Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA

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Human immunodeficiency virus (HIV)–1 amino acid sequence polymorphisms associated with expression of specific human histocompatibility leukocyte antigen (HLA) class I alleles suggest sites of cytotoxic T lymphocyte (CTL)–mediated selection pressure and immune escape. The associations most frequently observed are between expression of an HLA class I molecule and variation from the consensus sequence. However, a substantial number of sites have been identified in which particular HLA class I allele expression is associated with preservation of the consensus sequence. The mechanism behind this is so far unexplained. The current studies, focusing on two examples of "negatively associated" or apparently preserved epitopes, suggest an explanation for this phenomenon: negative associations can arise as a result of positive selection of an escape mutation, which is stable on transmission and therefore accumulates in the population to the point at which it defines the consensus sequence. Such negative associations may only be in evidence transiently, because the statistical power to detect them diminishes as the mutations accumulate. If an escape variant reaches fixation in the population, the epitope will be lost as a potential target to the immune system. These data help to explain how HIV is evolving at a population level. Understanding the direction of HIV evolution has important implications for vaccine development.

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Abbreviations used: BCL, B-lymphoblastoid cell line; ICS, intracellular cytokine staining; MTCT, mother-to-child transmission.

It is well established that CTLs exert a strong positive selection pressure on HIV, resulting in the appearance of escape mutations that allow the virus to evade these responses (1–7). CTLs are HLA class I restricted, as they only recognize their cognate peptide when it is presented in the context of the appropriate HLA molecule. A recent study by Moore et al. (3) exploited this property to show, via the identification of associations between particular HLA alleles and sequence polymorphism within the RT region, that escape mutation in HIV is common and restricted by a wide array of different HLA

molecules. Unexpectedly, 25 "negative associations" were also identified, linking the presence of a particular allele with preservation of the consensus sequence. This phenomenon was termed "negative selection pressure," to describe selection pressure that favored the conservation of WT virus in vivo (3). One suggested mechanism by which negative associations arise is through selection for the preservation of epitopes targeted by ineffective CTLs, whose presence is favored by the virus (8). An alternative hypothesis is that sites of negative selection in HIV represent residues in

which common HLA types have already selected a series of "optimized" mutations by passage through many infected individuals of the same HLA type (8). In this scenario, the consensus sequence would represent adaptation to high frequency alleles, with the positively selected escape mutations that are driven by those alleles embedded within it (3). Indeed, this mechanism has been proposed to contribute to clade-specific HIV-1 sequence differences (9).

To better understand the consequences of selection pressure exerted on HIV by CTLs, we have focused on three alleles, HLA-B*57, B*5801, and B*51, which are associated with effective suppression of viremia (10–15) and therefore likely to impose strong selection pressure on the virus. HLA-B*57 and B*5801 are closely related, targeting many of the same epitopes (16) and selecting for the same escape mutations (17). Through large-scale population sequencing of both C-clade and B-clade HIV, we identified two examples of negative associations: one associated with HLA-B*57/5801 and the other with HLA-B*51. We describe the process by which these phenomena arose and discuss their implications with regard to the evolutionary fate of CTL epitopes in HIV-1 infection.

RESULTS

HLA–B*57/5801 is associated with the conservation Nef residue 83

We initially analyzed proviral DNA sequences from 117 HIV-1 C-clade–infected study subjects recruited from Durban, South Africa, to seek sequence polymorphisms that were linked with expression of either HLA-B*57 or B*5801. The phenotypic frequencies of HLA-B*57 and of B*5801 in the Durban population are 6.4 and 10.1%, respectively. To increase the likelihood of identifying HLA-B*57/5801-associated sequence polymorphisms, a proportionately greater number of subjects expressing HLA-B*57 or B*5801 (together, 46 out of 117, 39%) were included in the study group. We initially focused on HIV-1 Nef, one of the most immunogenic regions of the HIV-1 proteome (18, 19). The sequence polymorphisms we identified in association with expression of HLA-B*57/5801 included a single strong association between HLA-B*57/5801 and conservation of the consensus sequence Gly, representing both the local consensus, generated from all proviral DNA and RNA sequences, and the Los Alamos HIV database C-clade consensus (http://www.hiv.lanl.gov) at residue 83 (Table I and Fig. 1 A). From 12 out of 18 (67%) HLA-B57+ individuals and 27 out of 28 (96%) HLA-B*5801+ individuals, we isolated proviral DNA sequences encoding the consensus Gly at residue 83 in Nef, compared with 25 out of the 71 (35%) HLA-B*57/5801− individuals sequenced (P = 1.4 × 10⁻⁷). The majority of viruses not encoding Gly at residue 83 instead expressed Ala.

As the function of HLA class I molecules is to present peptides on the cell surface for recognition by CTLs, we hypothesized that the association between HLA-B*57/

Table I. HLA-B57/5801 is associated with conservation of the C-clade consensus at residue 83 in Nef

	80 90	
Consensus	RPMTYKGAFDLSFFLKEKG	
HLA-B57 n = 18		
	`--------------------`	44%
	`-----------G-------`	17%
	`------A------------`	11%
	`-----QA------------`	6%
	`--------V----------`	6%
	`------Y-V----------`	6%
	`------E------------`	6%
HLA-B5801 n = 28		
	`--------------------`	75%
	`-----------G-------`	11%
	`----------IG-------`	4%
	`--------L--G-------`	4%
	`--------L---H------`	4%
	`------A------------`	4%
Non-B57/5801 n = 71		
	`------A------------`	34%
	`--------------------`	21%
	`------A----G-------`	20%
	`-----------G-------`	10%
	`------A-V----------`	6%
	`--------V----------`	4%
	`------A-V--H-------`	1%
	`------E------------`	1%
	`------A-I----------`	1%
	`------A---IG-------`	1%

