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Fine-mapping classical HLA variation associated with durable host control of HIV-1 infection in African Americans

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A small proportion of human immunodeficiency virus-1 (HIV-1) infected individuals, termed HIV-1 controllers, suppress viral replication to very low levels in the absence of therapy. Genetic investigations of this phenotype have strongly implicated variation in the class I major histocompatibility complex (MHC) region as key to HIV-1 control. We collected sequence-based classical class I HLA genotypes at 4-digit resolution in HIV-1-infected African American controllers and progressors ($n = 1107$), and tested them for association with host control using genome-wide single nucleotide polymorphism data to account for population structure. Several classical alleles at *HLA-B* were associated with host control, including B*57:03 [odds ratio (OR) = 5.1; $P = 3.4 \times 10^{-18}$] and B*81:01 (OR = 4.8; $P = 1.3 \times 10^{-9}$). Analysis of variable amino acid positions demonstrates that HLA-B position 97 is the most significant association with host control in African Americans (omnibus $P = 1.2 \times 10^{-21}$) and explains the signal of several *HLA-B* alleles, including B*57:03. Within HLA-B, we also identified independent effects at position 116 (omnibus $P = 2.8 \times 10^{-15}$) in the canonical F pocket, position 63 in the B pocket ($P = 1.5 \times 10^{-3}$) and the non-pocket position 245 ($P = 8.8 \times 10^{-10}$), which is thought to influence CD8-binding kinetics. Adjusting for these HLA-B effects, there is evidence for residual association in the MHC region. These results underscore the key role of *HLA-B* in affecting HIV-1 replication, likely through the molecular interaction between HLA-B and viral peptides presented by infected cells, and suggest that sites outside the peptide-binding pocket also influence HIV-1 control.

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INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) controllers are a subset of infected individuals who are able to maintain plasma viremia below 2000 copies per ml without antiretroviral therapy. These individuals generally remain healthy, with high CD4 counts and lower rates of HIV-1 transmission (1). Genome-wide association studies (GWASs) in individuals of European ancestry have consistently implicated variants in the major histocompatibility complex (MHC) as key determinants of HIV-1 virus load and disease progression (2–6). In addition to the MHC, variants in the *CCR5*/*CCR2* region have also been convincingly associated with control of HIV-1 replication and altered disease progression (7–9). Additional loci have been suggested to impact disease outcome, but most remain un-confirmed (3,6).

The importance of the classical HLA genes in HIV-1 disease outcome has been documented in numerous studies. In Europeans, alleles of *HLA-B* have been associated with both control, such as B*57:01, and lack of control, such as B*35:01 (10–12). Smaller studies in populations of African ancestry have also identified alleles, such as B*57:03, that associate with decreased plasma viremia (13–15). Although the mechanism by which these alleles mediate viral control or progression is not well understood, both genetic and immunologic data suggest that the interaction between the viral peptide and the host HLA protein is key, likely through influencing generation of HIV-1-specific CD8+ T cells (16).

Thanks to a large collaborative network of infectious disease physicians, we have assembled a multiethnic cohort of 1528 HIV-1 controllers and, in collaboration with the AIDS Clinical Trials Group (ACTG), 2975 HIV-1-infected individuals with progressive disease, the majority of whom were included in a recent GWAS (6). In that study, we focused on the European subset and identified four independent genome-wide significant single nucleotide polymorphism (SNP) associations. Using a novel procedure to impute classical HLA alleles and amino acid polymorphisms, we fine-mapped these SNP associations to specific amino acid positions in the peptide-binding cleft of HLA-B, specifically positions 67, 70 and 97, and validated their effects on virus load set point in an independent cohort of European ancestry (6). Thus, these amino acids play a key role in determining control/progression after HIV-1 infection, likely through the molecular interaction between HLA and the viral peptide. In addition, it has been proposed that an expression quantitative trait locus (eQTL) of *HLA-C* has an independent effect on host control, possibly involving a variant in the miRNA-148a-binding site (17–20), but this remains unconfirmed.

In the African American subset, we also identified four independent genome-wide significant SNP associations (rs2523608, rs2255221, rs2523590 and rs9262632) in the MHC, all of which differed from those observed in Europeans. Using a similar imputation strategy for classical alleles and amino acids, although with a much smaller reference panel ($N = 596$ individuals with SNP and 4-digit HLA data), we were able to confirm HLA-B*57:03 as the top classical allele association and position 97 as the top amino acid position (6). However, due to the limitations of our imputation reference panel (chiefly its small size), we were unable to implement the complimentary amino acid fine mapping in African Americans. Given the genetic diversity across continental populations in the MHC, such fine mapping has the potential to uncover classical alleles and amino acids not present in individuals of European ancestry.

Here, we build on and extend our previous work by obtaining high-quality sequence data at *HLA-A*, *HLA-B* and *HLA-C* in African American HIV-1 controllers and progressors. Through association testing of classical HLA alleles and individual polymorphic amino acid positions, we demonstrate disease-modifying effects of multiple HLA variants, including some that do not segregate at appreciable frequency in populations of European ancestry.

RESULTS

Comparison of sequence-based and imputation-based classical class I HLA types in African American HIV-1 controllers/progressors

In a previous study, we used an imputation framework to estimate genotype dosage of classical alleles and variable amino acid positions at the HLA class I locus in HIV-1 controllers/progressors. Although this approach was highly accurate in individuals of European ancestry (where a large reference panel was available), the results in African Americans were not as clear (owing to the relatively smaller reference panel) (6). In order to improve upon this, we obtained sequenced-based types at *HLA-A, HLA-B* and *HLA-C* in the majority of the African American sample with the available genome-wide SNP data (Supplementary Material, Table S1) using a combination of Sanger and next-generation sequencing (21). To evaluate the relative performance of both methods, we calculated association statistics of the classical HLA alleles for the imputed and sequence-based types and compared them. We observed good agreement in terms of the effect estimate (Fig. 1A–C) and significance (expressed as the Z-score) (Fig. 1D–F) of classical alleles identified by both approaches (Pearson $r^2 > 0.8$ for all genes).

However, we did observe greater HLA allelic diversity from the sequencing data, documenting 25 *HLA-A*, 34 *HLA-B* and 20 4-digit *HLA-C* alleles in this sample compared with 21 *HLA-A*, 26 *HLA-B* and 19 *HLA-C* alleles from imputation. Notably, HLA-B*58:02, previously associated with high virus load (13), was not present in our imputation reference panel so could not be included in the analyses based on imputation. In contrast, from the sequencing data, we found HLA-B*58:02 to be associated with progression [$OR = 0.4$, where an $OR < 1$ indicates progression and $OR > 1$ indicates control, $P = 4.9 \times 10^{-3}$]. Thus, direct sequencing of HLA alleles provides a more complete data set for fine-mapping of the genetic contributors to HIV-1 control in African Americans, when compared with imputation from a relatively small reference sample.

