivo data demonstrate that there is ultimately an increase in viral load concomitant with evasion of all three HLA-B*5703–restricted CTL responses. In HLA-B*5703–mismatched recipients, the previously described early benefit of transmitted HLA-B*5703–associated escape mutations is abrogated by the increase in viral load coincident with reversion. Rapid disease progression is observed in HLA-matched recipients to whom mutated virus is transmitted. These data demonstrate that, although costly escape from CTL responses can progressively attenuate the virus, high viral loads develop in the absence of adequate, continued CTL responses. These data underline the need for a CTL vaccine against multiple conserved epitopes.

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Abbreviation used: SGA, single genome amplification.

HLA-B*57 is the class I allele most strongly associated with control of HIV-1 infection (1–3). Because CTLs recognize viral peptides presented by HLA class I molecules on the surface of infected cells, this clear relationship between HLA-B*57 and lower viral loads may be a result of the specific HIV peptides that are displayed by this allele and the potent CTL responses targeting these peptides. It is hypothesized that the CTL responses appearing early in infection are those that most strongly influence clinical outcome (4), and HLA-B*57–restricted CTL responses have been shown to dominate in early HIV infection in individuals with this HLA allele (5). In SIV, Gag-derived CTL epitopes are presented early in the infection cycle of a cell (6), and HLA-B*5703 presents three epitopes in the capsid region of HIV-1 Gag p24: ISW9 (ISPRTLNAW;

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Gag 147–155), KF11 (KAFSPEVIPMF; Gag 162–172), and TW10 (TSTLQEQIAW; Gag 240–249).

In addition to the presentation of multiple Gag epitopes (7), the association of HLA-B*5703 with low viremia is related to the selection of multiple escape mutations within these epitopes (8). These HLA-B*5703–associated mutations reduce viral replicative capacity, as shown either from in vivo reversion to consensus after their transmission to HLA-B*57–negative individuals (8–11) or from in vitro viral growth assays (10, 12, 13). However, despite the decrease in replicative capacity conferred by these mutations, individuals with HLA-B*5703 eventually progress to disease, albeit at a slow rate (14). The cause for the loss of control of virus replication and the concomitant increase in viral load remains unclear.

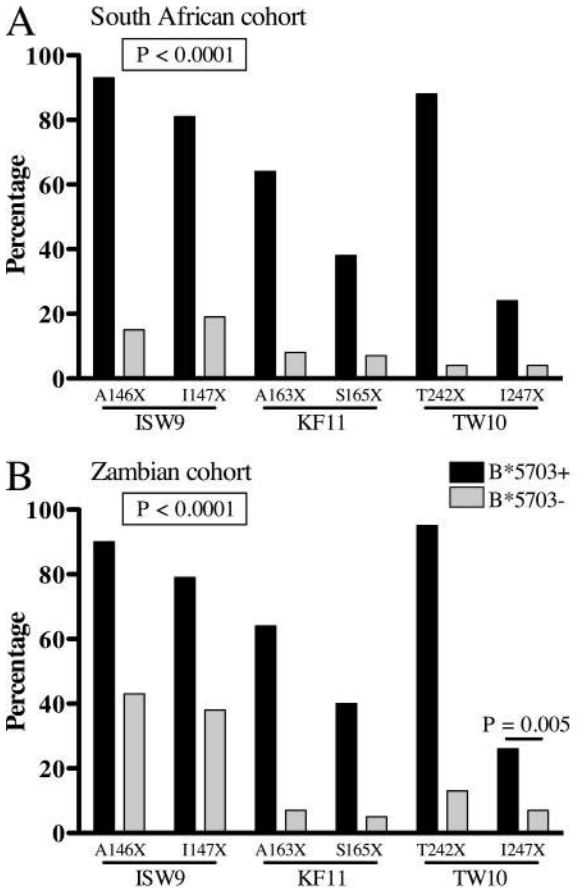
The influence on disease course of mutations selected in an HLA-B*5703 environment can be followed after transmission to HLA-mismatched recipients. Two studies examining HLA-B*5703–negative recipients, followed for up to 1 yr after transmission, have shown an advantage to being infected by a virus with two or more HLA-B*5703–associated mutations, which resulted in lower viral set points (9, 15). However, the long-term effects of infection by low fitness escape mutants have not been examined.

We studied two independent HIV-1 C clade–infected cohorts in Southern Africa, where HLA-B*5703 is the predominant HLA-B*57 allele, with two aims: first, to define more clearly the role of HLA-B*5703 in both control of virus replication and disease progression in HLA-B*5703–positive subjects; and second, to examine the long-term impact of HLA-B*5703 after transmission of a virus containing HLA-B*5703–associated mutations to HLA-matched and -mismatched individuals.

RESULTS

Predictable appearance of HLA-B*5703–associated escape mutations in distinct Southern African cohorts

Previous studies in Durban, South Africa have shown that early HIV-1 infection in HLA-B*5703–positive individuals is characterized by the selection of mutations in two Gag p24 epitopes, ISW9 (ISPRTLNAW; Gag 147–155) and TW10 (TSTLQEQIAW; Gag 240–249), followed by those in KF11 (KAFSPEVIPMF; Gag 162–172) (10). To characterize viral sequence changes occurring over time in Zambian transmission pairs where either the donor or the recipient expressed HLA-B*5703, we first undertook a cross-sectional analysis of viral p24 amino acid sequences generated from 178 HIV-infected (including 42 HLA-B*5703–positive) individuals in Lusaka, Zambia. For the purposes of these studies, the Zambian subjects studied included a high proportion of individuals expressing HLA-B*5703. The results are consistent with those observed in the study population of 645 HIV-infected individuals in Durban, South Africa (Fig. 1, A and B), with mutations in the three associated epitopes exhibiting comparable frequencies in the HLA-B*5703–positive populations in the two cohorts. The reason for the somewhat higher frequencies of A146X and I147X observed in the HLA-B*5703–negative population in Zambia is likely related to the higher prevalence of HLA-B*5703 in Lusaka, Zambia compared with KwaZulu-Natal, South Africa (16). Sequences from all HLA-B*5703–positive individuals in both cohorts contained at least one of these mutations (Fig. 1 C), and we inferred an



C

B*5703-associated mutations			B*5703-positive subjects	
ISW9 Gag 147-155 AISPRTLNAW	TW10 Gag 240-249 TSTLQEQIAW	KF11 Gag 162-172 KAFSPEVIPMF	South Africa (n = 42)	Zambia (n = 42)
			n	n
.x..... x..... xx.....	.....	.....	3	2
.x..... x..... xx.....	..x..... .....x.. ..x....x..	.....	12	13
.x..... x..... xx.....	..x..... ..x....x..	.x..... .x.x.....	27	27

