Virus specific CD8+ T cell responses better define HIV disease progression than HLA genotype†

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HLA alleles B57/58, B27, and B35 have the strongest genetic associations with HIV-1 disease progression. The mechanisms of these relationships may be host control of HIV-1 infection via CD8+ T-cell responses. We examined these immune responses in subjects from the Seattle Primary Infection Cohort with these alleles. CD8+ T-cell responses to conserved HIV-epitopes within B57/58 alleles (TW10 and KF11) and B27 alleles (KK10 and FY10) delayed declines in CD4+ T-cell counts (4–8x longer), while responses to variable epitopes presented by B35 alleles (DL9 and IL9) resulted in more rapid progression. Plasma viral load was higher in B57/58+ and B27+ subjects lacking the conserved B57/58 and B27 restricted responses. The presence of certain B57/58, B27, and B35 restricted HIV-specific CD8+ T-cells responses after primary HIV-1 infection better defined disease progression than HLA genotype alone suggesting that it is the HIV-specific CD8+ T-cells and not the presence of a particular HLA allele that determine disease progression. Further, the most effective host CD8+ T-cell responses to HIV-1 were prevalent within an HLA allele, high total allele fraction of the host CD8+ T-cell response, and targeting conserved regions of HIV-1. These data suggest that vaccine immunogens should contain only conserved regions of HIV-1.
INTRODUCTION

The toll of the HIV-1 pandemic continues to climb with a recent estimate of 33 million people living with HIV-1 (36). The time from HIV-1 infection to the onset of AIDS varies markedly among subjects, with the average being 8–10 years without antiretroviral therapy (28, 34). Some HLA class I alleles are associated with delayed progression to AIDS (e.g., B57/58 and B27), some are associated with rapid progression (e.g., B35Px alleles: B*3502, B*3503, B*3504, and B*5301) (10, 13), and others are associated with a more moderate course (e.g., B35PY alleles: B*3501 and B*3508). The mechanisms underlying these associations are unclear, although recent data have shown that unlike CD8+ T-cell responses restricted by other alleles, responses restricted by HLA B57/58 and B27 appear to be resistant to peripheral tolerance (14), as they retain their proliferative capacity throughout chronic infection. The differential function of cytotoxic T-lymphocytes (CTL) is very likely to be a critical component of host control of HIV-1 infection along with B-lymphocyte derived antibodies, helper T-lymphocyte function, natural killer-cell responses, and dendritic cell function (5). Better elucidation of the mechanisms of host control over HIV-1 disease progression would have enormous implications for future therapy and rational vaccine design. Therefore we assessed the breadth and magnitude of HIV-1-specific interferon-γ secreting T-cell responses in early HIV-1 infection in subjects possessing at least one of the above disease-altering HLA alleles. We examined the relationships between CD8+ T-cell responses restricted by these alleles and the decline in CD4+ T-cells counts and HIV-1 RNA viral load. These data provide the first clear evidence for CD8+ T-cell responses in early
infection directly altering HIV-1 disease progression. Furthermore, we show that the
most effective CD8+ T cell responses are those that are (i) prevalent within an HLA
allele, (ii) a high total allele fraction of the host CD8+ T-cell response, and (iii)
targeting conserved regions of HIV-1. These data suggest that next generation T-
cell-based HIV-1 vaccine candidates should contain only conserved regions of HIV-
1.
MATERIAL AND METHODS

Subjects. HIV-1 infected individuals were selected from the Seattle Primary Infection Clinic (PIC) cohort based on HLA genotype and sample availability. Appropriate Institutional Review Boards approved the studies and volunteers provided written informed consent.

Study Design. This study was designed as a four-armed nested case-control study comparing subjects with selected HLA-B alleles (B57/58, B27, B35Px, and B35PY) from the PIC cohort. The primary outcome was disease progression defined by declining CD4+ T-cell counts per mm$^3$ below thresholds (500, 350, and 200). Samples used in this study were drawn within 150 days of primary HIV-1 infection (PHI) (32). The PIC cohort estimated HIV-1 infection date was used as the date of infection; this is the symptom onset date for symptomatic subjects or the midpoint between last negative and first positive HIV test in those lacking symptoms. All subjects were antiretroviral therapy-naïve at the time of sampling. Most subjects were studied at a single time point n= 31, while some were sampled at two time points n=14.

CD4+ T-cell count and HIV-1 viral load assays. CD4+ T-cell counts were determined by the UW Laboratory Medicine clinical laboratory by combining percent CD4+ T-cells from flow cytometry and lymphocyte count from hemocytometry. HIV-1 viral load testing employed the Amplicor HIV-1 Monitor Test (Roche Molecular Systems, Inc.) with a lower limit of detection of 50 copies per mL before July 2003; thereafter, an in-house real-time RT-PCR method was used, with lower limit of
detection of 30 copies per mL (17). The HIV-1 plasma viral load was measured at
time point 1 for viral load analyses.