Multiple classical HLA alleles associate with HIV-1 control in African Americans

We next examined the impact of class I HLA genes on HIV-1 control using logistic regression, including covariates to control for population structure. The association signal was

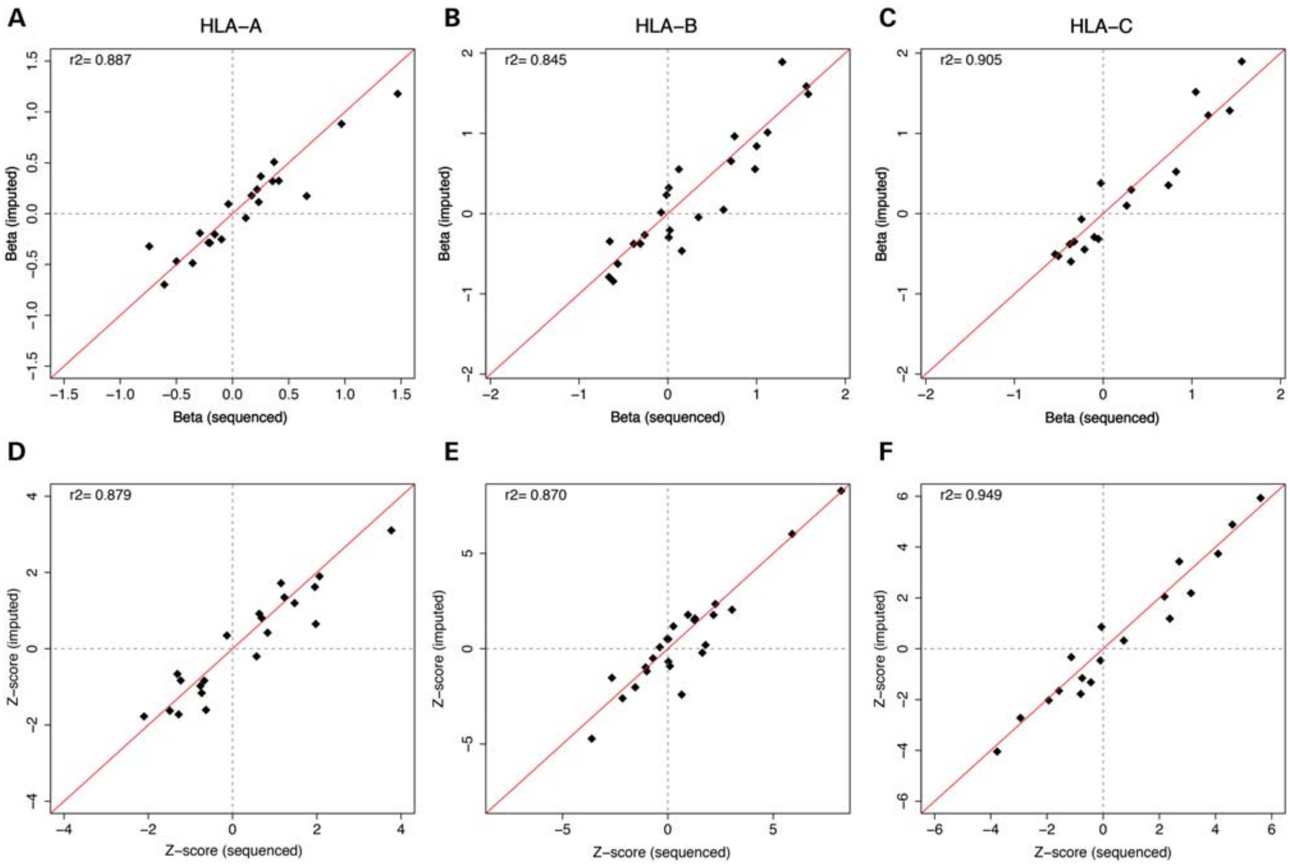

Figure 1. Comparison of sequence-based and imputed classical HLA allele association results. For each HLA allele called by both sequencing and imputation, the point estimate of the beta (**A**–**C**) and Z-score (**D**–**F**) from logistic regression models using sequence types (*x*-axis) or imputed dosages (*y*-axis) is plotted. Pearson r^2 is given for each comparison and the red line indicates a perfect correlation.

the strongest for *HLA-B,* where 14 alleles were nominally associated (all univariate $P < 0.05$, Table 1). The alleles B*57:03 (OR = 5.1; $P = 3.4 \times 10^{-18}$) and B*81:01 (OR = 4.8; $P = 1.3 \times 10^{-9}$) showed the strongest associations consistent with previous reports in populations with African ancestry (13–15). As in Europeans (6), B*57:01 (OR = 2.7; $P = 3.8 \times 10^{-2}$), B*14:02 (OR = 2.4; $P = 4.2 \times 10^{-3}$), B*27:05 (OR = 2.4; $P = 4.4 \times 10^{-2}$) and B*52:01 (OR = 2.1; $P = 2.7 \times 10^{-2}$) are associated with protection and B*35:01 (OR = 0.5; $P = 8.5 \times 10^{-3}$) is associated with progression in African American HIV-1 controllers/progressors. The remaining associated *HLA-B* alleles, B*45:01 (OR = 0.2; $P = 1.4 \times 10^{-5}$), B*39:10 (OR = 4.6; $P = 5.4 \times 10^{-4}$), B*53:01 (OR = 0.6; $P = 1.3 \times 10^{-3}$), B*57:02 (OR = 4.5; $P = 3.8 \times 10^{-3}$), B*58:02 (OR = 0.4; $P = 4.9 \times 10^{-3}$), B*15:10 (OR = 0.5; $P = 3.2 \times 10^{-2}$) and B*42:01 (OR = 0.6; $P = 3.6 \times 10^{-2}$), are all either absent or present only at low frequency in European populations.

To address which of these *HLA-B* alleles are independently associated, we next performed stepwise selection considering all nominally associated alleles. Of the 14 alleles listed above, 10 (B*57:03, B*81:01, B*45:01, B*39:10, B*57:02, B*14:02, B*52:01, B*27:05, B*57:01 and B*58:02) are independently associated (Table 1), demonstrating the key contribution of multiple *HLA-B* alleles to HIV-1 control and progression.