Figure 1. HLA-B*5703–associated polymorphisms in Gag p24 in two independent cohorts. (A and B) Association of polymorphisms within three B*5703-restricted epitopes (ISW9, KF11, and TW10), and the expression of HLA-B*5703 in (A) a South African cohort and (B) a Zambian cohort. All associations were statistically significant ($P < 0.0001$ [$P = 0.005$ for I247X in Zambia] using Fisher's exact test). Individuals expressing HLA-B*5801 were excluded from this analysis, as this allele also selects for escape mutations at residues 146, 147, and 242. (C) Sequence polymorphisms in three p24 HLA-B*5703–restricted epitopes, inferring the order of accumulation, and similar frequencies of these variants in the two cohorts. X represents a change away from HIV-1 C clade consensus.

order of appearance from these cross-sectional data: changes away from consensus were more common in and around ISW9 and in TW10 compared with KF11, and those in ISW9 were present before those in TW10, followed by KF11 (Fig. 1 C). This may be a result of the timing of CTL responses targeting these epitopes, or it may suggest that the later mutations are more costly to the virus and are therefore delayed. It is of interest to note that the order of appearance of mutations in these C-clade HLA-B*5703 African populations differs from that reported for a B-clade HLA-B*5701 Caucasian population, where escape mutations in TW10 were observed first, followed by those in ISW9 and KF11 (17).

HLA-B*5703–restricted p24 CTL responses are vital in the control of HIV-1 infection

The reversion of these individual HLA-B*5703–associated mutations upon transmission to HLA-mismatched recipients signifies the negative impact they have on viral replicative capacity (9–11). We sought to determine the impact not simply of the individual mutations but of the accumulation of these mutations on replicative capacity as they arise in vivo. Therefore, we introduced mutations sequentially into these epitopes in a C-clade recombinant p24 plasmid.

Using site-directed mutagenesis, three mutant viruses were constructed (Fig. 2 A): the first incorporated the A146P and I147L substitutions at positions −1 and +1 of ISW9; the second incorporated the T242N mutation in TW10, in addition to A146P/I147L; and the third added the KF11 mutations A163G and S165N to A146P/I147L/T242N. Jurkat cells were infected in triplicate at a multiplicity of infection of 0.01 with wild-type virus and the three mutant constructs. Viral spread was determined over 8 d by measuring the percentage of GFP-positive cells by flow cytometry. There was a significant decrease in replication as mutations in each HLA-B*5703 epitope were introduced, with very low numbers of infected cells observed in the virus with the complete spectrum of mutations (<1% of wild-type levels). Although we previously showed that S165N acts as a compensatory mutation for A163G in KF11 (10), its presence here cannot compensate for the loss of fitness imposed by mutations in all three epitopes. Thus, in natural infection, HLA-B*5703 CTL responses select for Gag mutations that progressively attenuate the virus.

To assess the impact of these escape mutations on control of viremia in HLA-B*5703–positive subjects, we compared viral loads in 42 chronically infected Zambian subjects (Fig. 2 B). In contrast to viral loads in individuals who had mutations in ISW9 alone or ISW9 and TW10, significantly higher viral loads were observed in those individuals with mutations in all three p24 epitopes ($P = 0.02$ using the Mann-Whitney U test). Data from two chronically infected HLA-B*5703–positive individuals studied, which were the only subjects in whom a mutation in KF11 arose during longitudinal follow up, also suggest that the selection of A163X in KF11 in subjects with a declining viremia may precipitate a higher final viral load (Fig. 2, C and D). Thus, the higher viral loads associated with escape from all three HLA-B*5703–restricted p24 CTL responses arise despite the considerable fitness costs associated with such changes shown from in vitro assays. This fits with data showing that the presence of a KF11 CTL response is associated with a 10-fold lower viral load in HLA-B*5703–positive individuals (7). Collectively, these findings confirm that the advantage to the virus conferred by escaping these CTL responses (the KF11 CTL response in particular) far outweighs the replication costs of the mutations. Other factors that may play a role in the higher viral loads are the length of time of infection and the prior selection of HLA-B*5703–associated compensatory mutations in ±20% of individuals, although no known compensatory mutations developed in these individuals during the time studied.

Early escape in HLA-B*5703–positive individuals

To better understand the relationship of HLA-B*5703 "footprint" mutations with in vivo viral replication, we tracked viral sequence changes in Gag p24 in five acutely infected HLA-B*5703–positive individuals from Zambia for a median time of 17 mo (range = 11–58 mo), and correlated the changes with variations in viral load. Because individuals in this cohort are sampled every 3 mo, the first time point is within 3 mo of transmission, and often the initial peak in viral load is missed. The presence of early CTL responses targeting the HLA-B*5703 epitopes ISW9 and TW10 was suggested by the relatively low median viral load of 10,309 copies/ml even at the first time point after seroconversion, and by the selection of CTL escape mutations (Fig. 3, A–E). Escape in was first detected at a median time after seroconversion of 13 mo (range = 4–18 mo). To determine what effect the escape mutations had on disease course in each individual, we calculated the median value of each individual's viral loads before escape was detected and compared this with the median value of the viral loads after escape (Fig. 3 F). Although escape mutations were being selected in the ISW9 and TW10 epitopes, there was no increase in viral load akin to that seen after the appearance of KF11 mutations (Fig. 3, B–D). This is consistent with a balance being maintained between the persistent wild type–specific immune response or de novo variant–specific responses, and the reduced replicative capacity of the virus. Examining these 5 HLA-B*5703–positive individuals together over 18 mo of infection (Fig. 3 G), we observed lower viral loads compared with the majority of 23 HLA-B*5703–negative subjects with acute infection (who were sampled every 6 mo), whose virus carried no HLA-B*5703 footprints (Fig. 3 H). This finding, in addition to the large difference between the initial viral loads in the HLA-B*5703–positive and –negative subjects (median = ∼13,000 vs. ∼43,000 copies/ml), is consistent with the known early impact of multiple Gag-specific CTL responses restricted by HLA-B*5703.

Early benefit of transmitted HLA-B*5703 escape mutations is abrogated by reversion in HLA-B*5703–negative individuals

Recent studies have found that the transmission of virus containing CTL escape mutations in p24 to HLA-mismatched

individuals resulted in lower viral loads in acute infection (9, 15), but the long-term effects of transmission of these variants on viral load are not known. To examine the longer-term consequences of transmission of HLA-B*5703 escape mutations to HLA-B*5703–negative subjects, we identified 11 Zambian transmission pairs in which the donor was HLA-B*5703 positive, the recipient was HLA-B*5703 negative, and their viruses were epidemiologically linked. Population sequencing of viral RNA from both partners at the time of transmission revealed that, in all cases, the donor transmitted a virus with an HLA-B*5703 footprint (and in six of those pairs for which single genome amplicons were sequenced, it appears that one virus founded the recipient's infection). In two recipients, these HLA-B*5703 mutations were stable during the time studied (7 and 20 mo), and their viral loads decreased (Fig. 4 A). Transmission of less fit variants, as well as early non–HLA-B*5703–restricted CTL responses, may have contributed to the decrease observed. In the nine other HLA-B*5703–negative recipients, studied for a median time of 44 mo (range = 16–62 mo), reversion of HLA-B*5703–associated mutations occurred (Fig. 4, B and C). In one individual (Fig. 4 B), the appearance of wild-type virus at time point four, creating a mixed population, was coincident with a large increase in viral load, to above the assay limit. Sequence data showed that the only change occurring in the HLA-B*5703 p24 epitopes was the reversion of N242T, which was complete by time point five (~7 wk later), after which the individual received antiretroviral treatment (this polymorphism was also the only change occurring in p24). A comparison of the median value of all viral loads for each individual before reversion with the median value of all viral loads after reversion showed an increase in seven out of nine individuals (Fig. 4 D; $P = 0.07$ using the Wilcoxon matched pairs test). In all cases, reversion of T242N in TW10 was observed. Additionally, A146P and/or I147L reverted in three cases. These reversions were detected at a median of 23 mo after seroconversion (range = 7–55 mo).