**Interferon-γ ELISpot.** Cryopreserved PBMC were thawed and rested
overnight at 37°C before use at 65,000-200,000 PBMC per well in interferon-γ
ELISpot assays (Mabtech) following the manufacturer’s guidelines. Synthesized
peptides corresponding to described HLA class I restricted CTL epitopes were
tested according to an individual’s HLA type. BioSyn, Corp., New England Peptide,
Inc., and Mimotopes synthesized the 8-11mer peptides. Peptides were used at a
final concentration of 2 µg ml⁻¹. PHA-P (0.5 µg ml⁻¹; Murex) served as a positive
control and media alone wells served as negative control. A positive interferon-γ
ELISpot result was designated when the number of spot forming cells (SFC) was
twice background (negative control) and at least 50 SFC per 10⁶ PBMC.

**HLA Class I typing.** Most HLA typing was performed by clinical laboratories
according to Clinical Laboratory Improvement Amendment (CLIA) accredited
techniques. Most was conducted at the Puget Sound Blood Center initially using
serology methods of lymphocyte lysis assays, later using sequence-specific primer
(SSP) polymerase chain reaction (PCR) at low resolution, and more recently by high
resolution SSP-PCR reagents from One Lambda, Inc. and Olerup SSP, Genovision,
now Qiagen Inc. A subset was analyzed at the Oklahoma Health Sciences
Laboratory using CLIA accredited sequence based typing (SBT) PCR (35).
Currently, a high throughput resequencing SBT-PCR is performed by Daniel E.
Geraghty at the Fred Hutchinson Cancer Research Center (31). A subset of
subjects were previously typed only at low resolution, so high resolution HLA-B35
typing was performed with LABType SSO Class I B Locus Typing Test, RSSO1B_011_BPI REV 1 One Lambda, Inc. on a Luminex instrument.

**Conservation Score Analysis.** For each 8-11mer peptide examined, the conservation score was represented by the frequency of occurrence of the peptide sequence in the HIV-1 M-group or B-subtype sequences in the Los Alamos HIV-1 sequence database of year 2005 (18). Only one sequence per subject was analyzed.

**Statistics.** Survival models were developed based on HLA genotype grouping alone and on the presence of good, moderate, or bad CTL responses using ESB1 and ESB2 categorizations. Kaplan Meier plots and Cox proportional hazard (PH) models were used to analyze the time from infection date to first time below threshold CD4+ T-cell counts. Subjects were censored when they left the cohort or initiated antiretroviral therapy (ART). The Cox PH models assumed censoring is independent of the CD4 decline event. Much of the censoring was due to ART initiation. However, at the time the data were collected (1994-2007), treatment was often initiated soon after diagnosis and for reasons other than CD4+ T-cell counts or viral load. Supplemental Fig. 1 illustrates the CD4 counts at the visit at least 3 days prior to ART initiation for those censored due to ART for the three thresholds; the majority of those beginning ART do not have CD4+ T-cell counts that would trigger ART initiation according to current guidelines (27); hence, the independent censoring assumption appears reasonable. Kruskal-Wallis tests were used to compare total ELISpot responses and viral loads across all groups and Wilcoxon-Mann-Whitney tests to compare two groups. All tests were two-sided with
a threshold of $p=0.05$. There were no adjustments for multiple testing. All analyses were conducted in R version 2.8.1.
RESULTS

Most subjects had symptomatic infection and 3 HIV-1-specific responses. We analyzed epitope specific HLA-B (B57/58, B27, and B35Px, and B35PY alleles, collectively referred to as ESB) restricted interferon-γ ELISpot responses in 45 subjects (B57/58 n=9, B27 n=7, B35Px n=12, and B35PY n=20) from the Seattle Primary Infection Clinic (PIC) cohort (Fig. 1). Like the parent cohort and the demographics of HIV-1-infection in the Pacific Northwestern United States, these subjects were mostly Caucasian men-who-have-sex-with men (MSM) (Table S1); we included one woman, one male self-identified as mixed race, and two males self-identified as Hispanic). Subjects were evaluated within 150 days of Primary HIV-1 Infection (PHI). Most had symptomatic PHI (n=40), while some were identified with asymptomatic infection by routine testing (n=5). The median time to evaluation was 48 days. A subset of 14 subjects was analyzed at a later time-point (time 2) at a median of 135 days post infection or onset of clinical symptoms of PHI (collectively referred to as DPI).