Adjusting for *HLA-B* effects, we next tested for the independent association of classical alleles at both *HLA-A* and *HLA-C* (Supplementary Material, Table S2). Of the 25 *HLA-A* and 20 *HLA-C* alleles tested, only A*31:01, C*05:01, C*08:04 and C*12:03 showed evidence of association after adjusting for either the 10 independent *HLA-B* alleles listed above or (more conservatively) all *HLA-B* alleles (Supplementary Material, Table S2). These data show that the major determinants of host control are localized to *HLA-B*, with a minor role for *HLA-A* and *HLA-C* alleles.

Variable amino acid positions in HLA-B associate with host control

To assess the extent to which specific amino acid positions within HLA-B may impact HIV-1 control, we tested each variable position by effectively grouping classical *HLA-B* haplotypes according to the amino acid carried at each position. In this analysis, a biallelic position corresponds to a 1-degree of freedom test, whereas positions accommodating more than two possible alleles require multiple degrees of freedom in an omnibus test (Supplementary Material, Table S3). Several positions in HLA-B are highly associated with host control (Fig. 2) with position 97 (omnibus $P = 1.2 \times 10^{-21}$) showing the most significant association in this

Table 1. Association results for classical *HLA-B* alleles sorted by OR

Allele	Frequency in progressors	Frequency in controllers	OR (95% CI)	*P*-values
B*57:03	0.035	0.145	5.1 (3.5–7.3)	3.4×10^{-18}
B*81:01	0.016	0.072	4.8 (2.9–8.0)	1.3×10^{-9}
B*39:10	0.005	0.025	4.6 (1.9–10.8)	5.4×10^{-4}
B*57:02	0.004	0.016	4.5 (1.6–12.6)	3.8×10^{-3}
B*57:01	0.006	0.015	2.7 (1.1–6.9)	3.8×10^{-2}
B*14:02	0.013	0.033	2.4 (1.3–4.5)	4.2×10^{-3}
B*27:05	0.007	0.016	2.4 (1.0–5.8)	4.4×10^{-2}
B*52:01	0.013	0.026	2.1 (1.1–4.2)	2.7×10^{-2}
B*15:01	0.006	0.010	1.8 (0.6–5.0)	2.8×10^{-1}
B*44:03	0.049	0.066	1.4 (0.9–2.0)	1.3×10^{-1}
B*41:02	0.008	0.010	1.3 (0.5–3.2)	6.1×10^{-1}
B*58:01	0.042	0.048	1.1 (0.7–1.8)	5.5×10^{-1}
B*13:02	0.010	0.012	1.1 (0.5–2.8)	7.7×10^{-1}
B*40:01	0.011	0.012	1.0 (0.4–2.5)	9.5×10^{-1}
B*15:03	0.050	0.049	1.0 (0.7–1.6)	9.4×10^{-1}
B*07:05	0.007	0.007	1.0 (0.3–3.0)	9.4×10^{-1}
B*44:02	0.018	0.016	0.9 (0.4–1.9)	7.8×10^{-1}
B*07:02	0.071	0.063	0.9 (0.6–1.3)	4.9×10^{-1}
B*14:01	0.012	0.010	0.8 (0.3–2.1)	6.8×10^{-1}
B*15:16	0.021	0.016	0.8 (0.4–1.6)	5.2×10^{-1}
B*08:01	0.032	0.025	0.8 (0.4–1.4)	3.9×10^{-1}
B*51:01	0.021	0.015	0.7 (0.3–1.5)	3.7×10^{-1}
B*42:02	0.007	0.005	0.7 (0.2–2.5)	5.9×10^{-1}
B*53:01	0.129	0.079	0.6 (0.4–0.8)	1.3×10^{-3}
B*42:01	0.054	0.033	0.6 (0.2–2.5)	3.6×10^{-2}
B*18:01	0.028	0.016	0.6 (0.3–1.2)	1.2×10^{-1}
B*49:01	0.020	0.012	0.6 (0.2–1.3)	1.7×10^{-1}
B*35:01	0.065	0.035	0.5 (0.3–0.8)	8.5×10^{-3}
B*15:10	0.036	0.018	0.5 (0.3–0.9)	3.2×10^{-2}
B*58:02	0.045	0.018	0.4 (0.2–0.8)	4.9×10^{-3}
B*78:01	0.011	0.003	0.3 (0.1–1.3)	1.1×10^{-1}
B*37:01	0.006	0.002	0.3 (0.0–2.3)	2.4×10^{-1}
B*50:01	0.013	0.003	0.3 (0.1–1.1)	6.6×10^{-2}
B*45:01	0.067	0.016	0.2 (0.1–0.4)	1.4×10^{-5}

ORs and *P*-values were computed by logistic regression including principal components to correct for population structure. ORs are for each allele compared with all others and expressed such that alleles at a higher frequency in HIV-1 controllers have OR > 1.

study. Position 97 accommodates six amino acid alleles and is more significant than the classical B*57:03 allele, the strongest association for HIV-1 host control reported to date in African Americans (15).

Given that *HLA-B* is among the most polymorphic genes in the human genome, we tested how likely such a result could emerge by chance tagging of classical *HLA-B* alleles with differential impact on HIV control. We performed a permutation analysis that effectively shuffles the amino acid haplotypes assigned to a given classical allele while holding classical allele type and phenotype constant per individual (6). The result of 10 000 such permutations showed that the association at position 97 is unlikely to be explained by spurious tagging (permutation $P = 0.025$). This provides further evidence of the importance of position 97 in HIV-1 control.

In order to identify additional amino acid associations within HLA-B, we used stepwise conditional haplotype analysis. Using this procedure, we further identified positions 245, 116 and 63 (Table 2 and Supplementary Material, Table S4). After controlling for these four positions (and the haplotypes they form), addition of further amino acids into the regression model failed to improve the goodness-of-fit ($P > 0.05$). A model including positions 97, 245, 116 and 63 explains 25.6% of the variation in HIV-1 control in this sample as calculated using Nagelkerke's approximation (22).

Relationship between classical HLA-B alleles and associated amino acid positions

The four amino acid positions in HLA-B form 18 haplotypes whose effects run from strong HIV control to progression (Table 3). Adjusting for the amino acid alleles at these positions, and the haplotypes they form, explains the association at the independently significant classical *HLA-B* alleles (conditional $P > 0.05$ for all). Position 97, which lies at the bottom of the beta-sheet in the canonical C pocket accommodates six amino acid alleles (Fig. 3A). This position explains the protective effect of the B*57 alleles (i.e. 57:01, 57:02 and 57:03) which all carry Val97 ($OR = 4.6$, $P = 8.0 \times 10^{-20}$) and of B*27:05, which carries Asn97 ($OR = 2.4$, $P = 2.2 \times 10^{-2}$).