The increase in viral load, coincident with reversions, in seven out of nine HLA-B*5703–negative recipients (Fig. 4 D) contrasts with the decreases in viral load seen in the two HLA-B*5703–negative recipients in whom reversion did not occur (Fig. 4 A), and with the lack of a consistent trend in the control group (Fig. 3 H). In addition, viral loads for these nine HLA-B*5703–negative individuals over 18 mo (Fig. 4 E) tended to be 10-fold higher than those in the five HLA-B*5703–positive individuals (Fig. 3 G) over the same period of time ($P < 0.0001$ using the Mann-Whitney U test) and continued to increase. It appears that reversion to a fitter phenotype once infection is established can result in a substantial increase in viral load.

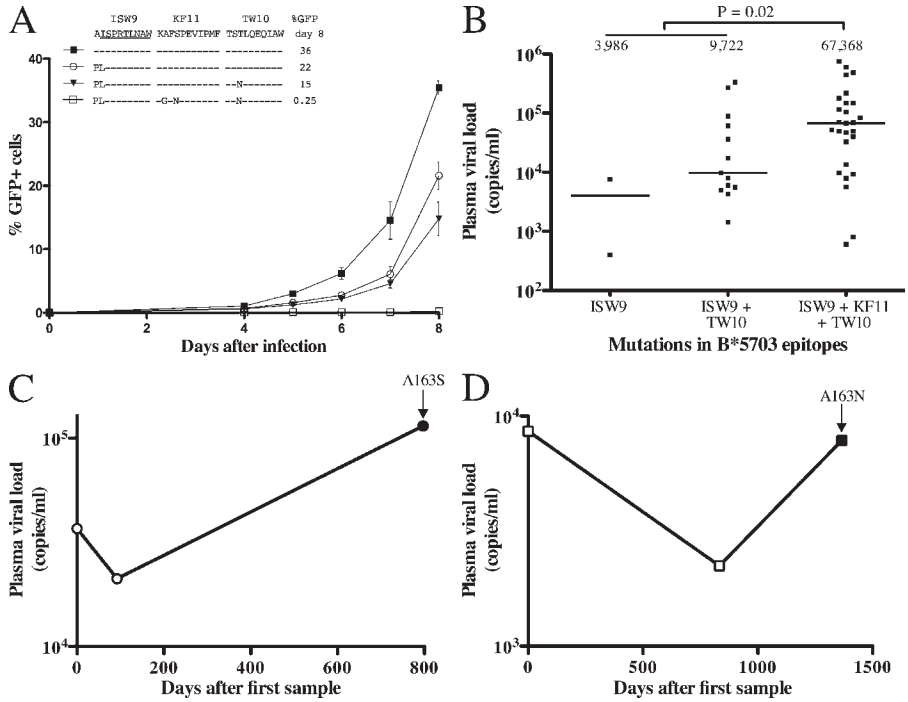


Figure 2. In vitro and in vivo effects of cumulative HLA-B*5703 mutations in HLA-B*5703–positive individuals. (A) Replication kinetics of NL4-3/GFP containing a wild-type C-clade p24 compared with three mutants containing an accumulation of polymorphisms in the three epitopes. Infectivity is expressed as the percentage of GFP-positive Jurkat cells over 8 d after infection. The results are from one experiment that was repeated three times, and means ± SEM are shown. (B) Correlation of escape mutations in HLA-B*5703 epitopes and viral loads in 42 HLA-B*5703–positive Zambian subjects. The median viral load for each group is shown (horizontal bars). (C and D) Plasma viral loads in two chronically infected HLA-B*5703–positive individuals, with an increase in viral load coincident with the appearance of A163X in KF11. Open symbols indicate wild-type epitope in the RNA population sequence; closed symbols indicate the first detection of a mutation of A163.

Reversion of HLA-B*5703–associated mutations dominates genotypic changes after virus transmission to HLA-B*5703–negative recipients

To better evaluate the relative role of reversion of the B*5703-associated mutations compared with acquisition of new mutations selected by the host HLA alleles, we studied the evolution of the transmitted virus from two HLA-B*5703–positive donors to their HLA-B*5703–negative recipients in further detail. Plasma samples from the donor and recipient at the time of the recipient's first positive antibody test were collected, as well as two later samples from the recipient. Fig. 5 shows phylogenetic analyses of single genome amplification (SGA)–derived Gag p24 sequences from these two pairs alongside analyses of the same sequences using the Highlighter program from LANL (available at http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html). The donor and recipient nucleotide sequences are compared with a master sequence, which in this case is the