Due to limited peripheral blood mononuclear cell (PBMC) availability, subjects were only screened for HIV-1-specific responses to previously reported epitopes based on HLA genotype (16). This screening depended on sample availability according to the sequential hierarchy: ESB, other HLA-B, HLA-A, and then HLA-C (Tables S2 and S3). The median number of epitopes detected at time 1 by screening was 3 (range 1–10) (Fig. 2a), and the mean response magnitude was 560 SFC per 10⁶ PBMC (median: 384; range 62–2060). The total magnitude of responses restricted by HLA-B57/58, B27, and B35 were not significantly different.
(Kruskal Wallis (KW) p=0.4, Fig. 2b). Details of the 226 detected responses from 59 PBMC specimens are reported in Tables S4 and S5, and summarized in Table 1.

**HIV-1-specific responses doubled in the first four months of infection.**

Fourteen subjects had a second assessment of CTL responses four months after infection. In these, the number of epitopes detected and the total magnitude of responses within a subject increased at the second time point. However, the median magnitudes of interferon-γ secreting ELISpot responses from time points 1 and 2 were no different (Wilcoxon signed-rank (WSR) p=0.8). The median number of responses increased from 2 to 5 (range –2 to 6) (WSR p=0.004, Fig. 2c). The total magnitude of responses increased from a median of 821 to 1986 SFC per $10^6$ PBMC (range –203 to 3325) (WSR p=0.02, Fig. 2d). This underscores that the early CTL responses to HIV-1 detected by HLA based screening increases about two-fold from one to four months of infection.

**HLA genotype was a predictor of HIV-1 disease progression.** To better understand the impact of epitope specific CD8+ T-cell responses restricted by HLA-B57/58, B27, and B35, we conducted survival analyses of subjects grouped by their HLA genotypes: B57/58, B27, B35Px, and B35PY. Kaplan Meier (KM) survival curves were constructed along with Cox proportional hazards (PH) models of time until CD4+ T-cell counts first declined below threshold values of 500, 350 and 200 (Fig. 3). In the KM plots, there was a significant difference in at least one of the HLA groups from time to CD4+ T-cell count below 500, 350, and 200 (Table 2). In a Cox PH estimate that included the three HLA groups, only the B35 group was significantly faster to decline below the 500 and 350 CD4+ T-cell count thresholds.
than the B57/58 group, with hazard ratios (HR) of 5.8 and 9.7. For the <200 CD4+
T-cell count threshold, the B57/58 and B27 groups had 0/15 events, and the B35 (Px
or PY) had 6/30 events; the HR was infinite.

ESB restricted responses also predicted HIV-1 disease progression. We
also conducted survival analyses of subjects grouped by two different models of
epitope-specific HLA-B restricted responses (ESB1 and ESB2). The ESB models
were based on the hypothesis that CTL responses to conserved regions of HIV-1
would result in delayed progression, while CTL responses to variable regions would
result in HIV-1 disease progression. We arbitrarily defined conserved epitopes as
those with a frequency of identical sequences in the Los Alamos HIV-1 sequence
database greater than 70% for M-group or B-subtype virus sequences. We also
limited our analyses to responses present in at least half of each HLA group so that
a sufficient number of subjects would be present in CTL response groupings. We
selected the HLA-B57/B58 restricted TW10 and KF11 responses and the HLA-B27
restricted KK10 and FY10 responses as hypothetical key prevalent and conserved
responses. Likewise, we selected the highly prevalent and variable B35 restricted
DL9 and IL9 responses as the hypothetical variable responses.

Subjects were grouped into strata of good, moderate, and bad based on the
presence or absence of beneficial or detrimental ESB responses: for ESB1 good
(B27KK10+, B57TW10+), moderate (B27KK10−, B57TW10−, B35IL9−/DL9−), and
bad (B35IL9+/DL9+) were used. For ESB2 good (B27KK10+/FY10+, B57TW10+/KF11+), moderate (B27KK10−/FY10−, B57TW10−/KF11−, B35IL9−/DL9−), and bad (B35IL9+/DL9+) were used. The ESB1 & ESB2 models were
developed to attempt to discriminate the effects of the major versus minor
prevalence responses compared to HLA genotype alone. The ESB1 & ESB2
models only differed in the handling of the secondary B27FY10 and B57KF11
responses, and there was no difference in the B35 individuals. The B35 subjects,
whether Px or PY, could only be placed into the bad group if they possessed the
detrimental response to the highly variable IL9/DL9 epitopes, otherwise they were in
the moderate groups.