5801 and Nef-83-Gly was linked to CTL activity. However, Nef-83 does not lie within any published HLA-B*57/5801-restricted epitope. Therefore, we used the BIMAS epitope prediction software (http://bimas.cit.nih.gov/molbio/hla_bind) to identify 8–11-mer peptides in this region capable of binding HLA-B*57 and B*5801, and this revealed a candidate epitope in the 9-mer KGAFDLSFF (KF9, Nef residues 82–90). Residue 83 lies at position 2 within this putative epitope, which is a primary anchor position for HLA-B*57/5801. However, according to elution data, the preferred residues at this position for HLA-B*57/5801 are Ala, Thr, and Ser, rather than Gly (16). Indeed, using the same software, KF9 with Ala at position 2 (KAAFDLSFF) has a 10-fold higher binding score than KGAFDLSFF, the putative epitope derived from the consensus sequence. This suggested a mechanism by which the observed "negative HLA association" might have arisen: that residue 83 lies within the HLA-B*57/5801-restricted epitope, KAAFDLSFF, and positive selection pressure, exerted through recognition by KF9-specific CTLs, induces escape mutation at this residue, from the WT Ala to Gly.
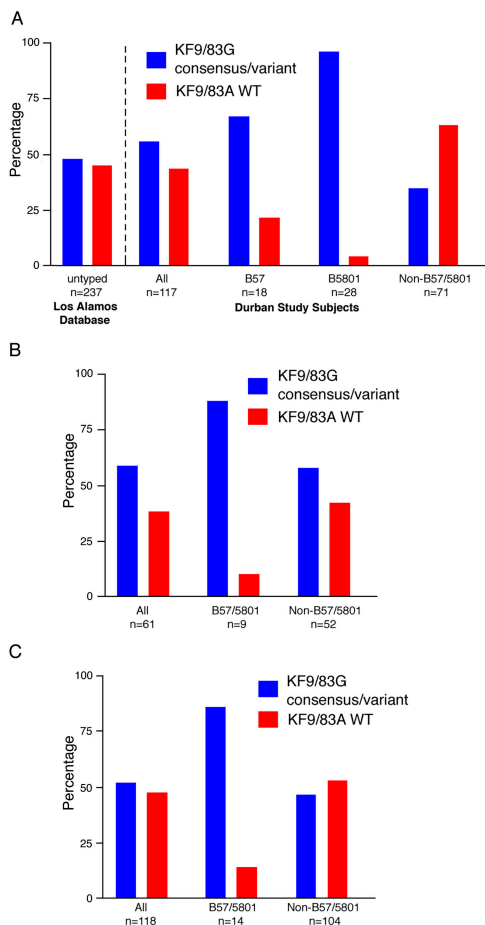
Figure 1. Selection pressure exerted on residue Nef 83 by HLA–B57/5801. (A) Association between the expression of HLA-B57/5801 and conservation of the C-clade consensus AA (Gly). Sequencing of Durban subjects done by population sequencing of proviral DNA. (B) Sequencing of viral RNA from Durban cohort reveals the same association. (C) The same association revealed in RNA sequence data from a separate, B-clade–infected cohort.

Table II. A83G mutation is selected for by HLA-B57/5801 and is stable on transmission

Consensus	B57/5801	Timepoint[a]		KAAFDLSFF	No. of clones
J120 Mother	−	6 yr	DNA	---------	pop. seq.[b]
J120 Child	+			-G-------	
J3 Mother	−	unknown	DNA	---------	pop. seq.
J3 Child	+			-G-------	
J143 Mother	−	4 yr	DNA	---------	pop. seq.
J143 Child	+			-G-------	
S30 Mother	−	6 wk		----G----	12/12
		3 mo	RNA	----G----	9/9
		6 mo		----G----	19/19
		9 mo		----G----	11/11
S30 Child	+	9 mo	RNA	-G--G----	10/10
6007 Man	+	unknown	DNA	-G-------	pop. seq.
6008 Woman	−			-G-------	
1048 Mother	−		RNA	-G-V-----	
1048 Child	−	6 wk	DNA	-G-V-----	pop. seq.
		2 yr		-G-V-----	
1060 Mother	−		RNA	-G-------	
1060 Child	−	1 yr	DNA	-G-------	pop. seq.
		2 yr		-G-------	
1043 Mother	+	unknown	DNA	-G-------	pop. seq.
1043 Child	+			-G-------	

[a]Timepoint postpartum/posttransmission.
[b]Population sequencing.

Positive selection for the A→G mutation at Nef–83 mediated by HLA-B*57/5801

We first sought to confirm our data by sequencing viral RNA from an additional 61 individuals of the same cohort. 8 out of 9 HLA–B*57/5801⁺ individuals expressed a variant from Nef-83-Ala compared with 30 out of 52 individuals lacking these alleles (Fig. 1 B), confirming the association between HLA-B*57/5801 and sequence polymorphism at residue 83. These sequences were from RNA, showing the association holds true for both circulating virus and proviral DNA. However, in this small cohort the differences were not significant (P = 0.074). The samples used were chosen at random, with no bias toward individuals expressing HLA–B*57/5801. This highlights the fact that even for alleles with a high phenotypic frequency, unless large cohorts are evaluated, it is difficult to detect associations between HLA and

sequence polymorphism where there is a high background of polymorphism within a population.

Next, we studied mother-to-child transmission (MTCT) pairs to determine whether the hypothesized events of an escape mutation at residue 83 in the face of HLA-B*57/5801 in fact occur (Table II). Four MTCT pairs were identified in which an HLA–B*57/5801⁻ mother transmitted HIV to her HLA–B*57/5801⁺ child. In each case, the maternal virus encodes Ala at residue 83 (KF9/83A), which mutates to Gly (KF9/83G) in the child. For the S30 MTCT pair, in whom transmission occurred between 6 wk and 3 mo postpartum, maternal virus was sequenced both before and after transmission, with all viral RNA clones expressing KF9/83A. However, in the child sequenced at 9 mo postpartum, <8 mo after transmission, all 10 RNA clones expressed KF9/83G. These data show that HLA-B*57/5801 selects for the A→G mutation at Nef-83 and furthermore that this change occurs rapidly after transmission. Therefore, Nef-83 is in fact subjected to positive rather than negative selection pressure, operating through HLA-B*57/5801, leading in the majority of cases to an Ala→Gly substitution.