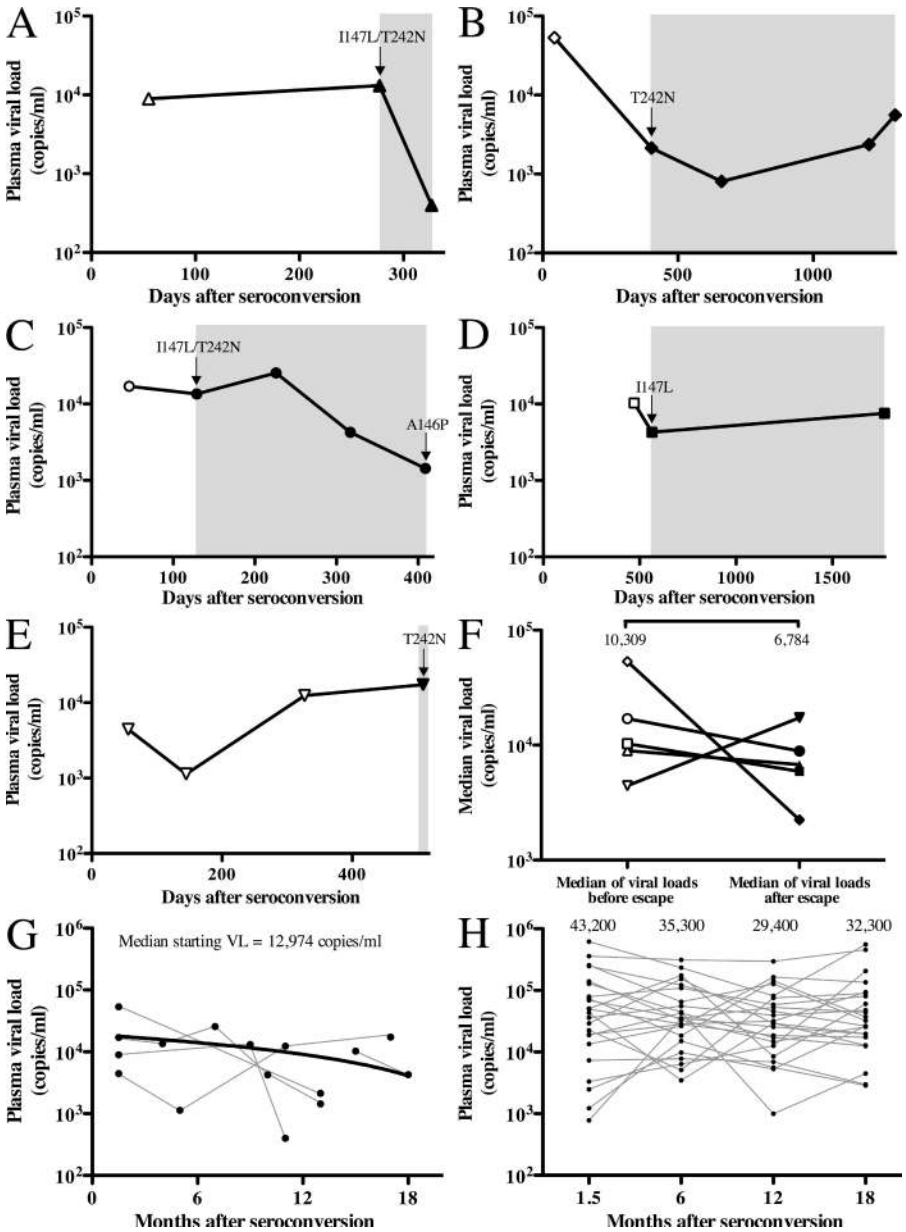


Figure 3. The effect of HLA-B*5703–restricted CTL responses on viral load in HLA-B*5703–positive individuals. (A–E) Plasma viral loads in five acutely infected HLA-B*5703–positive individuals in Zambia. Arrows indicate the time point at which escape was first detected at A146, I147, and T242 in the RNA population sequence. Closed symbols are escaped variants; shaded areas indicate the time period after escape. (F) A comparison of the median viral load for all time points before escape and for all time points after escape in five individuals from A–E. The symbols match those in A–E. (G) All data points from A–E over 18 mo, fitted with a nonlinear regression (line). (H) Plasma viral loads for 23 HLA-B*5703–negative subjects with acute infection, sampled every 6 mo, whose virus carried no HLA-B*5703 footprints. Medians are shown.

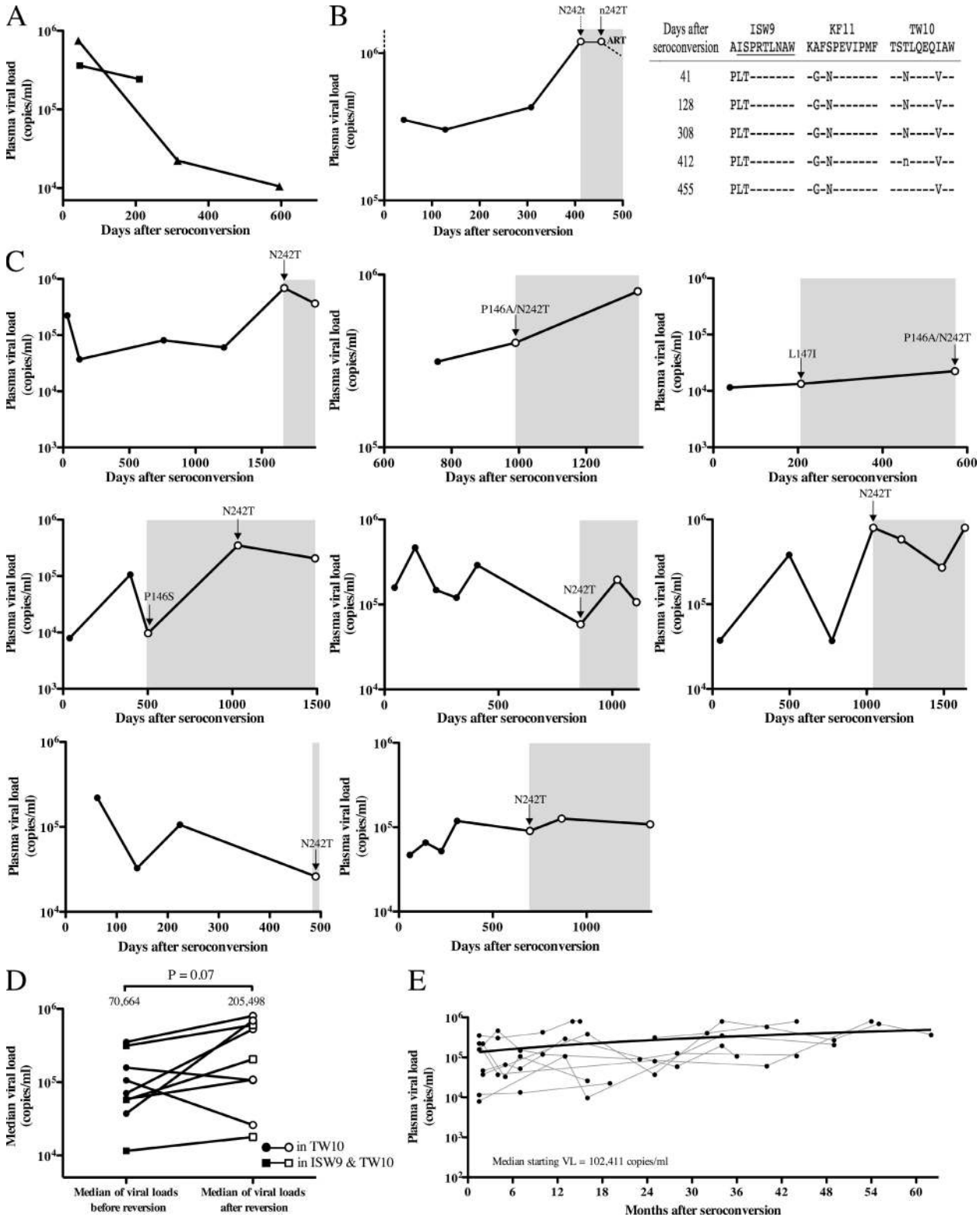


Figure 4. Longitudinal viral loads after transmission of escape mutants to 11 HLA-B*5703–negative individuals. (A) A decrease in viral load is seen in two HLA-B*5703–negative individuals after transmission of virus containing HLA-B*5703–associated mutations, the reversion of which did not occur. (B) Plasma viral load in one HLA-B*5703–negative recipient of virus with mutations in all three p24 epitopes targeted by this allele. Sequence data show the appearance of wild-type virus at day 412, which had outgrown the variant by day 455, and a concomitant rise in viral load. This reflects the only change in p24 in this individual over this time period. A dotted arrow and lowercase residue indicate a mixed population of wild type and variant.

donor's sequence on which the tree is rooted. Both donors transmitted virus containing the ISW9 mutations A146P/I147L, as well as T242N in TW10.