Position 116 also lies at the bottom of the beta-sheet in the canonical F pocket, spatially adjacent to position 97, and accommodates multiple amino acid alleles associated with host control (Fig. 3A). This position explains the protective alleles B*14:02 and B*39:10, which carry Phe116 ($OR = 2.0$, $P = 4.2 \times 10^{-4}$), and B*52:01, which carries Tyr116 ($OR = 1.7$, $P = 9.2 \times 10^{-8}$), and the risk alleles B*45:01 and B*58:02, which carry Leu116 ($OR = 0.4$, $P = 1.3 \times 10^{-6}$) and Ser116 ($OR = 0.6$, $P = 1.4 \times 10^{-6}$), respectively.

The biallelic position 63 (Asn63Glu) lines the edge of the peptide-binding groove. Carrying Glu63 is associated with HIV-1 control in this sample ($OR = 1.4$, $P = 1.5 \times 10^{-3}$), while Asn63 lies on several risk haplotypes. Position 245 outside of the groove in the $\alpha 3$ domain is also biallelic (Ala245Thr), with Thr245 being strongly protective ($OR = 5.1$, $P = 8.8 \times 10^{-10}$) (Fig. 3A). In this sample, only the classical HLA-B*81:01 allele carries Thr245 with all other alleles carrying Ala245. Interestingly, certain amino acid alleles at these positions lie on both protective and risk haplotypes (e.g. Glu63), highlighting that combinations (haplotypes) of amino acids, rather than any single position, may be required for durable HIV-1 control.

Replication of HLA-B association results in an independent sample

In order to replicate the above findings, we accessed an independent cohort of African American individuals with multiple measurements of plasma HIV-1 RNA concentration at set point. This sample is a subset of individuals enrolled in the US Department of Defense Human Immunodeficiency Virus Natural History Study (DoD HIV NHS) that were part of a previous GWAS (15). From this data set, we inferred amino acid variants in HLA-B in 216 individuals for whom classical allele types were available (using standard HLA definitions). Association testing of amino acid positions was performed using linear regression, including principal components based on GWAS data to correct for population structure, age and sex as covariates.

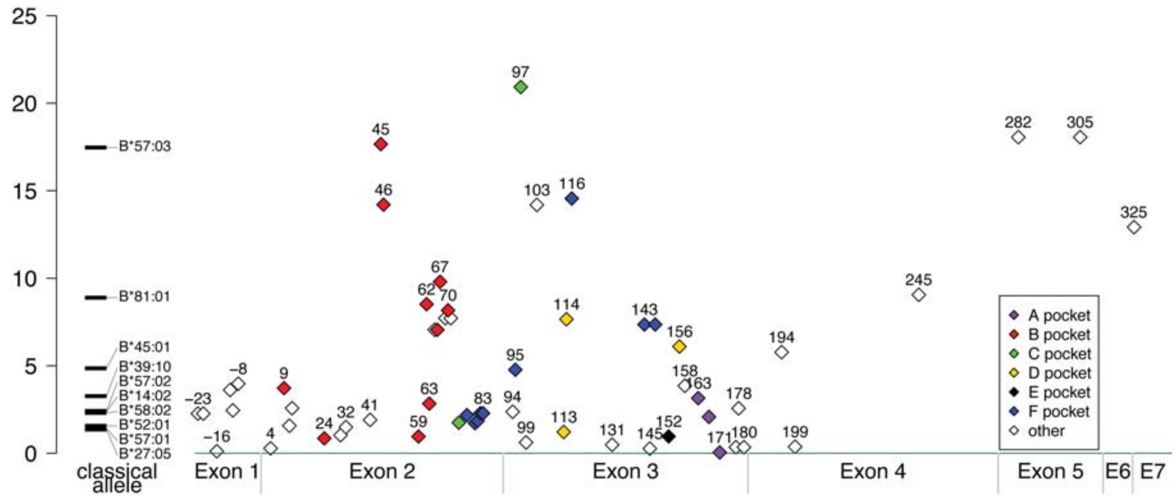

Figure 2. Association results ($-\log_{10}$ *P*-value) for all variable amino acid positions in HLA-B. African American HIV-1 controllers were compared with progressors at each position using logistic regression including covariates to correct for populations structure. For amino acid positions accommodating more than two possible alleles (such as position 97), the multi-degree of freedom omnibus test *P*-value is shown. Positions are colored according to canonical pockets of the peptide-binding groove. Classical 4-digit *HLA-B* allele association results are shown for comparison on the left.

Overall good agreement was observed between ORs from controllers/progressors and the beta coefficients in the DoD HIV NHS sample for the independent positions identified in controllers (Fig. 3B and Supplementary Material, Table S5). Again, HLA-B position 97 shows the strongest association of all variants tested (omnibus $P = 1.7 \times 10^{-7}$) with Val97 decreasing ($\beta = -0.868$, $P = 5.0 \times 10^{-9}$) and Arg97 increasing ($\beta = 0.148$, $P = 2.1 \times 10^{-2}$) virus load. Asn97, carried on the B*27:05 haplotype, was not observed in this sample. At position 116, both Tyr116 ($\beta = -0.140$, $P = 3.1 \times 10^{-2}$) and Leu116 ($\beta = 0.258$, $P = 1.2 \times 10^{-2}$) are nominally associated with the same effect directions as observed in controllers/progressors (Fig. 3B). However, Thr245 ($\beta = -0.469$, $P = 0.07$) and Glu63 ($\beta = -0.029$, $P = 0.64$) were not nominally significant, although both decrease virus load in the DoD HIV NHS sample (consistent with results in controllers/progressors). This lack of statistical replication may be due to the much smaller sample, which provides <45% power to detect a variant that explains 2% of the trait variance (the approximate effect size of Thr245) at nominal significance ($P < 0.05$). Overall, the consistency of the effect directions observed across the amino acids lends support to these positions mediating HIV-1 control.