The KM plots for ESB1 and ESB2 groups showed significant differences
between groups in the time to CD4+ T-cell count decline below 500, 350, and 200
(Fig. 3), with smaller p-values than comparable HLA only grouped KM plots (Table
2). In a Cox PH model of the three ESB1 groups, there was a significant difference
between the moderate and good groups in the time to decline below 500 with a HR
of 8.2 (Table 2). There was also some evidence of a difference between groups in
time to the 350 threshold: hazard ratio of 8.2, and a significant difference for ESB1
bad vs. good strata for declines below thresholds of 500 and 350 with HR of 16 and
25. For the 200 threshold, the bad, moderate, and good groups had 3/12, 3/22, and
0/11 events; the HRs for moderate vs. good and bad vs. good were infinite.

Similar results were seen in the Cox PH of the three ESB2 groups. There
was a significant difference between the moderate and good groups in time to
decline below 500 CD4+ T-cell counts with HR of 3.6, and a trend in the differences
in time to decline below 350 CD4+ T-cells HR=4.5. There was also a significant
difference for ESB2 bad vs. good strata for declines below thresholds of 500 and
350 with hazard ratios of 7.1 and 13. For the 200 threshold, the bad, moderate, and
good groups had 3/12, 3/19, and 0/14 events; the HRs for moderate vs. good and bad vs. good were infinite.

In both ESB1 and ESB2 models, the p-values for bad vs. moderate were less than 0.20 at all thresholds with ESB1 HRs of 2.0, 3.1, and 6.0 and ESB2 HRs 2.0, 2.9, and 4.8. This suggests a faster CD4+ T-cell decline in B35 individuals possessing the detrimental responses to the variable DL9 and IL9 epitopes.

**ESB restricted responses provided additional predictive gains.** Next, to understand the additive predictive effects of the CTL based models to the HLA model, a Cox PH model of the HLA groups (reduced model) was compared to an additional Cox PH model of the HLA groups and the ESB1 group (full model). For the time to CD4+ T-cell count decline below 500, the full model significantly improved upon the reduced model with a Nagelkerke $R^2=0.36$ vs. 0.22 (Table 2).

The results did not reach significance for the 350 threshold, although there was improvement in the $R^2$ with the full model (0.25 vs. 0.17). Interestingly, the ESB2 categories (full model) did not significantly improve prediction of time to CD4 decline for either the 500 or 350 thresholds.

These survival analyses demonstrate two key points. First, CD8+ T-cell responses to key HLA-B restricted epitopes determined disease progression in HIV-1-infected subjects better than HLA genotype alone. This was demonstrated in smaller log-rank p-values (KM plots), larger Cox PH HRs, and most convincingly by significantly improved prediction of time to CD4+ T-cell count decline when using ESB1 in addition to HLA groupings. Second, the presence of ESB responses to B27KK10/FY10 and B57TW10/KF11 delayed declines in CD4+ T-cell counts to 3.5–
8.2 times later or more than 8 years in HLA-B57/58 and B27 positive subjects, respectively.

**HIV-1 viral load differed by ESB group.** To evaluate the impact of CD8+ T-cell ESB responses on the control of viral replication, we examined the plasma HIV-1 viral load at the same time points. As also reported by others (7, 22, 25, 33), the median plasma viral loads were different by HLA grouping (KW p=0.001) with medians of the B57/58 group being lower than B35Px and B35PY groups (Wilcoxon-Mann-Whitney (WMW) p<0.0001 and p=0.0003, respectively, Fig. 4a). Likewise the median HIV-1 viral loads of subjects grouped by ESB1 and ESB 2 were different (KW p=0.004 and 0.01, respectively, Fig. 4b and data not shown). Interestingly, the four highest viral loads in the B57/58 subjects (WMW p=0.02) and highest viral load among the B27 subjects were in those lacking their respective ESB1 TW10 and KK10 responses. Together, these data demonstrate that altered disease progression in B57/58 and B27 subjects is at least partially attributable to the impact of ESB restricted CTL responses on viral replication.

**Conservation and dominance defined better HIV-1-response efficacy.** Our initial interest focused on the highly prevalent responses to conserved regions restricted by key HLA alleles (B57TW10/KF11 and B27KK10/FY9), which are known to be dominant in early HIV-1 infection (3) and to variable regions (B35DL9/IL9) also of relatively high dominance. Table 1 summarizes all responses present in at least 40% of subjects possessing a given HLA class I allele and in at least three subjects. This sorting allowed the focus to be on common CD8+ T-cell responses based on both HLA allele prevalence and response prevalence within an allele. A total of 19
epitopes met these criteria, representing 107 of 226 or 47% of the detected CTL responses. 

The lead B27KK10 epitope was highly conserved (87% M-group and 82% B-subtype) and dominant (67% of subjects' total interferon-γ ELISpot response to the HIV-1 peptides tested). The similarly conserved (86% M-group and 80% B-subtype), but subdominant (26%) B27FY9 response contributed to the B27 restricted response, together representing a median of 64% of the total response magnitude of the host CTL response to HIV-1. Likewise, the five B57/58 epitopes demonstrated the diversity of B57/58 in targeting multiple, but conserved regions of HIV-1. The B57/58 restricted epitopes represented a median of 86% of the total response magnitude of the CTL response to HIV-1. For HLA-B57/58 then, it appeared that subdominant CTL responses within an allele additively contributed to a cumulative dominant and effective response to conserved regions of HIV-1.