In the first transmission pair (Fig. 5 A), a heterogeneous population of virus in the donor is evident within the neighbor-joining tree, with a maximum of three synonymous and two nonsynonymous changes existing between sequences. At the time of seroconversion (month 0), the recipient's sequences were all identical in p24, with the exception of one synonymous difference between them and the donor's master sequence. At the second time point (15 mo), two nonsynonymous changes were dominant in the recipient's sequences: one was the reversion of A146P to consensus, and the other was a change from V to A, the consensus amino acid for subtype C at Gag residue 215. The significance of the latter reversion is unknown, although Gag-215 is located two amino acids upstream of the cyclophilin A binding loop, and polymorphisms in this loop can compensate for fitness costs resulting from HLA-B*5703–associated mutations (12). At 48 mo after transmission, sequences from the recipient contained four more nonsynonymous changes, two of which were in TW10. This included reversion of T242N. None of the changes were associated with the recipient's HLA alleles (HLA-A*3001/A*3004/B*4501/B*4501/Cw*0602/Cw*16). Overall, of the five nonsynonymous changes that were present in more than half of the recipient's sequences at 48 mo, three were reversions of HLA-B*5703–associated mutations. The viral load of the recipient was initially low (7,941 and 9,695 copies/ml at seroconversion and at 15 mo, respectively), consistent with previous findings (15), but at 48 mo, after reversion of both HLA-B*5703–associated escape mutations to consensus, the viral load rose to 205,498 copies/ml. This illustrative case described in detail shows the impact of reversion and supports the hypothesis that reversion to a more replication-competent virus may contribute to the large increase in viral load.

Similarly, in the recipient of the second transmission pair, reversions of HLA-B*5703–associated mutations were the only nonsynonymous changes occurring over 17 mo (Fig. 5 B). Again, a heterogeneous population was seen in the donor, but all of these nucleotide differences were synonymous changes. The population in the recipient at the time of seroconversion was homogeneous in p24, and 5 mo later, the only change in 8 out of 10 sequences was the reversion of I147L to consensus. Two other sequences at this time point were identical to those at seroconversion. 17 mo after seroconversion, two more nucleotide changes resulted in changes at the amino acid level, and both of them involved reversion of HLA-B*5703–associated mutations (A146P and T242N). The viral load in this recipient also increased marginally on reversion of I147L, from 11,545 to 13,376 copies/ml, and increased again to 22,415 copies/ml at 17 mo, after reversion of the A146P and T242N mutation. These longitudinal analyses of two HLA-B*5703–negative recipients, who were both infected by virus containing HLA-B*5703 escape mutants from their respective donors, show that reversion of HLA-B*5703–associated escape mutants dominates the viral sequence changes within Gag p24. This suggests that these reversion events may be a contributing factor to the subsequent increases in viral load. In addition, the lack of polymorphisms in p24 selected by the recipients' own HLA-B alleles contrasts with the early imprinting that HLA-B*5703 has on the virus, underscoring the strength of HLA-B*5703–restricted immune responses.

Rapid progression after transmission of HLA-B*5703 escape mutants to HLA-matched recipients

Previous studies in relation to HLA-B*27, another HLA allele associated with slow progression in HIV infection (18, 19), have suggested that transmission of viruses encoding escape mutations in the key HLA-B*27 p24 epitope to HLA-B*27–positive subjects may adversely affect the ability of the recipient to achieve successful control of viremia (20). This prompted us to investigate the effect the transmission of a virus containing HLA-B*5703 Gag escape variants would have on disease progression in HLA-matched recipients. We identified two such transmission pairs in Zambia for which samples near the time of seroconversion of the recipient were available. In one pair, Z80, both partners were HLA-B*5703 positive, whereas in the other pair, Z48, the donor was HLA-B*5703 positive and the recipient was B*5801 positive. HLA-B*5801, like HLA-B*5703, is associated with slow progression to AIDS (7, 21), is very similar in its peptide-binding motif to HLA-B*5703 (22), differing by only five amino acids in total (23), and presents two of the three p24 epitopes presented by HLA-B*5703 (i.e., ISW9 and TW10).

Both HLA-B*5703–positive donors transmitted virus containing escape mutations in all three HLA-B*5703 p24 epitopes: A146P/I147L in ISW9, A163N in KF11, and T242N in TW10 (Fig. 6). A polymorphism in the cyclophilin A binding loop, V223X, which is associated with T242N (12), was also transmitted to both recipients.

PBMCs were not available to establish the HIV-specific CTL responses that were made in these subjects. However, on the basis of the transmitted viral sequences, it seems likely that Z80M (the recipient) did not have the same opportunity to generate responses to any of the three HLA-B*5703–restricted epitopes—ISW9, KF11, or TW10—that would have been possible had she been infected with virus encoding the nonmutated epitopes, because the mutations

(C) Plasma viral loads in eight HLA-B*5703–negative recipients. Arrows indicate the time point at which reversion to the wild-type epitope was first detected in the population RNA sequence (e.g., N242T indicates the time point at which the virus had fully reverted from N to T at residue 242, as detected in the RNA population sequence). Shaded areas indicate the time period after reversion. (D) A comparison of the median viral load for all time points before reversion and for all time points after reversion in nine HLA-B*5703–negative individuals from B and C, showing an increase overall ($P = 0.07$ using the Wilcoxon matched pairs test). (E) All data points from B and C over 62 mo, fitted with a nonlinear regression (line).

