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ABSTRACT
In individuals with HIV-1 infection, depletion of CD4+ T cells is often accompanied by a malfunction of CD8+ T cells that are persistently activated and/or exhausted. While the dynamics and correlates of CD4 counts have been well documented, the same does not apply to CD8 counts. Here, we examined the CD8 counts in a cohort of 497 Africans with primary HIV-1 infection evaluated in monthly to quarterly follow-up visits for up to 3 years in the absence of antiretroviral therapy. Statistical models revealed that (i) CD8 counts were relatively steady in the 3- to 36-month period of infection and similar between men and women; (ii) neither geography nor heterogeneity in the HIV-1 set-point viral load could account for the roughly 10-fold range of CD8 counts in the cohort (P > 0.25 in all tests); and (iii) factors independently associated with relatively high CD8 counts included demographics (age ≤ 40 years, adjusted P = 0.010) and several human leukocyte antigen class I (HLA-I) alleles, including HLA-A*03:01 (P = 0.013), B*15:10 (P = 0.007), and B*58:02 (P < 0.001). Multiple sensitivity analyses provided supporting evidence for these novel relationships. Overall, these findings suggest that factors associated with the CD8 count have little overlap with those previously reported for other HIV-1-related outcome measures, including viral load, CD4 count, and CD4/CD8 ratio.

IMPORTANCE
Longitudinal data from 497 HIV-1 seroconverters allowed us to systematically evaluate the dynamics and correlates of CD8+ T-cell counts during untreated primary HIV-1 infection in eastern and southern Africans. Our findings suggest that individuals with certain HLA-I alleles, including A*03 (exclusively A*03:01), persistently maintain relatively high CD8 counts following HIV-1 infection, a finding which may offer an intriguing explanation for the recently reported, negative association of A*03 with HIV-1-specific, broadly neutralizing antibody responses. In future studies, attention to HLA-I genotyping data may benefit in-depth understanding of both cellular and humoral immunity, as well as the intrinsic balances of these types of immunity, especially in settings where there is emerging evidence of antagonism between the two arms of adaptive immunity.

CD8+ cytotoxic T lymphocytes (CTLs) are critical to early immune control of HIV-1 infection, and many studies have documented the dynamics and evolution of HIV-1-specific CTLs that target viral epitopes in the context of differential presentation (restriction) by the highly variable human leukocyte antigen class I (HLA-I) molecules (1–5). More often than not, the immune protection provided by CTLs is transient, as CTL escape mutations are abundant in the circulating viruses, even in the presence of favorable HLA-I variants like B*57 and B*81 (6–8). Concomitantly, depletion of CD4+ helper T cells can exacerbate the losing battle for CTLs, leading to the accumulation of activated and exhausted CD8 cells (9–13), as well as a persistent reversion of the CD4/CD8 T-lymphocyte ratio (14–16). Moreover, the orchestration of cellular and humoral immunity can be problematic when CTL impairment occurs early, as broadly neutralizing antibodies usually take years to develop (17–19).

In the clinical realm, attention to the dynamics and functions of CD8 cells per se has been rather limited, as much of the decision-making process relies almost exclusively on the HIV-1 viral load (VL) and CD4+ T-cell (CD4) counts following diagnosis of HIV-1 infection. However, the new era of early and intensified antiretroviral therapy (ART) is likely to change this paradigm for three reasons. First, the CD4 count alone is unable to fully gauge immunologic health after ART (20–22). Second, CD8 cells are essential to the eradication of residual HIV-1 reservoirs after ART initiation (23–26). Third, CD8 cells can be induced to enhance the efficacy of vaccination (27), as reported recently in nonhuman primate models (28, 29). To this end, it is worthwhile to take a step back.
and examine the dynamics and correlates of CD8 counts before ART initiation, especially in regions where such data remain sparse.

Our findings, based on evaluations of 497 HIV-1-infected Africans with multiple pre-ART visits, now suggest that the independent correlates of the CD8 count have little overlap with those previously seen with the set-point VL, CD4 count, and CD4/CD8 ratio. The underlying biology deserves further investigation and may have implications beyond cellular immunity.

MATERIALS AND METHODS

Study population, laboratory techniques, and outcome measures. Our work here focused on 497 HIV-1 seroconverters (SCs) from Kenya, Rwanda, Uganda, and Zambia who were enrolled under a uniform study protocol developed and implemented by the International AIDS Vaccine Initiative (IAVI). The study design and research procedures, including written informed consent and laboratory testing (e.g., viral sequencing and HLA genotyping), were approved by the institutional review boards at IAVI, Emory University, and the University of Alabama at Birmingham. Clinical and laboratory tests, including centralized, T-cell immunophenotyping during monthly to quarterly follow-up visits, have been described in detail elsewhere (16, 30–33). ART initiation followed appropriate national guidelines (34), but post-ART data were too sporadic (limited to 56 person visits) to allow meaningful analysis. To facilitate a direct comparison with earlier statistical models for establishing correlates of the set-point VL, CD4 count, and CD4/CD8 ratio in primary HIV-1 infection (16, 32, 33), the SCs included in this study must have had at least three virologic and immunologic outcome measures in the 3- to 24-month period after the estimated date of infection (EDI). In addition, all SCs had fully resolved HLA-I genotypes, as also reported earlier (16, 32, 33).