Amino acid positions in HLA-B explain the majority of associations in HLA-A and HLA-C

We next assessed the extent to which amino acid positions in HLA-A and -C contribute to HIV-1 control. Adjusting for effects at HLA-B positions 63, 97, 116 and 245, we individually tested each variable position in HLA-A and HLA-C for association and an improved model fit. Of the amino acids tested, only position 304 in HLA-C showed an improved goodness-of-fit ($P = 5.6 \times 10^{-3}$). Position 304 is located in the transmembrane domain and is biallelic (Cys304Met), with Met304 occurring at a higher frequency in HIV-1 controllers than progressors (OR = 1.5, $P = 3.5 \times 10^{-3}$). Interestingly, this position was also identified by our previous analysis in European HIV-1 controllers and validated in an independent sample from the Swiss HIV Cohort Study (6). Including position 304 to the multivariate amino acid model increased the explained variance in host control from 25.6 to 26.4% in this sample.

Conditional analysis of associated classical *HLA-A* and *-C* alleles in the presence of the four amino acid positions in HLA-B and position 304 in HLA-C shows no residual signal at A*31:01, C*05:01, C*08:04 and C*12:03 ($P > 0.05$). Thus, these five positions are collectively able to explain all classical allele effects in the class I region. The improved model fit and consistency with results in Europeans suggest an interesting role for position 304 in HLA-C (or an unrecognized functional variant in LD with it), and requires further investigation.

Amino acids in HLA proteins explain the majority of the SNP association signal

In order to resolve the relation between the tag SNPs previously identified in African American HIV-1 controllers/progressors by GWAS (6) and the amino acids highlighted above, we performed haplotype analysis while accounting for the admixture in this population (see Supplementary note and Fig. S1). This analysis showed a complex structure of LD between the SNPs and amino acids with alleles at each SNP tagging multiple protective and risk HLA variants (Supplementary Material, Table S6).

We next tested for evidence of residual association at the four tag SNPs accounting for the effects of the identified amino acids. Three of the four SNPs showed evidence for an improved model fit (model comparison $P < 0.05$, Supplementary Material, Table S7) with rs2523608 (the strongest associated SNP) demonstrating the largest improvement ($P = 3.6 \times 10^{-3}$). This suggests that additional MHC variation,

Table 2. Association results and model comparisons of the independent amino acid positions in HLA-B identified by stepwise conditional haplotype analysis (see Materials and Methods)

Step	Position	Alleles	Location	Position *P*-value	Model comparison *P*-value
1	97	V/N/S/T/W/S	C pocket	1.2×10^{-21}	—
2	245	T/A	a3 domain	8.8×10^{-10}	4.1×10^{-12}
3	116	F/Y/D/S/L	F pocket	2.8×10^{-15}	1.0×10^{-10}
4	63	E/N	B pocket	1.5×10^{-3}	1.1×10^{-3}

Positional *P*-values are given where a 1-degree of freedom test is used for the biallelic positions 245 and 63 while positions 97 (six alleles) and 116 (five alleles) require multiple degrees of freedom. Model comparison *P*-values are calculated using the LRT where a model including the position identified in that step (and all previous steps) is tested against the model without the newly implicated position.

possibly outside of classical HLA genes, may contribute to HIV-1 control.

Residual SNP association in African American HIV-1 controllers/progressors is not due to a miRNA-binding site deletion

A proposed explanation for the SNP signal not accounted for by amino acids in HLA-B is an *HLA-C* eQTL effect (19) mediated through an insertion/deletion polymorphism (rs67384697) in a microRNA-binding site in the 3′ UTR of *HLA-C* (20). However, this effect has not been investigated in populations with African ancestry. As all classical *HLA-C* alleles can be categorized as carrying either the deletion (high *HLA-C* expression) or insertion (low expression) polymorphism [(20,23) and Supplementary Material, Table S8], we used *HLA-C* types to infer genotype at this site and tested for association with HIV-1 control. Using either an additive (per allele) or dominant (del/del + del/ins versus ins/ins) model, we found no association between carrying the deletion and HIV-1 control ($P_{\text{additive}} = 2.9 \times 10^{-1}$ and $P_{\text{dominant}} = 2.8 \times 10^{-1}$). The lack of association was maintained in a model including the four independent amino acid positions in HLA-B ($P_{\text{additive}} = 7.0 \times 10^{-2}$ and $P_{\text{dominant}} = 1.1 \times 10^{-1}$). Although limited power in this sample may explain the lack of evidence for association of the deletion carrying high expression *HLA-C* alleles with HIV-1 control, this result does suggest that an *HLA-C* eQTL effect mediated through rs67384697 is not sufficient to explain the residual SNP signal.

DISCUSSION

The present study demonstrates the importance of HLA-B in durable host control of HIV-1 in African Americans. Improving upon our previous HLA allele imputation analysis, we show multiple classical *HLA-B* alleles are associated with control/progression, all of which can be explained by variants at amino acid positions 63, 97, 116 and 245 in the HLA-B protein. We also show good consistency of the effect direction of these amino acids in an independent cohort, where all alleles that associate with HIV-1 control also decrease virus load set point. Together, these four positions account for >25% of the observed trait variance. Controlling for these main effects, we observed a residual signal mapping to amino acid position 304 in HLA-C and further residual SNP signals, suggesting a role for additional MHC variation in mediating HIV-1 control.

Multiple independent SNP associations with HIV-1 control have been observed in the MHC region in African Americans (6,15). In order to fine map these SNP associations, we obtained classical class I HLA types in 1107 African American HIV-1 controllers/progressors. Compared with our previous effort where we imputed classical allele and amino acid types, a higher level of HLA diversity was observed at all class I genes through sequencing. Additionally, sequencing provides direct genotypes rather than genotypic probabilities (calculated by imputation), reducing uncertainty (particularly for low frequency alleles). Although the sequence-based types analyzed here are an improvement over the previously imputed types (particularly for fine-mapping), it should be noted that the consistency of association results of the alleles called by both methods suggests that a larger reference panel that captures more classical HLA allele diversity in populations of African ancestry (such as the one generated in this study) would improve the performance of imputation.

The 10 independent classical *HLA-B* alleles identified in controllers are consistent with previous work. In a study of 1211 HIV-1-infected individuals from South Africa B*57:03, B*57:02, B*81:01 and B*39:10 (which all associate with HIV-1 control) decreased virus load, while B*58:02 and B*45:01 (associated with progression) increased virus load (14). Only B*14:02 (associated with control) failed to show the same trend in the South African sample, possibly due to low power or clade-specific effects. The remaining alleles, B*57:01, B*27:05 and B*52:01, were not observed in the South African sample. However, the effect direction in African American HIV-1 controllers is consistent with Europeans for each of these alleles. Taken together, these results confirm the causal role of HLA-B in controlling HIV-1 replication and highlight the impact of multiple classical alleles at this locus on host control.