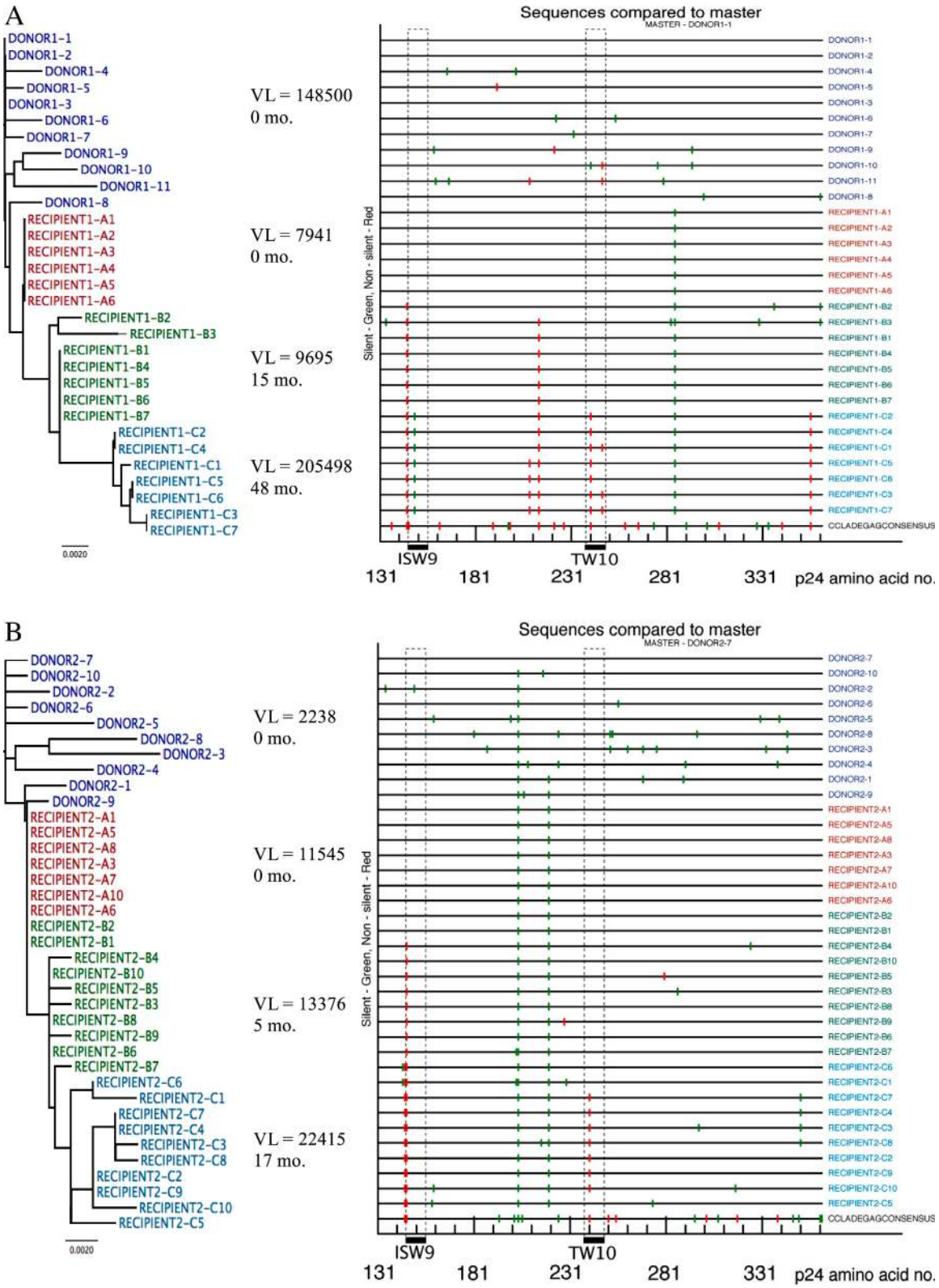


Figure 5. Reversion of Gag p24 HLA-B*5703–associated mutations dominates early infection in HLA-B*5703–negative recipients. (A and B) Neighbor-joining trees of SGA-derived p24 sequences (231 amino acids) from (A) transmission pair 1 and (B) transmission pair 2. Both donors are HLA-B*5703 positive, and both recipients are HLA-B*5703–negative. Trees are rooted to the first donor sequence. (A) Donor 1 transmitted a virus containing polymorphisms in all three Gag p24 B*57 epitopes: A146P/I147L, A163N, and T242N. Clones from recipient 1 from time points A (day of seroconversion),

observed in the ISW9 and KF11 epitopes affect peptide processing and epitope presentation (24, 25). The presence of an additional mutation, A248T, in the Z80M sequence subsequent to transmission within the TW10 epitope may suggest that either a CTL response was generated to the transmitted T242N mutant epitope, as has been previously described in HLA-B*5703/B*5801–positive infants born to infected mothers (26), or that this represents selection of a compensatory mutation to T242N, as previously described (12). It is notable that within 11 mo of seroconversion, this recipient's viral load had reached a level similar to the median viral load of the chronically infected HLA-B*5703–positive individuals with mutations in all three epitopes (Fig. 3 B). This suggests that the HLA-B*5703 allele was doing little to protect against a relatively rapid increase in viral load. It is uncertain whether these events are causally linked to clinical outcome, as this individual died from chronic gastroenteritis <14 mo after seroconversion (CD4 cell counts are unavailable).

An unexpectedly rapid disease course for an individual expressing the HLA-B*5801 allele was also observed in the recipient Z48F (Fig. 6 B). Viral set point at 4 mo after transmission was extremely high at 153,451 copies/ml. As in recipient Z80M, this HLA-B*5801–positive recipient received a virus with the ISW9 and TW10 epitopes mutated, and it is therefore probable that optimal responses to wild-type ISW9 and TW10 epitopes could not have been generated. The reversion of A146P to consensus in this recipient's virus supports the prediction that no ISW9-specific response was made. The combined observations at 17 mo after transmission of the compensatory mutation H219Q (11, 12), reversion of A146P, and an increase in viral load (from 153,451 to 613,919 copies/ml in just 13 mo) are consistent with an increased viral replicative capacity. This rapid increase in viral load would not normally be associated with an individual carrying HLA-B*5801 (2) at only 17 mo after infection.

Collectively, the final viral loads for these two individuals are substantially higher than the viral loads observed in five HLA-B*5703–positive individuals (Fig. 3, A–E) measured at a similar time after infection who did not receive escape variants (<400–17,351 copies/ml; median = 2,131 copies/ml). Thus, the unfavorable outcomes in the two recipients (Z80F and Z48M) appear to be linked, albeit anecdotally, to the inaccessibility of p24 CTL epitopes on which the association with immune control of HIV infection may be likely to hinge (8).