KF9/83G persists in the absence of HLA-B*57/5801

To understand how Gly might have replaced Ala as the consensus sequence at residue 83, we next sought to determine
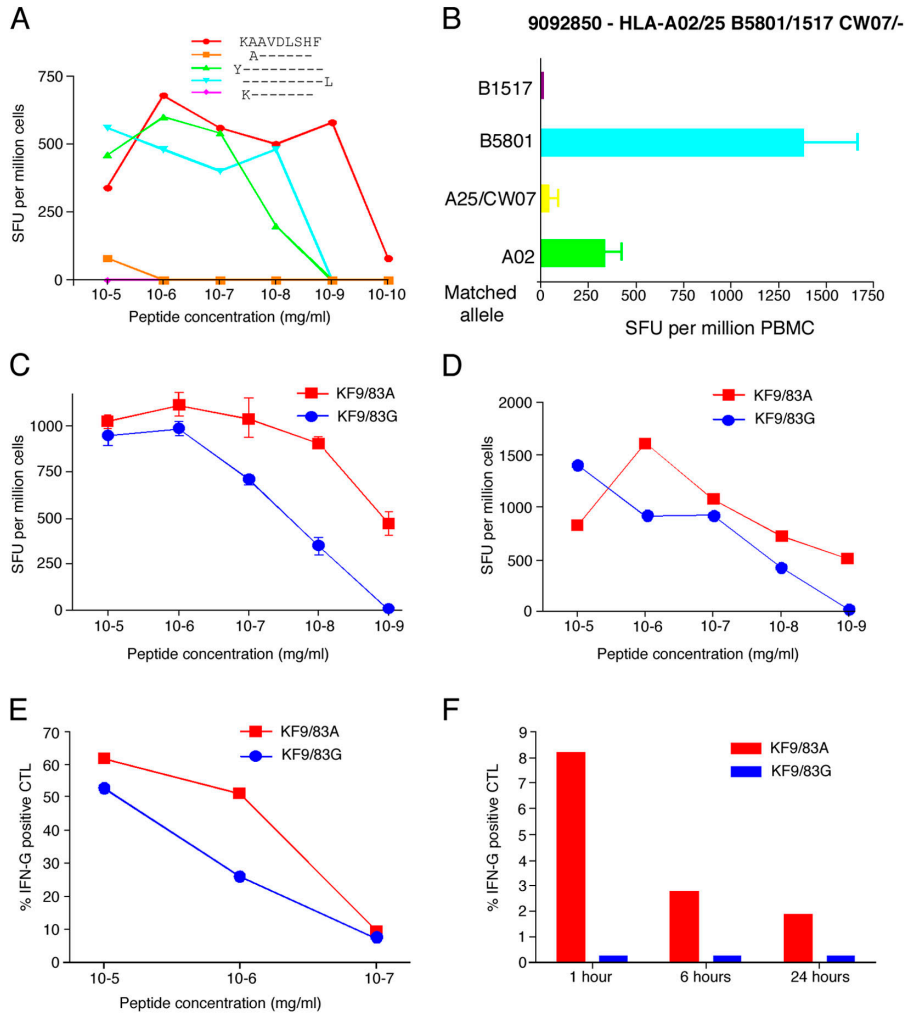
Figure 2. KF9 is an HLA–B*57/5801 epitope. (A and B) Optimization and restriction of B-clade KF9 (KAAVDLSHF) using fresh PBMCs isolated from the B-clade–infected 9092850 (B*5801/1517) by IFN-γ ELISPOT. (C–E) Recognition of KF9/83A and KF9/83G peptides by: (C) KF9-specific CTL line from B-clade–infected 0001726HW (B*57/7) by ELISPOT, (D) fresh PBMCs from 9092850 by ELISPOT, and (E) KF9-specific CTL line from the C-clade–infected SK059 (B*5801/-) by ICS. (F) Ala to Gly substitution at position 2 increases off-rate of KF9. Recognition of HLA-B*5801+ BCLs, pulsed with either KF9/83A or KF9/83G for 1, 6, or 24 h, by KF9-specific CTL line (SK059) by ICS. Background staining of unpulsed BCLs at each time point was subtracted.

if KF9/83G is stable in the absence of HLA-B*57/5801. We identified one previously published horizontal transmission pair (17) in which the HLA-B*5801+ subject 6007 transmitted HIV to the HLA-B*57/5801− subject 6008. In both individuals, virus encodes the KF9/83G mutation (Table II). In addition, we identified two MTCT pairs, 1043 and 1060, in which neither mother nor child possesses HLA-B*57/5801. In both cases, both mother and child display virus encoding KF9/83G, which is seen to be stable in the child for at least 2 yr after transmission (Table II), despite the absence of HLA-B*57/5801. In a final MTCT pair, 1043, both express HLA-B*5801 and both carry virus encoding KF9/83G. These data, combined with the fact that we see a high frequency of KF9/83G in individuals that lack HLA-B*57/5801 (Fig. 1), show that KF9/83G, selected for by HLA-

B*57/5801, is transmitted and is then stable in the absence of the selection pressure that drove it. Data from a cohort of B-clade–infected subjects in Perth, Australia, demonstrate the same effect (Fig. 1 C), with 52% of the cohort as a whole expressing KF9/83G as the consensus compared with 86% (12 out of 14) of HLA-B*57/5801+ subjects that express KF9/83G (P = 0.009).

KF9 is an HLA–B*57/5801–restricted epitope

Next, we sought to confirm that KF9 is indeed an HLA-B*57/5801-restricted epitope, and further, that KF9/83G represents an escape mutation. In a subsequent screening of HLA-B*57/5801+ subjects with the KF9/83A 9-mer, 20 out of 51 showed detectable responses by ex vivo ELISPOT (median magnitude of 300 sfu/million PBMCs; not de-

picted). In addition, we identified one B-clade–infected individual, 9092850-RI, whose PBMCs recognized the B-clade version of KF9 (KAAVDLSHF). Furthermore, by peptide pulsing fresh PBMCs from HLA-B*57/5801+ individuals who do not make ex vivo responses to KF9 and culturing in the presence of cytokines, we generated KF9-specific CTLs from one B-clade–infected, HLA-B*57+ individual. Using fresh PBMCs from 9092850-RI, we confirmed that KF9 is the optimal epitope (Fig. 2 A) and that the restriction element was indeed HLA-B*5801 (Fig. 2 B). These data show that KF9 is an HLA-B*57/5801-restricted epitope and that KF9/83G occurs at the primary anchor position 2 within it.

An Ala→Gly change at position 2 affects peptide off–rate from HLA–B*57/5801

To determine whether KF9/83G is an escape mutation, we titrated out KF9 peptides containing either Ala (KF9/83A) or Gly (KF9/83G) at position 2, using either fresh PBMCs or cultured cells from each subject, and tested recognition via IFN-γ production by ELISPOT assay or intracellular cytokine staining (ICS; Fig. 2, C–E). In each case, the KF9/83A peptide was slightly better recognized at low peptide concentrations than the KF9/83G peptide, consistent with the hypothesis that KF9/83G represents an escape mutation in this epitope. However, in the three individuals tested in this way, the KF9/83G variant appears to be recognized to some degree. It remains unclear as to whether such differences represent escape mutation or cross-recognition of variant epitopes (20). Given that Gly is not a preferred residue at position 2 in the B*57/5801 peptide-binding motif (16), we hypothesize that this change would affect peptide binding affinity or off-rate, effects that might not be detected by ELISPOT or ICS, as these are systems in which the presenting cells and peptide are normally coincubated for the duration of the assay. Therefore, we conducted further assays to determine the effect of the Ala→Gly change at position 2 on the binding of KF9 to HLA-B*57/5801. EBV-transformed B cells (BCLs) expressing HLA-B*5801 were pulsed for 1 h with either the KF9/83A or KF9/83G peptides. The cells were then washed and tested for recognition by a B*5801-restricted KF9-specific CTL line via ICS. In contrast to the direct ICS/ELISPOT assays, although the BCLs pulsed with KF9/83A were strongly targeted, recognition of those pulsed with the KF9/83G variant was completely ablated (Fig. 2 F). When the cells were incubated for an additional 5 or 23 h, rewashed, and tested, the BCLs pulsed with KF9/83A were still recognized, although the magnitude of response was seen to decrease in a time-dependent manner. As expected, after further incubation, BCLs pulsed with the KF9/83G variant peptide remained untargeted. These data suggest that the Ala→Gly change at position 2 within KF9 affects peptide off-rate, with the KF9/83G variant form dissociating from HLA-B*5801 more readily than the KF9/83A peptide.