Responses to variable HIV-1-regions lacked efficacy. In contrast, the B35DL9/IL9, B13RI9, B27VL9, and B51EI9 represented responses to variable HIV-1 regions, all with low conservation scores (<13% M-group and <38% B-subtype). In the case of the B35DL9/IL9 responses, these were associated with more rapid progression in the survival models above, suggesting that these CTL responses to variable regions were ineffective at limiting disease progression.

The three A03RK9, B08RL9, and B35PYNY9 epitopes had moderate conservation scores (24–45% M-group and 53–66% B-subtype), moderate prevalence (43–50%), and low dominance (11–29%). While numerous, the A03RK9 and B35PYNY9 responses did not demonstrate a significant effect on HIV-1 disease
progression in our data (not shown). Future studies may be able to determine the threshold for effective conservation and dominance in the context of prevalence defined sample-size. Low HLA allele prevalence and low response prevalence prevented such analyses on the less conserved A29SY9, B08Ei8, and B51Li9 epitopes (35–56% M-group and 72–86% B-subtype). Interestingly, the highly conserved Nef B08FL8 response has been associated with long-term non-progression and escape mutations have been associated with lower CD4+ T-cell counts and higher viral loads (12, 23, 24). Together these data characterized the effective host CD8+ T-cell responses to HIV-1: prevalent within an HLA allele, a high total allele fraction of the host CTL response, and targeting conserved regions of HIV-1.
The HLA predicted CD8+ T-cell interferon-γ ELISpot responses we detected after primary HIV-1 infection matched the number of responses found in similar PHI cohorts (medians = 2–4, range = 0–7) (1, 4, 8). The longitudinal changes in the CD8+ T-cell responses in the first few months after infection resulted in more responses of a similar magnitude, and an increasing total magnitude. We provided data showing that targeting of conserved versus variable epitopes correlated better with HIV-1 disease progression in persons with specific HLA alleles than HLA genotype alone. To our knowledge these are the first data showing that possession of HIV-1 specific CD8+ T-cell responses in early infection directly correlates with HIV-1 disease progression in terms of CD4+ T-cell count decline below clinically relevant thresholds. Recently, Streeck et al saw an association with CD8+ T-cell response and setpoint viral load only after primary HIV infection (which we and many others have observed, see below), but they saw no association of CD8+ T-cell responses to change in CD4+ T-cell count after PHI (p=0.52) (33). The association that they did find with CD8+ T-cell responses and CD4+ T-cell count was only observed in chronic infection, and it was not associated with a similar correlation to setpoint viral load (p>0.22). The study by Altfeld et al did look at the correlation of total HIV-specific CD8+ T-cell responses during early infection to progression, but it did not find a significant correlation with any outcome other than death (3). In addition, this study did not look at (i) specific epitope/allele associations with disease outcomes, only the total HIV-specific IFN-γ-secreting T-cell response; or (ii) whether possession of HIV-specific T-cell responses restricted by protective or non-protective
alleles correlated better with survival than having the protective alleles alone. Our data showed, for the first time, a direct correlation of CD8+ T-cell responses induced during PHI with disease progression (decline in CD4+ T-cell count below threshold) and an associated correlation to viral load.

Our analysis of HIV-1 plasma viral load confirmed our survival results and matched prior reported associations of CTL responses to viral load (12, 23, 24, 33). It also demonstrated that plasma viral load is a possible correlate of CTL control of HIV-1 disease progression. Despite a nested case-control design looking at a limited number of HLA alleles (about 17% total allele prevalence) (9), we were also able to identify other responses (e.g., B08FL8, A03RK9, A29SY9, B08EI8, B08RL9, B35PYNY9, B51LI9) that might contribute to differential control of HIV-1 infection. Recently, viral escape has been confirmed in some of the epitopes described here (7, 37). Likewise, other groups have confirmed CTL driven viral escape in long-term non-progressors, after primary HIV-1 infection, and in chronic HIV-1 disease (15, 19, 26, 37).