DISCUSSION

In this paper, we have investigated the role of HLA-B*5703–restricted Gag CTL responses and the consequent mutations selected to evade these responses in two distinct HIV-1 C clade–infected populations. Both HLA-B*5703–positive donors and their HLA-matched and -mismatched transmission recipients were analyzed. Broad CTL responses that target epitopes in Gag p24 are associated with decreased viral loads (7, 27), and HLA-B*5703 CTLs target three peptides in this conserved region. We show that the escape polymorphisms are selected in similar frequencies in these p24 epitopes in HLA-B*5703–positive individuals in both Zambia and South Africa, where this HLA allele is associated with lower viral loads (2, 21). We also noted that the order of HIV escape in C clade differed from that in HIV-1 B clade–infected individuals with the B*5701 allele, suggesting that mutational pathways may be B*57 allele or clade specific. Although sequential addition of the observed HLA-B*5703–associated mutations in the three Gag p24 epitopes results in an incremental reduction in viral fitness in vitro, changes in all three epitopes are found in the majority (64%; 54 out of 84) of chronically infected HLA-B*5703–positive individuals. Indeed, higher viral loads were observed in HLA-B*5703–positive individuals with mutations in all three Gag p24 epitopes in the absence of any consistent compensatory mutations. This does not reflect the malleability of p24, because it is a highly conserved region (28), but rather the highly effective nature of HLA-B*5703–restricted immune responses in selecting escape mutations. Despite the lack of actual CTL data, this strong pressure is evident in the early appearance of escape mutations (ISW9 mutations were observed in all B*5703 individuals at the seroconversion time point, and TW10 mutations were first detected here at 4–18 mo after seroconversion; median = 13 mo) compared with the relatively slower reversion rate (7–55 mo; median = 23 mo), as well as a 10-fold lower median viral load in acute infection in HLA-B*5703–positive compared with HLA-B*5703–negative individuals. This is an observation consistent with other studies showing that, first, HLA-B*57–restricted CTL responses are effective and large in magnitude (5, 29), and second, that compensatory mutations are sometimes selected to help minimize this cost (12). Thus, these studies suggest that although the fitness costs associated with HLA-B*5703–driven Gag escape mutations may contribute to successful containment of HIV in HLA-B*5703–positive subjects, durable control of viremia depends also on the maintenance of effective CTL responses.

These data are therefore consistent with recent SIV studies in the Burmese macaque model showing, first, that multiple Gag-specific CTL responses can be responsible for control of viral replication but that the accumulation of CTL mutations in each of the epitopes can result in viral evasion from this control (30), and second, that although the sequential

B (15 mo), and C (48 mo) are shown. The Highlighter analysis, in which green indicates a synonymous nucleotide change and red indicates a nonsynonymous change, shows that the predominant amino acid changes in the recipient sequences are reversions of B*5703–associated polymorphisms. Epitopes ISW9 and TW10 are boxed. (B) Similarly, donor 2 transmitted a virus containing A146P, I147L, and T242N. Clones from recipient 2 from time points A (day of seroconversion), B (5 mo), and C (17 mo) show that reversions of A146P, I147L, and T242N are the only nonsynonymous changes in Gag p24.

addition of escape mutations substantially reduced in vitro viral fitness, those same viruses encoding multiple Gag mutations could still replicate to high levels and result in progression in vivo (31).

Nevertheless, despite the high viral loads seen in chronically infected HLA-B*5703–positive individuals, the median viral load of this subgroup of the population (~67,000 copies/ml) remains lower than that seen in the rest of the population (~110,000 copies/ml), showing that carriage of this HLA allele is beneficial despite the frequent selection of escape mutations and evasion of the cellular immune response. In addition, because of high reversion rates, HLA-B*5703 epitopes are likely to persist throughout the epidemic, providing stable targets for individuals with this allele.

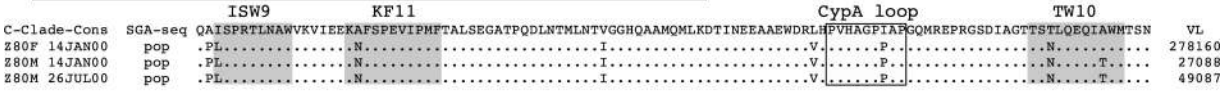
The second aspect of the data presented in this paper addressed the impact of HLA-B*5703–associated mutations on disease outcome in HLA-matched and -mismatched transmission recipients. Two recent studies reported lower viral set points in HLA-B*5703–negative recipients who were infected by virus with two or more HLA-B*5703–associated mutations (9, 15). Our data suggest that this benefit may only be transient in HLA-B*5703–negative recipients, because reversion of HLA-B*5703–associated mutations coincides with an overall increase in median viral load. Although viral load might be expected to increase in the natural disease course in these HIV-infected individuals, studied for between 16 and 62 mo, this observation contrasts with the lack of change in viral load coincident with epitope escape and CTL responses in the HLA-B*5703–positive individuals, studied for a similar length of time (11–58 mo), and a stable viral set point seen in a control group under no influence of HLA-B*5703. Detailed analysis of two HLA-B*5703–negative recipients revealed the extent to which reversion of these mutations dominates the genotypic changes in early infection. These data are consistent with previously published findings in other HLA types that reversion is prevalent in early infection (32). The observation that reversion is the main driving force of p24 evolution in these HLA-B*5703–negative recipients contrasts with the changes in p24 selected by HLA-B*5703 CTL responses dominating early infection in HLA-B*5703–positive individuals. Although early viremia may be influenced significantly by the escape mutations transmitted (9, 15), the findings presented in this paper suggest that long-term control of viremia ultimately rests more on the host's own HLA class I alleles and the CTLs generated.

The importance of HLA-B*5703–restricted CTLs is again suggested by the observation of rapid progression in two HLA-B*57/5801–positive recipients of virus containing HLA-B*5703–associated mutations in Gag (death at 14 mo after transmission in one case, and a viral load of >600,000 copies/ml by 17 mo after transmission in the second). Previous mother-to-child transmission studies involving HLA-B*27–positive infants support the hypothesis that transmission of viruses encoding escape mutations in the key Gag epitopes can dislocate the association between a protective MHC class I allele and control of immunodeficiency virus infection (20). A recent study of Mamu-A*90120-1a–positive Burmese macaques addresses this issue more directly (33). These macaques were previously vaccinated with a DNA prime/Gag-expressing Sendai virus vector boost and contained viral replication after a SIVmac239 challenge (34). However, when the vaccinated macaques were infected with virus containing multiple Gag CTL mutations that had been selected in other Mamu-A*90120-1a–positive macaques, they generated small or no CTL responses to these epitopes, and failed to control viral replication despite the variants having a lower replicative ability compared with the wild type (33). This emphasizes the central role of Gag-specific CTL responses in the early containment of viral replication and suggests that the benefit associated with certain HLA alleles may be lost in individuals who are infected by virus from HLA-matched donors.

These results initially appear to be in contrast with a recent case study of a single HLA-B*5703–matched transmission pair (35) that reported sustained, excellent virological control in the recipient despite the apparent transmission of

A

Partner	Sex	Status	HLA-B	First positive Ab test
Z80F	Female	Donor	0702/5703	
Z80M	Male	Linked recipient	5301/5703	11 September 1999



B

Partner	Sex	Status	HLA-B	First positive Ab test
Z48M	Male	Donor	5301/5703	
Z48F	Female	Linked recipient	4201/5801	19 May 1999

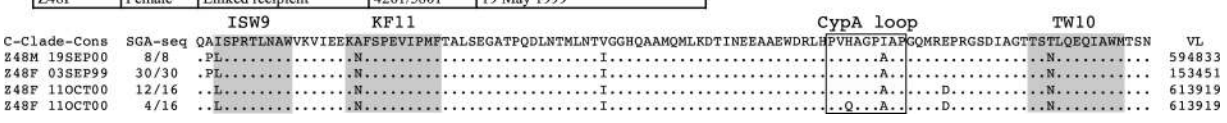


Figure 6. Transmission of virus containing HLA-B*5703–associated mutations from an HLA-B*5703–positive donor to HLA-B*5703– or HLA-B*5801–positive recipients. (A and B) Gag p24 sequences (residues 145–253) from (A) an HLA-B*5703–positive female donor (Z80F) aligned with sequences from the HLA-B*5703–positive male recipient, and (B) an HLA-B*5703–positive male donor (Z48M) aligned with sequences from the HLA-B*5801–positive female recipient. HLA-B*5703 epitopes are highlighted, and the cyclophilin A binding loop is boxed. Viral loads are indicated. Ab, anti–HIV-1 antibody; Pop., population RNA sequences; SGA seq., sequences derived from SGA from viral RNA.

an HLA-B*5703 escape mutant. However, in this case, the recipient also carried the HLA-B*27 allele and mounted a large CTL response toward the p24 epitope KK10, which is associated with long-term control of viremia (19). Furthermore, the HLA-B*5703–associated mutations may have been selected by the recipient's own potent CTL responses early in infection, as the sequences were obtained at least 5 yr after the likely transmission event.