Table III. Escape mutation in HLA-B57–restricted epitope KF11

Consensus	KAFSPEVIPMF	
HLA-B57	–G–N–––––––	14%
	–G–K–––––––	8%
	–G–––––––––	3%
	–N–––––––––	8%
	–S–––––––––	3%
	–S––––T––––	3%
	–––––––––––	62%
HLA-B5801	–G–N–––––––	6%
	–G–––––––––	2%
	–N–––––––––	2%
	––––––I––––	4%
	–––––––––––	86%
Non-B57/5801	–G–N–––––––	5%
	–N–––––––––	1%
	–S–––––––––	1%
	–––N–––––––	2%
	––––––I––––	7%
	–––––––––––	84%

To confirm the impact of this Ala→Gly substitution at residue 2 within HLA-B*57/5801-binding peptides, we next focused on the Gag epitope KAFSPEVIPMF (KF11), which dominates the HLA-B*57–restricted HIV-specific CTL response during chronic infection (13, 14). We have identified an identical Ala→Gly mutation selected at position 2 in KF11. Sequencing of HLA-B*57+ individuals from the Durban cohort reveals positive selection for a mutation at position 2 within KF11 (Gag residue 163), the majority of which involve an Ala→Gly substitution (KF11/163G; Table III and Fig. 3 A). 9 out of 37 HLA-B*57+ individuals expressed KF11/163G compared with 8 out of 149 HLA-B*57− subjects (P = 0.0002). All four viruses isolated from HLA-B*57/5801− individuals that expressed KF11/163G also displayed at least one other known footprint of this allele (A146P, I147L. H219Q, and G248X; reference 17) compared with 30 out of 105 that express these footprints but lack the KF11/163G mutation (P = 0.008; references 17 and 21; not depicted). This indicates that the KF11/163G mutation in these individuals owes its origin to HLA-B*57. Hence, we hypothesize that like KF9/83G, KF11/163G is transmitted and stable in the absence of HLA-B*57.

As observed for KF9, titration of the KF11/163A and KF11/163G peptides in an IFN-γ ELISPOT assay reveals only minor differences in response to these peptides (Fig. 3 B). However, we hypothesized that KF11/163G also represents an escape mutation, as identical mutations observed in two separate HLA-B*57–restricted epitopes are unlikely to be selected unless they both provide a selective advantage. Therefore, we conducted the same binding assay for KF11, using HLA-B*57+ BCLs pulsed with either KF11/163A or
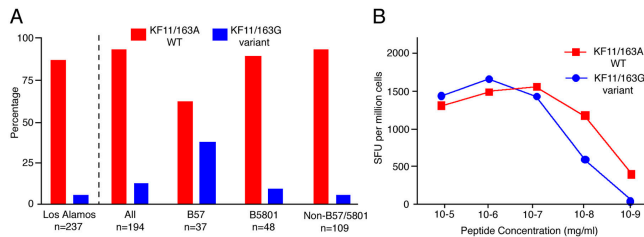
Figure 3. Identical changes observed in a second HLA–B57–restricted epitope, KF11. (A) Association between expression of HLA-B57 and an Ala to Gly substitution at position 2 within KF11 (KF11/163G). (B) Recognition of KF11/163A and KF11/163G peptides by KF11-specific CTL lines (9308196RI) by IFN-γ ELISPOT.

KF11/163G peptides, with recognition by a KF11–specific CTL line tested via ELISPOT. Unlike KF9, after 1 h, both the KF11/163A- and KF11/163G-pulsed BCLs were recognized, indicating that both peptides were still bound to HLA-B*57 on the surface of the BCLs (Fig. 4 A). However, when we incubated the cells for an additional 11 h, washed, and retested them, recognition of the KF11/163G-pulsed BCLs was almost completely ablated, with only a very small response observed at the highest E/T ratio (Fig. 4 B). In contrast, the KF11/163A-pulsed cells were still well recognized. Indeed, even after 36 h, KF11/163A-pulsed cells remained well recognized, whereas there was no response detected to the KF11/163G-pulsed BCLs (Fig. 4 C). The same results were observed when the experiment was repeated using a KF11 clone derived from a separate HLA-B*57⁺ individual (Fig. 4, D and E). In this case, recognition of pulsed

BCLs was determined in a chromium release assay, showing that the KF11/163G mutation affects killing of target cells and not only IFN-γ production. Although these assays not only measure peptide off-rate and include the contribution of TCR sensitivity to WT and variant peptide forms, they also demonstrate clear-cut differences in recognition that were not apparent when peptides were coincubated for the duration of the assay with target cells. These data indicate that an Ala→Gly change at position 2 within two separate HLA-B*57–restricted epitopes has the same effect on peptide recognition, with the Gly variant form being less well recognized than the WT Ala form. We hypothesize this is due to an increase in peptide off-rate.

Even relatively small changes in off-rate can greatly affect the ability of a ligand to trigger T cells (22). Therefore, subtle changes in peptide off-rate associated with an Ala→Gly substitution at position 2 within B*57-restricted epitopes might be expected to reduce recognition of the epitope on the surface of an infected cell. To test this, we transfected an HLA-B*57⁺ BCL line with synthetic mRNA constructs encoding the KF11/163A epitope or the KF11/163G or KF11/163N variants (Table III). Constructs were derived from a 162-residue fragment of p24 from the HIV molecular clone NL4-3, expressing either WT KF11/163A or variant KF11 epitope at the NH_2 terminus and tagged with the B*57-restricted epitope ISW9 (ISPRTLNAW, Gag 147–155) at the COOH terminus as a positive control. Transfected BCLs were used to stimulate KF11- or ISW9-specific CTL clones in an IFN-γ ELISPOT assay (Fig. 5 A). Both ISW9 and KF11 clones recognized BCLs transfected with WT KF11/163A mRNA. However, KF11 clones failed to
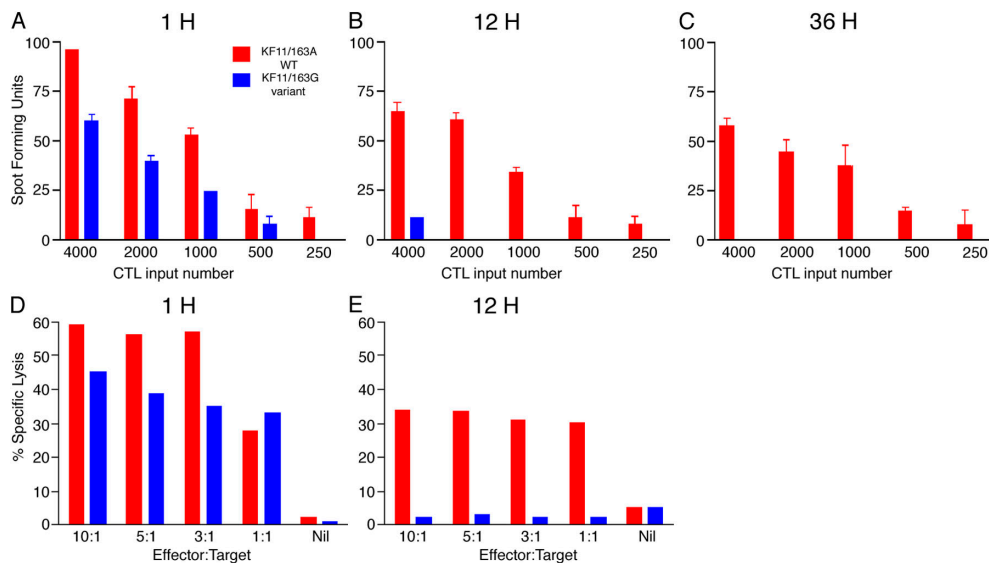