For instance, the B57TW10 response resulted in complete escape early in infection, while the B08FL8 response resulted in 50% escape by two years (6). The B57/58KF11 response, with its 97% conservation score and its subsequent limited escape, clearly persisted as a critical component of the host response in B57/58 subjects (11, 30). It remains to be determined whether it is more important to have persistent responses to conserved epitopes like B57/58KF11 or to have responses that result in escape like B57TW10 that produce potentially less fit viruses.
While our study population was limited, we demonstrated a differential effect on HIV-1 disease progression based on possession of certain CTL responses. This was supported by statistical analyses that showed a significant additional predictive effect of CTL responses over genotype alone. Future efforts to expand these survival results to more HLA alleles and other epitopes will clearly require larger numbers to include both rare HLA genotypes and lower-prevalence responses, so that more dominant, conserved responses that alter HIV-1 disease progression can be identified.

The most thorough mapping of CTL responses would use autologous viral sequences of a distribution of a subject’s viral pool (2). A step down from this would be to use potential T-cell epitopes, and then consensus viral sequences (21). Our approach using only HLA-matched optimal epitopes was a limitation of this study. It would also have been beneficial to use other methods for determining the magnitude of HIV-specific responses, such as tetramer staining. In addition, an assessment of epitope sequences in each individual’s virus would have allowed us to determine if the B35-restricted responses were not protective due to the fact that the epitopes rapidly mutated when under T cell pressure. However, sample availability and lack of available tetramers for every epitope specificity limited the assays that could be performed. Our calculation of conservation score equally weighted all positions in the peptide epitope; a strategy giving greater weight to the first and last two amino acids could provide different results as these positions are more important in peptide-MHC binding affinities (20, 29). In our efforts to associate a phenotype with the highly reported HLA-genetic associations of HIV-1 disease progression, the
definition of conserved epitope (>70%) was arbitrary. An exploration of the survival effects in lesser conserved B57/58 and B27 epitopes might provide further insight. Also, extending this approach to highly conserved epitopes restricted by alleles not associated with delayed progression may further confirm the CTL-effect on progression (e.g., B08FL8, A03RK9, A29SY9, B08E18, B08RL9, B35PYNY9, B51LI9).

Our measure of CD8+ T-cell function was limited to interferon-γ production in an ELISpot assay. Future efforts might examine more cytokines (e.g., tumor necrosis factor-α), effector markers (e.g., perforin and granzymes), and CTL proliferative capacity (via carboxyfluorescein diacetate succinimidyl ester staining) through other methods (e.g., intracellular cytokine staining, viral suppression assays, and T-cell functional assays). These expanded efforts will almost certainly require new longitudinal cohort enrollment and follow-up, as these methods are not possible with the limited cell numbers in currently stored historical cohorts.

In conclusion, we conducted a nested case-control study that uniquely demonstrated HIV-1 disease progression outcome differences among subjects with HLA B57/58, B27, and B35 alleles based on their possessing CTL responses to conserved or variable regions of HIV-1. CTL responses within B57/58 and B27 restricted alleles to conserved epitopes resulted in delayed declines in CD4+ T-cell counts, while responses within B35 restricted alleles to variable epitopes resulted in more rapid disease progression. HIV-1 plasma RNA viral load matched these CTL effects. These data provide the first phenotypic mechanisms of the well-established disease non-progression genotypes of HLA-B57/58, B27, and B35 in a clinical
cohort. Our data suggest that vaccine immunogens should be designed to remove variable and non-immunogenic regions of the proteome so that the host immune response only has the option of responding to conserved, immunogenic regions of HIV-1.
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FIGURE LEGENDS

FIG. 1. Study Design. Primary Infection subject (n=45) selection is shown. Inclusion was limited by available HLA genotype data, presence of an epitope-specific HLA-B (ESB) restricted allele of interest HLA-B57/58, B27, B35Px, or B35PY, by sample availability, and cell viability >60%. Four subjects lacked ELISpot responses and four had poor cell viability. One subject possessed both B27 and B57, and two subjects possessed both B35Px and B35PY.

FIG. 2. CD8+ T-cell interferon-γ ELISpot responses. A median 3 (range 1–10) number of interferon-γ ELISpot responses were detected at median 48 days post infection (DPI) by HIV-1 with testing of HLA predicted screening of 45 subjects, and the distribution is shown (a). The total interferon-γ ELISpot responses were no different by HLA group (b) (Kruskal-Wallis p=0.36). In a subset of 14 subjects also evaluated at a second later time point median 135 DPI the median number of responses increased significantly (Wilcoxon signed-rank (WSR) p=0.004) from 2 to 5 responses, a change of median 3 (range –2 to 6) (c). There was also a significantly greater total response magnitude at the second time point (WSR p=0.02) (d).