In summary, these analyses of the evolution and transmission of HLA-B*5703–associated CTL mutations underline the importance of CTL responses in the control of HIV replication in both HLA-B*5703–positive and –negative individuals. The presence of escape mutations that reduce viral replicative capacity is not sufficient to provide long-term protection against HIV disease progression in HLA-B*5703–positive individuals, because the virus can still replicate to high levels in the absence of functional CTLs. In HLA-mismatched recipients, the reversion of these escape mutants, together with increases in viremia, suggest a limited long-term benefit of these mutations unless these individuals, too, can generate effective CTLs against the virus. Further, the unfavorable outcome in HLA-matched recipients of virus escaped in all three HLA-B*5703 p24 epitopes is likely linked to their inability to mount effective CTL responses against these key epitopes. Therefore, although mutations that affect fitness can assist in decreasing viral replicative capacity, in the absence of continued CTL pressure, it is likely that disease progression will occur. Thus, for an HIV vaccine to be truly effective in the long term as opposed to the short term, it will need to stimulate broad CTL responses against multiple epitopes that will continue to control viremia in the face of sequential escape.

MATERIALS AND METHODS

Study cohorts. The South African cohort consisted of 645 HIV C clade–infected patients recruited from clinics in Durban, South Africa (2, 7). The 178 HIV-1 C clade chronically infected Zambian subjects were selected from the Zambia-Emory HIV Research Project cohort of HIV-1 serodiscordant cohabiting heterosexual couples. This cohort was established in 1994 in Lusaka, Zambia to provide voluntary HIV testing, counseling, health care, and follow up (36). HIV-negative partners are monitored for antibody seroconversion at 3-mo intervals, and epidemiological linkage of the viruses in subsequent HIV-1 transmission pairs (8.5% of couples per year) is confirmed by phylogenetic analyses of *env* sequences (37). Plasma viral loads for all subjects were determined using the Amplicor HIV-1 Monitor Test (version 1.5; Roche). HLA class I typing was performed as previously described (38, 39). The patients in both cohorts were antiretroviral therapy naive, and all gave written informed consent. All research protocols were approved by the ethics committees in Durban, South Africa and Lusaka, Zambia, and by the Oxford University and Emory University Institutional Review Boards.

Isolation, amplification, and sequencing of HIV-1 proviral DNA and viral RNA. Genomic DNA from South African samples was extracted from PBMCs, amplified, and sequenced, as previously described (11). Viral RNA from Zambian samples was extracted using a robotic system with the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). Amplification and population sequencing of Gag was performed as previously described (15); for SGA and sequencing, we used previously described methods (40), but with the *gag*-specific primers from the population sequencing (15). Sequences were analyzed using Sequencher 4.8 (Gene Codes Corp.)

Site-directed mutagenesis. The plasmid p83-2 encoding the 5′ genomic half of HIV-1 strain NL4-3 (41), substituted with the p24 capsid coding region from a C clade–infected child (13), was modified using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). This p24 region encoded the mutations A146P, I147L, and S252N. First, the ISW9 (A146P/I147L) mutant virus was constructed by reverting S252N using 5′-GCATGGATGACAAGTAATCCACCTATCCCAGTAGG-3′ (HXB2 nt 5,044). The wild-type virus was then constructed by reverting A146P and I147L using 5′-GGGGCAAATGGTACATCAGGCCATATCACCTAGAAC-3′ (nt 1,225 and 1,228). The ISW9/TW10 mutant virus was constructed by introducing T242N to the ISW9 mutant using 5′-GTGACATAGCAGGAACTACTAGTAACCTTCAGGAAC-3′ (nt 5,014), and A163G and S165N were introduced to this plasmid to construct the ISW9/TW10/KF11 mutant using 5′-AGTAGAAGAGAAGGGTTTCAACCAGAAGTA-3′ (nt 1,277 and 1,283). Mutagenesis sites are underlined. The mutants were verified by sequencing after each step, and were subcloned into new p83-2 plasmids using the unique restriction sites SapI (nt 1,098) and ApaI (nt 2,005) to avoid carryover of any unwanted mutations in the mutagenized plasmid. The p24 coding region sequences for each mutant were verified before the generation of viral stocks.

Generation of infectious viral stocks. Viral stocks were generated in Jurkat cells using previously published methods (13). In brief, the plasmid carrying the 5′ half of the genome (p83-2 and constructed mutants) and plasmid p83-10 (41), carrying the 3′ half of the genome of HIV-1 NL4-3 (which also encoded the GFP in frame with Nef), were linearized using Eco RI and electroporated into Jurkat cells. Cells were grown for 7 d, at which time the supernatants were collected. The 50% tissue culture infective dose was determined for each viral stock using the Spearman-Karber method.

Viral replication assays. 7.5×10^6 Jurkat cells were infected at a multiplicity of infection of 0.01, incubated at 37°C for 2 h, washed twice, and resuspended in 15 ml R10 (RPMI 1640 [Sigma-Aldrich], 10% fetal calf serum, 10% L-glutamate, and 10% penicillin/streptomycin). Cultures were divided into 3 wells of 2.5×10^6 cells each and incubated at 37°C. 200 µl of culture was collected every day for 8 d, and the cells were fixed in 2% formaldehyde. The percentage of GFP-expressing cells was measured by FACS analysis using a flow cytometer (FACSCalibur; BD) and analyzed using FlowJo software (version 8.6.3; Tree Star, Inc.). Experiments were performed in triplicate.

Statistical analysis. The analysis of polymorphisms associated with HLA-B*5703 was undertaken using Fisher's exact test (Fig. 1, A and B). The Wilcoxon matched pairs test was used to compare the two paired groups in Figs. 3 F and 4 D. Median plasma viral loads in Fig. 2 B were analyzed using the Mann-Whitney U test, and nonlinear regressions were fitted in Figs. 3 G and 4 E. All statistical analyses were undertaken using Prism (version 5.0; GraphPad Software, Inc.).

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