Figure 4. Ala to Gly substitution at position 2 also increases off–rate of KF11. Recognition of HLA-B*57⁺ BCLs pulsed with either KF11/163A or KF11/163G peptides (A–C) for 1, 12, and 36 h using a KF11-specific CTL (9308196RI) measured by IFN-γ ELISPOT. At each time point, effector cells were titrated out and tested for recognition of 25,000 pulsed BCLs (D and E) for 1 and 12 h using a KF11 clone measured by percent of killing in a ⁵¹Cr assay. For all assays, background staining of unpulsed BCLs at each time point was subtracted.
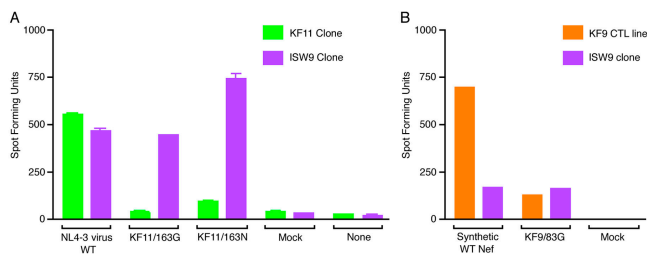
Figure 5. Ala to Gly substitution at position 2 affects presentation of endogenously processed peptides. (A) KF11 clone tested against either HLA-B57+ BCLs transformed with mRNA containing either the KF11/163A or KF11/163G epitope, and a second B57 restricted epitope ISW9, no mRNA (Mock), or untransformed BCLs. (B) KF9-specific CTL line (SK059), tested against either HLA-B5801+ BCLs transformed with mRNA containing either KF9/83A or KF9/83G and ISW9, or untransformed BCLs. In all cases, recognition was measured by IFN-γ production in an ELISPOT assay.

Figure 6. Negative association in a second, HLA–B*51–restricted epitope LPPVVAKEI (LI9). (A) A mutation from Ile to Val (LI9/31V) at position 4 within in LI9 is selected for in B*51+ donor and is transmitted to B*51− recipient. LI9/31V is stable in the recipient, still being found in 100% of the RNA clones isolated 8 mo after transmission. (B) LI9/31V is associated with the expression of HLA-B*51 in both B-clade and C-clade. In B-clade, this is seen as a negative association, as LI9/31V has become the consensus. However, in the C-clade epidemic in Durban, where HLA-B*51 is rare, LI9/31I is still the consensus and hence this is seen as a positive association.

recognize BCLs transfected with either KF11/163G or KF11/163N variant mRNA. As expected, recognition of ISW9 was unaffected by these distal mutations in the KF11 epitope. Therefore, the consequence of the Ala→Gly substitution at position 2 within KF11 is that the peptide is no longer presented at sufficient levels on the cell surface to induce an IFN-γ response. Similar assays were performed using KF9-specific CTLs and mRNA transcripts encoding WT KF9/83A or variant KF9/83G, and these showed the equivalent result (Fig. 5 B). These data show that the KF9/83G and KF11/163G variants are indeed escape mutations that would allow the virus to evade these CTL responses.

Ile→Val at integrase–31 is an HLA–B*51 negatively associated B–clade polymorphism

To determine whether the mechanism by which the negative association between HLA-B*57/5801 and Nef-83 arose is a more general phenomenon, we next sought other examples of associations between HLA alleles and conservation of the consensus. We focused on another allele, HLA-B*51, because, like HLA-B*57/5801, it is associated with slow progression in B-clade infection (11, 12) and hence likely to impose strong selection pressure on the virus. Initially, we studied a B-clade–infected horizontal transmission pair, in which the donor expressed HLA-B*51 and the recipient did not, to follow escape mutations arising in the former that were transmitted and stable in the latter. We observed such a mutation at position 4 in the integrase epitope, LPPVVAKEI ("LI9": residues 28–36; reference 23), involving an Ile→Val substitution at residue 31 (LI9/31V), where Val is the B-clade consensus amino acid in this position (http://www.hiv.lanl.gov). 22 out of 22 clones sequenced from viral RNA at the first time point in the B*51+ donor encoded Ile at residue 31 (LI9/31I), but at the time of transmission 18 mo later, 11 out of 11 clones sequenced now encoded Val. This LI9/31V mutation was transmitted to the B*51− recipient and was still found in 100% of the clones isolated from
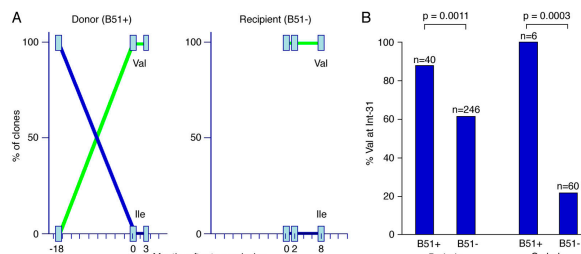
the latest sampling point 8 mo after transmission (Fig. 6 A). In a second similar transmission pair, the B*51− recipient continued to carry virus expressing the same LI9/31V mutation at least 14 mo after transmission (not depicted).