Testing individual amino acid positions has proved fruitful in mapping key residues that contribute to complex phenotypes with strong evidence for classical HLA allele associations (6,24,25). To define the specific amino acids that contribute to the observed classical allele signal, we tested variable positions within HLA-B for association. This strategy amounts to grouping classical alleles at each position based on the amino acid residue they carry, and tests the overall contribution of that position to control or progression. Using this strategy, position 97 shows the strongest signal of any

Table 3. Haplotype structure relating amino acid alleles at the independently associated positions to significant classical *HLA-B* alleles ordered by OR. ORs were calculated taking the haplotype with the lowest frequency difference between cases and controls as reference. *P*-values are for each haplotype tested against all others. Only haplotypes with frequency above 1% were included accounting for >95% of the haplotype diversity

Classical allele	63	97	116	245	Frequency in progressors	Frequency in controllers	*P*-values	OR (95% CI)
81:01	N	S	Y	T	0.012	0.059	7.6×10^{-8}	4.8 (2.2–10.7)
57:02 57:03	E	V	Y	A	0.031	0.163	1.3×10^{-15}	4.4 (2.4–8.1)
39:10	N	R	F	A	0.009	0.037	9.2×10^{-5}	4.2 (1.7–10.0)
57:01	E	V	S	A	0.010	0.013	4.5×10^{-5}	2.6 (1.1–6.2)
27:05	E	N	D	A	0.009	0.023	1.5×10^{-2}	2.6 (1.0–6.5)
52:01	E	T	Y	A	0.017	0.030	7.4×10^{-2}	1.6 (0.7–3.6)
14:02	N	W	F	A	0.023	0.044	3.2×10^{-2}	1.6 (0.8–3.3)
	E	R	Y	A	0.027	0.014	4.2×10^{-3}	1.2 (0.5–2.7)
	E	T	L	A	0.011	0.012	7.2×10^{-1}	Reference
	E	R	S	A	0.078	0.125	6.5×10^{-1}	0.9 (0.5–1.6)
	E	R	D	A	0.072	0.086	9.7×10^{-1}	0.8 (0.5–1.5)
	E	S	Y	A	0.037	0.013	1.7×10^{-1}	0.8 (0.4–1.7)
	N	S	Y	A	0.148	0.142	1.1×10^{-1}	0.7 (0.4–1.1)
	N	R	S	A	0.268	0.138	4.6×10^{-8}	0.4 (0.3–0.8)
	N	R	Y	A	0.035	0.021	1.2×10^{-1}	0.4 (0.2–1.0)
	N	T	Y	A	0.030	0.018	7.8×10^{-2}	0.3 (0.1–0.8)
58:02	E	W	S	A	0.046	0.015	1.1×10^{-3}	0.2 (0.1–0.6)
45:01	E	R	L	A	0.101	0.029	2.2×10^{-8}	0.2 (0.1–0.4)

variant tested. Position 97 sits at the bottom of the peptide-binding cleft (Fig. 4A) and is critical for HLA protein conformation and folding, affecting both epitope presentation and surface expression (26). This single position accommodates six amino acid alleles with disparate effects on host control. This result is consistent with observations in European HIV-1 controllers, and was replicated in the independent DoD HIV NHS sample underscoring the key effect of position 97 across continental populations.

We further identified positions 116 and 63, also in the HLA-B peptide-binding groove (Fig. 4A), as independent contributors to HIV-1 control. Position 116 is located in the canonical F pocket, sits adjacent to position 97 on the floor of the beta-sheet and contributes to epitope selection and binding. In addition to influencing epitope specificity, studies of variation at this position have shown that certain alleles (particularly Tyr116) can determine the peptide loading pathway used (27), highlighting the importance of this position to antigen processing. Position 63 lies in the α1 domain and contributes to the shaping of the B pocket. Like position 97, this position was identified in HIV-1 controllers of European ancestry as independently contributing to host control (6). That three positions in the binding groove are identified in this sample as key mediators of HIV-1 control supports the hypothesis that the peptide/HLA-B interaction is critical in determining disease course. Functional studies designed to measure the impact both on the virus and the host CD8+ T cell response as residues at these positions vary (while keeping the amino acid sequences at all other positions constant) would be of great interest, as the nature of the CD8+ T cell response and the impact that HLA has on viral fitness have been implicated in mediating HIV control (28–31). Such studies would be most useful for informing vaccine trials as to the types of responses that are beneficial, as inducing the controller phenotype in individuals that would otherwise progress could have a large impact on the pandemic (1).

In addition to the positions within the peptide-binding groove, the newly implicated non-groove position 245 in HLA-B is located in the α3 domain (Fig. 4B) and is monomorphic in Europeans with all classical alleles carrying alanine. Thr245 defines the strongly protective B*81:01 allele. Variation at this position, although outside of the peptide-binding cleft, has been shown to affect the binding kinetics between HLA class I and CD8α homodimers (32,33) as this position contributes to the shape of the α3 domain, which directly interacts with CD8 (Fig. 4B). HLA-B*48, present in Asian and Native American populations, also carries Thr at position 245 and has been shown to bind CD8α with lower affinity than alleles with Ala245, an effect that was ablated upon a Thr245Ala substitution (33). Interestingly, B*81:01 and B*42:01 carry the same amino acid alleles at positions 63, 97 and 116 but have opposite impacts on HIV control, with B*42:01 being a putative risk allele ($OR = 0.6$, $P = 0.04$). Although these alleles present the same epitopes (including the immunodominant Gag TL9 epitope), B*81:01 has been shown to have an increased ability to present variant epitopes and has a higher functional avidity than B*42:01 (34,35). Although these two alleles vary at more than just position 245 (and thus other positions cannot be ruled out as contributors to the observed differences of effect), it is possible that the interaction between CD8 and the α3 domain may partially explain these functional differences. Further studies are warranted to determine the precise effect of variation at position 245 on the MHC/CD8 interaction and the functional consequences this has to the general anti-HIV response. Such analyses could potentially lead to the development of therapies that seek to mimic this function in individuals with progressive disease.