FIG. 3. Survival curves. Kaplan-Meier (KM) plots survival fraction models are shown for time to decline in CD4+ T-cell counts below thresholds of 500, 350, or 200 CD4+ T-cells per µL. Subjects were grouped according to HLA genotype (B57/58, B27, B35Px, and B35PY) (first row) or by possession of particularly good, moderate, or bad interferon-γ ELISpot responses. For ESB1 (second row) good (B27KK10+, B57TW10+), moderate (B27KK10–, B57TW10–, B35IL9–/DL9–), and bad
(B35IL9+/DL9+) were used. For ESB2 (third row) good (B27KK10+/FY10+, B57TW10+/KF11+), moderate (B27KK10−/FY10−, B57TW10−/KF11−, B35IL9−/DL9−), and bad (B35IL9+/DL9+) were used. Censoring was due to treatment initiation or dropout. For the KM plots, at least one HLA stratum was significantly different for time to CD4+ T-cell below 500, 350, and 200 (log-rank p=0.01, 0.008, 0.02). For ESB1 and ESB2, there was also a significant difference for time to CD4+ T-cell below 500, 350, and 200 in at least one of the groups (log-rank p=0.0004, 0.005, 0.01 for ESB1 and p=0.002, 0.006, 0.007 for ESB2, respectively).

FIG. 4. HIV-1 viral loads. The median HIV-1 RNA plasma viral loads by HLA grouping (a) were different (Kruskal-Wallis (KW) p=0.001) with medians of the B57/58 group being lower than B35Px and B35PY (Wilcoxon-Mann-Whitney p<0.001 and p=0.003, respectively). Likewise the median viral loads were different by the ESB1 groupings (b) (KW p=0.004).
<table>
<thead>
<tr>
<th>HLA allele</th>
<th>Response Prevalence #/# (%)</th>
<th>Response Dominance (%)</th>
<th>HXB2 Site Peptide Abbr.</th>
<th>M</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>A03</td>
<td>5/10 (50)</td>
<td>29</td>
<td>Gag p1720-28</td>
<td>RK9</td>
<td>45</td>
</tr>
<tr>
<td>A29</td>
<td>3/3 (100)</td>
<td>67</td>
<td>Env gp160209-217</td>
<td>SY9</td>
<td>35</td>
</tr>
<tr>
<td>B08</td>
<td>3/7 (43)</td>
<td>17</td>
<td>Gag p24128-135</td>
<td>EL8</td>
<td>56</td>
</tr>
<tr>
<td>B08</td>
<td>3/7 (43)</td>
<td>21</td>
<td>Nef p105-97</td>
<td>FL8</td>
<td>91</td>
</tr>
<tr>
<td>B08</td>
<td>3/7 (43)</td>
<td>13</td>
<td>Env gp16048-856</td>
<td>RL9</td>
<td>33</td>
</tr>
<tr>
<td>B13</td>
<td>3/3 (100)</td>
<td>58</td>
<td>Nef p105-114</td>
<td>RL9</td>
<td>56</td>
</tr>
<tr>
<td>B27</td>
<td>7/7 (57)</td>
<td>26</td>
<td>Pol Im p125-194</td>
<td>FY9</td>
<td>88</td>
</tr>
<tr>
<td>B27</td>
<td>5/7 (71)</td>
<td>67</td>
<td>Gag p24131-140</td>
<td>RL9</td>
<td>82</td>
</tr>
<tr>
<td>B27</td>
<td>3/7 (43)</td>
<td>19</td>
<td>Vpr p131-39</td>
<td>VL9</td>
<td>12</td>
</tr>
<tr>
<td>B35Px</td>
<td>7/2/12 (50)</td>
<td>36</td>
<td>Env gp16078-86</td>
<td>DL9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>(58-75)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B35PY</td>
<td>6/12 (50)</td>
<td>37</td>
<td>Pol RT p293-301</td>
<td>IL9</td>
<td>9</td>
</tr>
<tr>
<td>B51</td>
<td>3/4 (75)</td>
<td>47</td>
<td>Vpr p105-37</td>
<td>E9</td>
<td>13</td>
</tr>
<tr>
<td>B51</td>
<td>3/4 (75)</td>
<td>55</td>
<td>Pol Im p125-36</td>
<td>IL9</td>
<td>37</td>
</tr>
<tr>
<td>B57/58</td>
<td>4/9 (44)</td>
<td>13</td>
<td>Nef p116-124</td>
<td>HW9</td>
<td>36</td>
</tr>
<tr>
<td>B57/58</td>
<td>4/9 (44)</td>
<td>20</td>
<td>Gag p2415-23</td>
<td>ISW9</td>
<td>59</td>
</tr>
<tr>
<td>B57/58</td>
<td>7/9 (78)</td>
<td>27</td>
<td>Pol RT p244-252</td>
<td>IVW9</td>
<td>14</td>
</tr>
<tr>
<td>B57/58</td>
<td>6/9 (67)</td>
<td>34</td>
<td>Gag p24140-40</td>
<td>KF11</td>
<td>84</td>
</tr>
<tr>
<td>B57/58</td>
<td>6/9 (67)</td>
<td>42</td>
<td>Gag p24106-117</td>
<td>TW10</td>
<td>40</td>
</tr>
</tbody>
</table>