To determine whether there is an association between the occurrence of Val at this position and B*51 expression, we compared viral sequences in 40 B*51+ and 246 B*51− B-clade–infected persons and found that although the clear majority (61%) of the B*51− subjects carry virus expressing LI9/31V, a significantly greater proportion (88%) of B*51+ persons carry virus expressing LI9/31V (P = 0.0011). This suggests that as for KF9/83G, the Ile→Val substitution that now forms the consensus in B-clade infection is driven by CTL escape in B*51+ subjects. To further test this hypothesis, we examined the situation in the C-clade–infected cohort in Durban, in which B*51 is rare. In contrast to B-clade virus, the C-clade consensus amino acid at integrase-31 is Ile, with 68% of HLA-B*51− subjects from this cohort expressing LI9/31I (Fig. 6 B). We sequenced the virus in six B*51+ C-clade–infected subjects, and in each case the LI9/31V mutation was present (P = 0.0003). Thus, we have observed a second clear case in which the negative association with an HLA allele and an amino acid occurs as a result of the positive selection of an escape mutation that is transmitted and apparently stable in the absence of the selecting HLA allele.

DISCUSSION

A substantial number of the described links between HLA class I expression and HIV sequence polymorphism involve associations for conservation of the consensus sequence rather than variation away from it. The mechanism that operates to produce these associations has been unclear. The term "negative selection pressure," which was proposed to explain their existence, suggests that selection pressure is operating against sequence change at these positions. However,
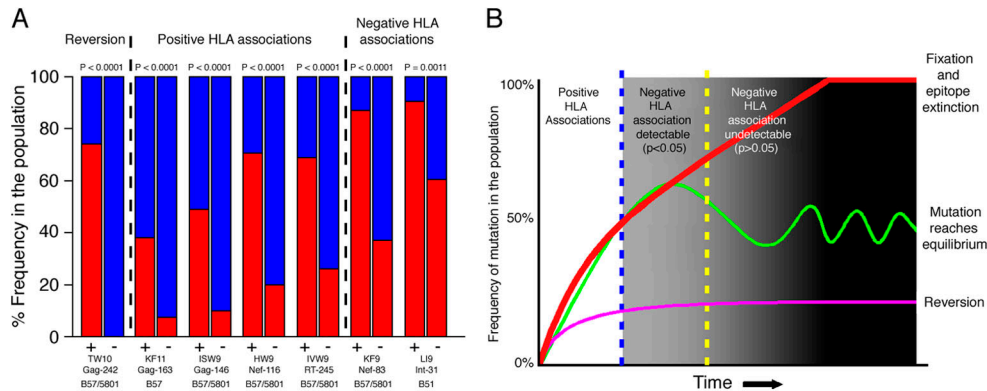
Figure 7. The evolutionary fate of CTL epitopes. (A) Five HLA-B*57/5801-restricted epitopes and one B*51-restricted epitope in which escape mutation is transmitted and stable in the absence of the selecting allele. This is observed as a positive association between the selecting allele and sequence polymorphism or a negative association between the selecting allele and sequence conservation depending on whether or not the escape mutation has spread in the population to the point at which it has replaced the consensus sequence. The B*57/5801-restricted TW10 reverts on transmission and hence will not spread at the population level. Mutations in KF11, KF9, and IVW9 (B-clade only; all occurring at primary anchor positions) and in ISW9 (which affect processing) remove the epitope as a potential target and hence might be expected reach fixation. Conversely, mutations in HW9 and LI9 occur at nonanchor residues and are likely to remain as targets to the immune system. (B) Three potential outcomes of CTL escape mutation are: (a) escape mutations that revert on transmission: frequency of escape mutation will not exceed the phenotypic frequency of the selecting allele. The epitope will remain a potential target to the immune system and positive selection pressure will always be evident. (b) Escape mutation that is stable but does not affect processing/presentation. Frequency of mutation will equilibrate at 50%, the epitope will remain a target but there will be no clear consensus and evidence of selection pressure maybe lost. (c) Escape mutation that is stable and affects epitope processing/presentation. Epitope will become extinct as mutation reaches fixation, when all evidence of selection pressure and indeed the epitope itself will be lost.

the data presented here show that KF9/83G, which forms the consensus sequence, actually represents an escape mutation from the WT KF9/83A, driven by targeting of the previously undescribed HLA-B*57/5801-restricted epitope, KF9. This KF9/83G mutation is transmitted and persists in the absence of HLA-B*57/5801, and as a result, its subsequent accumulation at the population level has lead to replacement of Ala by Gly as the consensus sequence. Similarly, the HLA-B*51–associated escape mutant LI9/31V in the epitope LI9 is transmitted and persists after transmission to the point where the escape variant LI9/31V now represents the consensus sequence in B-clade infection. The same escape mutation is observed in the C-clade–infected population in Durban, with all HLA-B*51+ individuals sequenced expressing LI9/31V. However, in this C-clade population, in which HLA-B*51 is rare, LI9/31I remains the consensus. Thus, the same LI9/31V variant is identified as a "positive association" in C-clade infection, and as a "negative association" in B-clade infection.

These data suggest that, like the more typically observed "positive" associations, "negative" HLA associations with sequence polymorphism can also be the result of positive selection. The apparent differences are only evident because sequences are viewed as an evolutionary snapshot and relate simply to the extent to which an escape mutation has accumulated in the population. An escape mutation that does not undergo reversion (17, 24, 25), but which has yet to accumulate in the population to the extent that the consensus sequence has switched to the variant form, as in the case of LI9/31V in C-clade infection, is seen as a positive HLA association. The same mutation analyzed later in the epidemic will be seen as a negative HLA association because the variant will have become the consensus sequence (Fig. 7, A and B). We hypothesize that this is a primary mechanism by which associations between the expression of particular HLA alleles and sequence conservation, such as those identified by Moore et al. (3), might arise. Therefore, these negative associations represent the genetic footprints of immune responses, and their presence supports the hypothesis that HLA-restricted CTL responses are driving HIV evolution at the population level (3, 8, 17, 26).

Recent studies have demonstrated that not all escape mutations will increase in frequency in the population over time. Escape mutations that occur at a fitness cost to the virus revert to WT on transmission to individuals lacking the HLA allele that drove their selection (17, 24, 27). Only mutations that have a minimal effect on viral fitness, and are therefore selectively neutral, are likely to persist in the absence of the relevant HLA allele and thereby accumulate at the population level (21). A recently described example is the HLA-B*57/5801–associated processing escape mutation A146P within p24 Gag (Fig. 7 A). The frequency of this escape mutation is increasing over the course of the epidemic, and in vitro competition experiments comparing the fitness of viruses that were isogenic other than for the change en-

coding the A146P substitution demonstrated no reduction in replicative capacity resulting from this mutation (21).