The role of *HLA-C* in HIV-1 control is less clear. After conditioning on positions 97, 245, 116 and 63 in HLA-B, only the dimorphic position 304(Met/Val) located in the transmembrane domain of HLA-C remains associated. Including this position in a regression model shows a better goodness-of-fit

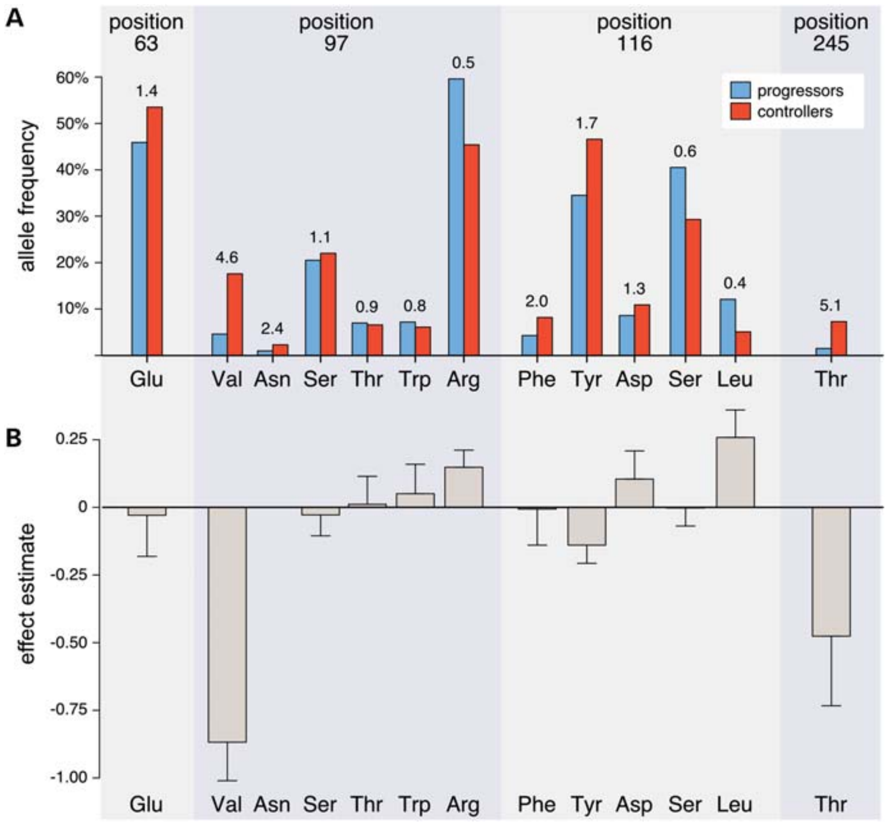

Figure 3. Frequency and effect sizes for key HLA-B amino acids in HIV-1-infected African Americans. (**A**) Allele frequency differences between controllers (in orange) and progressors (in blue) for amino acids at positions 63, 97, 116 and 245 in HLA-B. Numbers above bars are ORs where OR >1 indicates a protective effect. (**B**) Beta estimates of alleles at positions 63, 97, 116 and 245 in the DoD HIV NHS sample. Asn97 was not observed in this sample. The beta estimates are from linear regression models including covariates and are given in $\log_{10}$ units of virus load set point.

than the HLA-B only amino acid model and explains the signal of the three associated *HLA-C* alleles (C*12:03, C*05:01 and C*08:04) which all carry the protective Met304 residue. Interestingly, this position was also selected by stepwise regression in HIV-1 controllers/progressors of European ancestry, with Met304 associating with control (6). After conditioning on all five amino acid positions, no classical alleles remain significantly associated. Thus, these five amino acid positions fully explain all classical allele associations in this sample.

Although the mechanism by which variation at position 304 in HLA-C may mediate HIV-1 control is not obvious, the transmembrane domain has been implicated in influencing HLA class I cell surface levels and immune response to alloantigens (36,37), supporting a mechanism other than peptide presentation. In individuals of European ancestry, an *HLA-C* expression effect, influenced by a microRNA-binding site insertion/deletion polymorphism, has been proposed to contribute to HIV-1 control (20). Using *HLA-C* classical allele type as a proxy for the insertion/deletion (20,23), we did not observe evidence for association in this African American sample. Further studies, including full sequencing of the *HLA-C* 3′ UTR, are required to fully understand the impact of *HLA-C* variation on HIV-1 control in multiple populations.

The results presented here underscore the value of genetic studies in multiple populations to confirm potentially causal variants and to discover novel associations. We employed HLA allele and amino acid association testing to resolve SNP signals within the MHC of African American HIV-1 controllers and progressors. Our results strongly support the role of position 97 in the HLA-B-binding cleft as the key mediator of HIV-1 control. We provide additional evidence that positions 245, 116 and 63 in HLA-B and position 304 in HLA-C also contribute to host control. Taking these effects into account, residual evidence for association exists in the MHC region. Understanding this residual association will require larger samples with full characterization of variation across this region. Community-wide meta-analyses and sequencing studies are needed to more fully capture host genetic variation that mediates HIV-1 control.

MATERIALS AND METHODS

Sample collection

Details of the sample collection have been previously reported (6). Briefly, 1528 HIV-1 controllers, defined as individuals that maintain plasma virus load below 2000 copies/ml in the absence of antiretroviral therapy, with a minimum of three determinations spanning at least a 12-month period, were recruited through outpatient clinics (http://www.hivcontrollers.org/membermap). A total of 2975 antiretroviral