- Only responses prevalent in at least 40% of those with an allele and in at least 3 subjects are shown.
- Prevalence is the fraction or percentage of subjects with a response within a given HLA allele.
- Dominance is the percentage of the subject's total response magnitude derived from this specific response.
- Conservation is calculated as frequency of complete epitope match in aligned M-group (n=615-1224) and B-subtype (n=97-243) sequences.
- B27 includes one subject with combined B27 and B57 without B27 restricted responses.
- B35PY includes two subjects with combined B35Py/PY with both B35Px/PY responses.
- B35PY includes one subject with B35PY homozygosity.
- B57/58 includes two subjects with B58.
### TABLE 2. Survival model results.

<table>
<thead>
<tr>
<th>Kaplan-Meier p-value</th>
<th>CD4+ T-cell Count Thresholds</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA(^a)</td>
<td>500</td>
</tr>
<tr>
<td>ESB1(^c)</td>
<td></td>
</tr>
<tr>
<td>ESB2(^d)</td>
<td></td>
</tr>
</tbody>
</table>

Cox PH\(^b\) HR (p-value)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA(^a)</td>
<td></td>
</tr>
<tr>
<td>B35 vs. B57/58</td>
<td>5.8 (0.006)</td>
</tr>
<tr>
<td>ESB1(^c)</td>
<td></td>
</tr>
<tr>
<td>M vs. G</td>
<td>8.2 (0.007)</td>
</tr>
<tr>
<td>B vs. G</td>
<td>16 (0.0007)</td>
</tr>
<tr>
<td>B vs. M</td>
<td>2.0 (0.13)</td>
</tr>
<tr>
<td>ESB2(^d)</td>
<td></td>
</tr>
<tr>
<td>M vs. G</td>
<td>3.6 (0.03)</td>
</tr>
<tr>
<td>B vs. G</td>
<td>7.1 (0.001)</td>
</tr>
<tr>
<td>B vs. M</td>
<td>2.0 (0.15)</td>
</tr>
</tbody>
</table>

Cox PH\(^e\) R\(^2\) (p-value)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA(^a)+ESB1(^c) (full)</td>
<td>0.36 (0.01)</td>
</tr>
<tr>
<td>HLA(^a)+ESB2(^d) (full)</td>
<td>0.25 (0.4)</td>
</tr>
<tr>
<td>HLA(^a) (reduced)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\(^a\) Human Leukocyte Antigen model grouping: B57/58, B27, B35(Px or PY)
\(^b\) Cox Proportional Hazards (PH) model Hazard Ratio (HR) and Wald test p-value
\(^c\) Epitope specific HLA-B restricted model 1 grouping: B-Bad, M-Moderate, G-good
\(^d\) Epitope specific HLA-B restricted model 2 grouping: B-Bad, M-Moderate, G-good
\(^e\) Cox PH model Nagelkerke R\(^2\) improvement of prediction with full over reduced model, p-value from likelihood ratio test
\(^f\) B57/58-0/9 B27-0/6 B35-6/30 events; HR was infinite, likelihood ratio test p-value
\(^g\) B-3/12 M-3/22 G-0/11 events; HR was infinite, likelihood ratio test p-value
\(^h\) B-3/12 M-3/19 G-0/14 events; HR was infinite, likelihood ratio test p-value
FIG. 1. Study design.

Primary Infection Clinic (PIC) cohort n=268

- No HLA data n=31
- No ESB allele n=148

PIC with HLA data n=234

- B35 n=52
  - Not tested\(^a\) n=17
  - B35 n=35
    - Low Viability n=2
    - No Response n=3
  - B35 n=33

- B27 n=15
  - Not tested\(^a\) n=6
  - B27 n=9
    - Low Viability n=1
    - No Response n=1
  - B27 n=8

- B57/58 n=21
  - Not tested\(^a\) n=12
  - B57/58 n=9
    - Low Viability n=0
    - No Response n=0
  - B57/58 n=9

ESB+ with Responses Included in Analysis

\(^a\) Not tested due to sample availability
\(^b\) B35 includes two subjects with combined B35PxyPY with both B35PxyPY responses
\(^c\) B27 includes one subject with combined B27 and B57/58 without B27 restricted responses
(FIG. 2. CD8+ T-cell interferon-γ ELISpot responses.)
(FIG. 3. Survival curves.)

Survival Fraction

HLA

ESB1

ESB2

CD4 <500

CD4 <350

CD4 <200

Years after Infection

Survival Fraction

Years after Infection
(FIG. 4. HIV-1 viral loads.)