A further important feature of transmitted escape mutants is that the immunological significance and pattern of accumulation of these variants will vary. Escape mutations that interfere with TCR recognition likely only represent escape at the individual and not the population level (17, 28). There is a vast array of unique TCRs that can be generated by TCR gene rearrangement and N-region substitutions (29), which in combination with a high degree of cross reactivity, suggest that the T cell repertoire is broad enough to respond to essentially all foreign peptides that contain appropriate MHC anchor residues (30). Therefore, although variation in the TCR contact residues may result in loss of peptide recognition in an individual, it is likely that if transmitted to other individuals sharing the restricting HLA allele, they will be able to generate a response to this variant form. This is perhaps best exemplified by escape mutation in the HLA-A*02–restricted Gag epitope SLYNTVATL (SL9), in which responses have been observed to all of the commonly observed polymorphisms (31, 32), and, although HLA-A*02$^+$ individuals are often infected with a virus encoding variants of SL9 at positions 3, 6, and 8 in the epitope, their ability to generate primary responses to SL9 is unaffected (33). Thus, despite persistent selection of stable escape mutations, SL9 remains a frequently targeted epitope. Conversely, epitopes in which mutation disrupts either the processing or presentation are effectively removed as possible CTL targets (4, 21, 34) and hence, if the escape does not revert, individuals subsequently infected with this virus will no longer be able to generate a response. If such mutations spread up to the point of fixation, the epitope can be defined as "extinct," as it no longer exists as a potential target to the immune system. This principle has been demonstrated in EBV, in which an immunodominant HLA*A11-restricted epitope has been lost in viral strains isolated from communities in New Guinea, in which HLA*A11 is highly prevalent (phenotypic frequencies of 25–50%; reference 35).

Consideration of these factors should allow one to predict the evolutionary fate of a given CTL epitope (Fig. 7, A and B). Epitopes such as the HLA-B*57/5801–restricted TW10 in which the escape mutation reverts (17) will remain targets to the immune system. The frequency of the escape mutation in the population is likely to stabilize at some level below the phenotypic frequency of the selecting allele, depending on how rapidly and consistently escape occurs, and the positive selection operating will always remain apparent. Conversely, epitopes such as KF9, KF11, the HLA-B*57/5801–restricted Gag epitope, ISW9 (21), and RT epitope IVW9 (RT 244–252), in which the escape affects processing and/or presentation and does not revert, are likely to be those that tend toward extinction over time. In between these two extremes, there are epitopes such as SL9, in which the escape mutation does not revert but where the epitope is still presented and thus remains a target for the immune system. Potential examples of such epitopes from our data include the HLA-B*57/5801-restricted Nef epitope HW9 (Nef 116–124) and the HLA-B*51–restricted LI9. In both of these the escape mutation is stable in the absence of the selecting allele but does not occur at a primary anchor residue. Under such circumstances, one might expect the frequency of the mutation in the population to stabilize at ∼50%, with no clear consensus discernible.

The precise frequency in the population of any given mutation will depend on several factors. The frequency of the selecting allele may have an effect, and indeed it has been proposed that high frequency alleles are more likely to drive escape variants that are embedded in the consensus as "negative HLA associations" (3). However, the degree to which different alleles drive immune escape in HIV infection differs substantially and is not simply related to the allele frequency (15). In particular, HLA-B alleles as a whole tend to drive escape more than HLA-A alleles, and there are likely to be differences between individual alleles in this respect, just as there are between the individual alleles and viral set point (15). Other HLA–dependent factors that will influence the frequency of an escape variant would include duration of epidemic, timing of generation of the escape variant within the infection, and likelihood of transmission of the variant (related to HLA-associated viral load). Furthermore, the clustering of epitopes restricted by many different alleles into regions of high immunogenicity means that the frequency of KF9/83G and LI9/31V are likely to be affected not only by HLA-B*57/5801 and B*51, respectively, but also by other alleles, the identity of which are likely to vary from one population to another. Finally, for mutations such as KF9/83G and LI9/31V that are essentially selectively neutral, there are important HLA–independent influences, such as genetic drift and founder effects, which are likely to have a strong impact on the frequency of these variants within different populations. These latter influences of genetic drift and founder effects would not themselves explain the negative HLA associations observed here and previously (3), although they may influence the observed frequency of particular amino acids and therefore contribute to the determination of a consensus sequence.

An additional discussion point that emerges from these data concerns the inadequacy of standard assays, involving coincubation of peptide with target cells for the duration of the assay, for use in analyzing variant recognition. The use of assays that include assessment of peptide off-rate appear to be a more faithful representation of the in vivo recognition of variants, as assessed by minigene transfection of targets (36) or, in these studies, by the mRNA transfection assays described. These indicate that the off-rate assay can reveal clear-cut differences in recognition between variants that are debatable when viewed from the perspective of the standard assay. Peptide optimization occurring within the ER ensures that only ligands with slow HLA dissociation rates are selected (37, 38). It is therefore likely that the increase in pep-

tide off-rate associated with A→G substitution at position 2 in B*57/5801-restricted epitopes is such that this variant is out-competed in the ER by alternative B*57/5801-restricted peptides. These studies thus highlight the limitations of commonly used assays, which rely on coincubation of targets with peptides for the duration of the assay and may greatly underestimate the true level of escape mutation being driven by CTL selection pressure.

The importance of understanding the direction of HIV evolution in the development of HIV vaccines is clear. A vaccine that induces CTL response to epitopes no longer in circulation will be redundant and perhaps even harmful in inducing immunodominant responses incapable of recognizing the circulating forms of the virus. This concept has been considered in the context of whether a consensus based on circulating forms might have advantages over an ancestral reconstruction for vaccine antigen design. The observation of negative HLA associations, which are positively selected escape variants that might be in the process of disappearing by becoming embedded within consensus sequence, indicates that the process of adaptation of HIV to host HLA alleles is certainly occurring. This suggests that even epitopes such as KF11, which is presently the dominant HLA-B*57–restricted response in chronic infection, may become extinct as the epidemic evolves. In considering the epitopes that should be incorporated into today's HIV vaccine, one could argue that only those epitopes that revert after transmission should be included, because these not only incur a cost to viral replicative capacity when escape occurs, but they also are likely to remain in circulation and accessible as epitopes many years into the epidemic. Epitopes with negative HLA associations, on the other hand, might be the epitopes least likely to make an effective contribution to a vaccine, because, in cases where epitope processing and presentation has been affected, the challenge virus will not be recognized by corresponding CTLs. Additionally, even if the variant epitope remains a target, the cost to viral fitness of any subsequent escape mutation induced is likely to be low. Thus, understanding the evolutionary events subsequent to transmission is of direct relevance to HIV vaccine design.

MATERIALS AND METHODS

Subjects studied. C-clade HIV samples were collected from Cato Manor antenatal clinic, Durban, South Africa. All subjects are antiretroviral therapy naive. B-clade samples were collected from cohorts encompassing Europe and North America, with the majority also being antiretroviral therapy naive at the time of analysis. Additional B-clade samples were obtained from the Hemophilia Growth and Development Study in the United States (39). This study was approved by the University of Natal Review Board, the Oxford Research Ethics Committee, and the MGH Review Board. All individuals gave informed consent for participating in this study. HLA class I typing was performed on genomic DNA by PCR single-stranded conformation polymorphism (40).