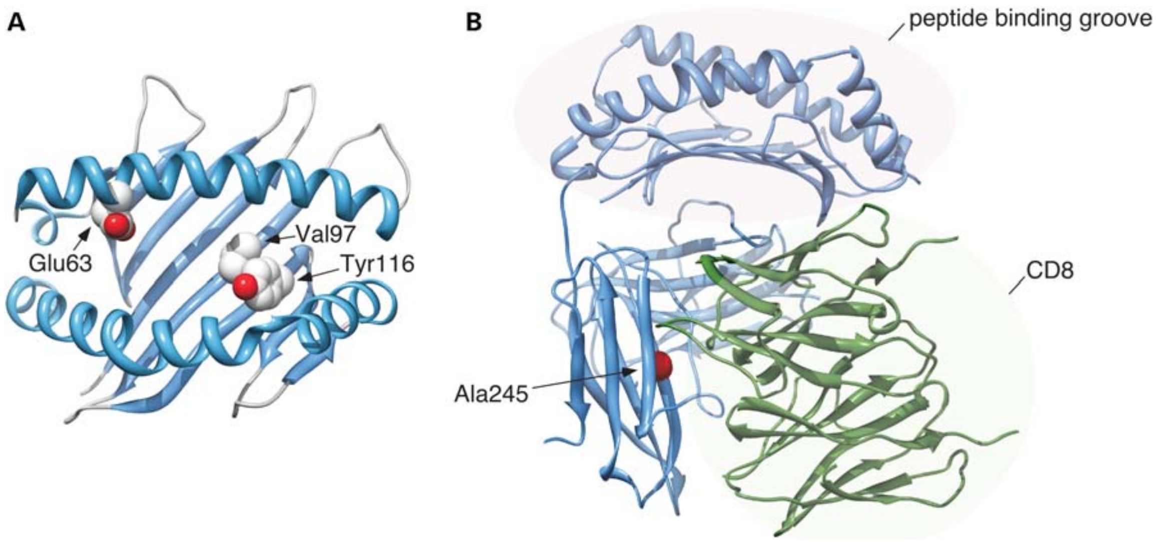

Figure 4. Three-dimensional structure of HLA-B highlighting key amino acid positions. (**A**) The HLA-B protein based on Protein Data Bank entry 2BVP looking in to the peptide-binding groove (44). The independently associated pocket positions 63, 97 and 116 are highlighted. (**B**) Side view of the HLA-B molecule (blue) interacting with CD8 (green) based on Protein Data Bank entry 1AKJ with position 245 highlighted (45). Variation at position 245 has been shown to influence the binding kinetics between the CD8α subunit and the HLA molecule (32,33). The figure was prepared with UCSF Chimera (46).

naïve individuals with progressive HIV-1 infection were recruited through the AIDS Clinical Trials Group (protocols ACTG384, A5095, A5142 and A5202) and consented for genetic testing under protocol A5128. All progressors were recruited prior to standardized screening for B*57:01 to exclude individuals at high risk of abacavir hypersensitivity. All subjects gave written informed consent and institutional review boards for all participating centers approved the study protocol. Ancestry was assessed using genome-wide SNP data over a set of high-quality (>99% call rate), unlinked SNPs using EIGENSTRAT (38) to project controllers and progressors onto HapMap 3 populations (39). For the present analysis, only individuals clustering with the African Americans from the southwestern US (ASW) population were retained.

Classical HLA class I allele and amino acid imputation and sequencing

Methods for imputing classical HLA alleles and amino acids in this sample have been previously described (6). Briefly, a reference panel was constructed from a subset of the HIV-1 controllers ($n = 596$) with both genome-wide SNP data and 4-digit HLA types. To eliminate bias due to sample overlap an iterative, leave-one-out, imputation procedure was performed, where a sample was removed from the reference panel when imputing alleles in that subject. Imputation was performed using default parameters in BEAGLE (10 iterations of phasing/imputation, testing 4 pairs of haplotypes for each individual at each iteration) (40). Alleles with low imputed frequency (<1%) were excluded from the analysis.

Sequenced-based HLA classical allele types in HIV-1 controllers were obtained on DNA samples using standard Sanger sequencing of exons 2 and 3 and/or single-stranded conformation polymorphism PCR. The standardized protocols for HLA genotyping by this method, according to International Histocompatibility Working Group (http://www.ihwg.org), were followed. In progressors, *HLA-A*, *B* and *C* types were obtained by sequencing exons 2 and 3 on the 454 FLX Titanium platform coupled to a novel HLA calling algorithm that has shown good concordance with standard Sanger methodology (21). Amino acid variants for all classical alleles were inferred using definitions for HLA allele sequences from the EMBL-EBI Immunogenetics HLA database (41). In individuals with classical allele types having incomplete protein sequence information, the genotypes at the unresolved positions were set to missing. Comparison of association results from imputation and sequencing were performed in the R statistical package version 2.12 (http://www.r-project.org).

Association testing and stepwise regression modeling

Classical HLA alleles and amino acid residues were tested for association with HIV-1 control using logistic regression in PLINK version 1.07 (42). For sequencing data, classical alleles and amino acid genotypes were tested directly. For imputed types, genotype dosages calculated by BEAGLE were used (to account for imputation uncertainty). To correct for population stratification, we added the top principal component calculated on genome-wide SNP by EIGENSTRAT (38) as a covariate in all models. This top PC was selected for inclusion because it is the only one that significantly associates with the phenotype ($P < 0.05$) and we have previously shown that its inclusion is sufficient to correct for inflation in this sample (6).

For classical HLA alleles, given the prior evidence for a primary role for *HLA-B*, we considered all nominally significant ($P < 0.05$) alleles as associated. To determine independence of effects for these alleles, we used simultaneous forward and backward stepwise regression in the statistical package R version 2.12 (http://www.r-project.org). To assess the association evidence at *HLA-A* and *-C*, we tested classical alleles at these genes controlling for the effects at either the independent *HLA-B* alleles or all *HLA-B* alleles. We only considered *HLA-A* and *-C* alleles with $P < 0.05$ in the univariate test and after controlling for *HLA-B* alleles as associated.

To determine independent effects of amino acid positions, we performed stepwise conditional haplotype testing followed by model comparison. In the first step, we built a model including population covariates and the top associated position (position 97 in HLA-B) and tested all remaining positions in that model. The top association in that analysis was then added to the regression model and the two models were compared using the likelihood ratio test (LRT). This procedure was repeated (i.e. comparing a model containing the next top association to one containing all previous positions) until the addition of the top associated position failed to show a significant model improvement compared with the previous model (LRT $P > 0.05$).

Using the resulting positions in HLA-B, we then tested all positions in HLA-A and -C. We required a multivariate $P < 5 \times 10^{-4}$ (to account for the number of positions tested) and an improved model fit than the HLA-B alone model using the LRT to consider a position as associated.

Permutation of amino acid results

To further address the significance of the top position in HLA-B, we devised a permutation scheme where, at each permutation, a given classical allele was randomly assigned one of the amino acid sequences that corresponds to a 4-digit type while case/control status and classical allele types were held constant. Association was then tested for each position. This randomization was carried out 10 000 times and a *P*-value was assigned based on how often the permuted result was more significant than what was observed in our sample. This test gives a quantitative indication of how likely the alleles of a given position divide the classical HLA alleles into differential risk groups by chance.

Replication set of African American individuals with virus load set point measurements

We acquired classical *HLA-B* allele types from an additional set of African American individuals recruited as part of the US Department of Defense HIV-1 Natural History Study (DoD HIV NHS) with multiple measurements of virus load at set point ($n = 216$). Genome-wide identity-by-state analysis was performed to ensure no overlap between this sample and the HIV controller/progressor sample. Details of HLA typing and association testing in this replication set have been published elsewhere (15). Association of HLA allele and amino acid types with virus load set point was tested using linear regression including the top principal component from EIGENSTRAT, age and sex as covariates. These covariates were previously shown to be sufficient to control for inflation in this sample (15). Power for variant detection was calculated using the online genetic power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) (43).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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