Sequencing of proviral DNA and viral RNA. Genomic DNA was extracted from whole blood via the Puregene DNA isolation kit (Gentra). We amplified HIV gag sequences by nested PCR as described previously

(17), using the following primers: Gag-specific primers and Nef-specific primers: 5′-TTCAGCTACCACCGATTGAGA-3′, 5′-TGAGGGTTG-GCCACTCC-3′ for first round PCR and 5′-TTCAGCTACCAC-CGATTGAGA-3′, 5′-TGAGGGTTGGCCACTCC-3′ for second round PCR; RT-specific primers: 5′-GAAGGACAGTACTAAGTGGAG-3′, 5′-CTGGCTACATGGACTGCTAC-3′ and 5′-ACTGCATTCACC-ATACCTAG-3′, 5′-TTATGTGCTGGTACCCATGA-3′; and INT primers: 5′-CATGGTGGACAGACTATTGGC-3′, 5′-CCTGCCATC-TGTTTTCCATA-3′ and 5′-CCTCCCCTAGTAAAATTATGG-3′, 5′-GCTGTCTCTGTAATAAACCCG-3′. PCR product was purified by PEG precipitation and then either directly sequenced (population sequencing) or cloned as described previously (41) using Topo TA cloning kit (Invitrogen). Viral RNA was obtained from plasma using the Nucleospin RNA extraction kit (Mackery-Nagel). This was converted to cDNA, amplified, and sequenced as described previously (17). All sequencing used the BigDye ready reaction termination mix V3 (Applied Biosystems) for Gag using Gag-specific primers as described previously (17) and for Nef, RT, and INT using the two second round PCR primers and the following four additional primers: Nef: 5′-ACCTGAGGTCTGACTGGAAAG-3′, 5′-GATAAACATGGGGCACTTAC-3′, 5′-AGCAGTCTTTGTAATAC-TCCG-3′, and 5′-ACCTCAGGTGCCTTTAAGAC-3′; RT: 5′-TGG-GTCATAATATACTCCATGTA-3′, 5′-CAGACATAGTACCACT-AACTGAA-3′, 5′-CATAGAAAGTTTCTGCTCCT-3′, and 5′-CTC-CCCTAGTAAAATTATGGTA-3′; and INT: 5′-TCACTAGCCATT-GCTCTCC-3′, 5′-ATTGGAGGAAATGAACAAGTAGA-3′, 5′-GCC-CACCAACAGGCTGC-3′, and 5′-GAAACAGGACAAGAAACAGC-3′. All sequences were analyzed on the ABI 3700 DNA analyzer. All residue numbers are from HXB2 reference sequence. Total number HIV-1 viruses sequenced as follows: Gag, 194 C-clade; RT, 113 B-clade; INT, 286 B-clade and 66 C-clade; Nef, B-clade 81 and C-clade 117. We constructed neighbor-joining trees of all the sequences together with reference sequences from the Los Alamos Database (http://www.hiv.lanl.gov) to verify the viral clades and to exclude the possibility of contamination with laboratory HIV strains.

Cells lines. KF11-specific CTL lines were generated as described previously (42) and maintained in R10 medium (RPMI 1640 [Sigma-Aldrich], 10% fetal calf serum, 10% L-glutamate, and 10% penicillin/streptavadin) with the addition of 25 ng/ml human recombinant IL-15 (PreproTech). KF9 lines were generated by peptide pulsing 5×10^{-6} PBMCs with 100 mM KF9 peptide for 1 h and resuspended in R10. After 1 wk in culture (at 37°C, 5% CO_2), the medium was replaced two to three times a week with R10 containing 50 U/ml IL-2 and 5% T-stim (Becton Dickinson). KF11 and ISW9 clones were generated by limiting dilution, and characterized and maintained as described previously (43).

Definition of KF9 epitope. KF9 optimization and restriction was confirmed (14) using fresh PBMCs from the HIV B-clade–infected subject 9092850 (HLA-B*57/1517). Responses were tested by IFN-γ ELISPOT (42). Fresh PBMCs isolated from HLA-B*5801$^+$ individuals from the Durban cohort were tested for recognition of the optimal KF9 peptide by IFN-γ ELISPOT. Fresh PBMCs or KF9-specific CTL lines were tested for recognition of KF9/83A and KF9/83G by IFN-γ ICS (42).

Peptide off-rate assay. For KF9, HLA-B*5801$^+$ BCLs were pulsed with 0.1 μM KF9/83A or KF9/83G peptide, incubated for 1 h (at 37°C, 5% CO_2), and washed three times with PBS. The pulsed BCLs were split into aliquots and either tested for recognition by KF9-specific CTL via ICS or incubated for an additional 5–23 h, rewashed, and then tested. For KF11, HLA-B*57$^+$ BCLs were pulsed with 0.1 μM WT or A163G KF11 peptide, incubated, and washed as described above. Pulsed BCLs were split into aliquots and either tested for recognition at different E/T ratios by KF11-specific CTLs via IFN-γ ELISOT (in triplicate), or incubated for an additional 11–36 h, rewashed, and tested. The experiment was repeated using a KF11 clone, with recognition tested by ^{51}Cr release (21).

Processing and presentation. BCLs were transfected with synthetic mRNA transcribed from a PCR product template. For KF9, the forward primer was 5′-TAATACGACTCACTATAGGGAGAGCCACCATGG-CTGGCAAGTGGTC-3′ and the reverse primer was 5′-TTACCATG-CATTTAAAGTTCTAGGTGATATTGCAAAGAAGCTAAGATCGA-AAGCT**X**CC-3′, where X = G for KF9/83A or C for KF9/83G. The PCR template was a synthetic gene encoding the 2001 consensus B Nef sequence (http://www.hiv.lanl.gov) purchased as a plasmid from DNA 2.0. For KF11, the forward primer was 5′-TAATACGACTCACTATAG-GGAGAGCCACCATGGTAGTAGAAGAGAAG**XY**TTTC-3′, where XY = GC for KF11/163A, GG for KF11/163G, and AA for KF11/163N, and the reverse primer was 5′-TTACCATGCATTTAAAGTTCTAGGT-GATATAGCTTGCTCGGCTCTTAGAGT-3′ and the PCR template was HIV clone NL4.3. Synthetic mRNA was transcribed from PCR template using the Message Machine transcription kit (Ambion), and a 3′ poly A tail was added with the Poly(A) Tailing Kit (Ambion). 10^6 APCs were transfected (300 V/0.5 mS, square wave) in a 0.2-cm cuvette with synthetic RNA using a GenePulserII electroporator (Bio-Rad Laboratories) using 10 ug mRNA. Transfected LCLs were immediately mixed with CTLs at a ratio of 1:1 and incubated overnight in an IFN-γ ELISPOT.

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