Statistical analysis. Using software packages in SAS, version 9.4 (SAS Institute, Cary, NC), data analyses focused on pre-ART CD8 counts, with further consideration being given to earlier work that analyzed the pre-ART VL, CD4 count, and CD4/CD8 ratio (16, 32, 33). We began with a full assessment of log10-transformed CD8 counts in the 3- to 36-month period after EDI, using Pearson’s correlation coefficients (r), local regression (LOESS) curves, mixed models for repeated measurements, analysis of variance (ANOVA) of cross-sectional data (i.e., visit-specific data or mean CD8 counts over a given time period), and logistic regression models for cross-sectional data. Association analyses targeted HLA variants that were adequately prevalent (present in ≥5% of the study population), with a focus being on individual alleles that met two thresholds of statistical significance, i.e., a P value of <0.05 and a q value of <0.10. Summary statistics included (i) P values and associated false discovery rates (FDR; q values) when multiple testing was applied and (ii) the effect size of individual factors on the CD8 count, as measured by mean regression beta estimates (Δ), the standard error (SE) of Δ, and the degree of variance explained by each factor (R²). In multivariable models, statistical adjustments were made for demographics (sex and age), geography (eastern versus southern Africa), and three categories (low, medium, and high) of the set-point VL that have clinical and epidemiological implications (32). The final statistical models were also subjected to sensitivity analyses that were restricted to data from the 3- to 24-month period after EDI. For individual correlates of the CD8 count (with log10 transformation), the statistical significance was accepted at the level of a P value of <0.05 and a q value of <0.10 in the initial screening models, followed by an adjusted P value of <0.05 in multivariable tests.

Bioinformatics. Several public databases were surveyed for further evidence of genomics data pertinent to HLA/major histocompatibility complex (MHC) gene expression and effective tagging of individual HLA alleles by single nucleotide polymorphisms (SNPs). Specifically, MHC SNPs that tag HLA class I alleles in Africans (35) were first queried in HaploReg, version 4.0 (http://www.broadinstitute.org /mammals/haploreg/haploreg_v4.php, last accessed on 2 September 2016) (36) for patterns of linkage disequilibrium (LD) uncovered by The 1000 Genomes Project and for functional properties annotated by the ENCODE project (37, 38). SNPs already associated with immune disorders and/or gene expression quantitative trait loci (eQTLs) (39) were checked in the NCBI Global Cross database (http://www.ncbi.nlm.nih.gov) and the SCAN database (http://www.scandb.org/newinterface/index.html, last accessed on 11 March 2016). Findings on HLA-I variants were interpreted in light of these bioinformatics data, with further reference being made to a panel of fine-mapped, causal SNPs linked to various genome-wide association studies (40).

RESULTS

Steady CD8 counts in 497 SCs. In the 3 to 36 months after EDI, CD8 counts were available for a total of 4,131 person visits. Overall, CD8 counts ranged from 2.40 to 3.30 log10 (a roughly 10-fold range), being relatively stable within individuals and similar between 185 women and 312 men (P > 0.60) (Fig. 1). For example, the linear correlation between the first CD8 count after 3 months of infection and the last count before 36 months was quite strong for both men (Pearson r = 0.77, P < 0.0001) and women (r = 0.78, P < 0.0001). Evaluation of other demographic features revealed that longitudinal CD8 counts differed between individuals
in two age groups (P = 0.027 for individuals >40 years old versus individuals ≤40 years old) but were similar between individuals from eastern and southern Africa (P = 0.47). Differences between age groups were confirmed by analysis of the mean CD8 counts (with log10 transformation) during the 3- to 36-month intervals (P = 0.018) (Fig. 2).

**HLA variants as genetic correlates of CD8 counts.** In the study cohort, 12 HLA-A, 16 HLA-B, and 9 HLA-C variants had to be observed in at least 25 (5%) individuals to facilitate statistical screening for potential associations with repeated measures of CD8 counts (Table 1). After statistical adjustments for potential confounding by age, sex, geography, and duration of HIV-1 infection, individual alleles that met the thresholds of statistical significance included A*03 (exclusively A*03:01 (Δ = 0.08 ± 0.03, P = 0.003, q = 0.048), B*15:10 (Δ = 0.06 ± 0.02, P = 0.005, q = 0.059), and B*58:02 (Δ = 0.07 ± 0.02, P < 0.001, q = 0.015). The only variant that appeared to have a negative impact on CD8 counts was B*58:01, but the borderline statistical significance (P = 0.049) had a high probability of false discovery (q = 0.394) (Table 1).

Visualization using LOESS curves indicated steady differences between subjects with and without these HLA variants (e.g., for the A*03-positive [A*03+] versus A*03-negative [A*03−] groups in Fig. 3). In multivariable models, all three genetic correlates were independent of other potential confounders (adjusted P-value range, <0.001 to 0.013) (Table 2). An alternative model for mean CD8 counts led to almost identical results for the HLA variants of interest (adjusted P-value range, <0.001 to 0.013) (Table 2). In contrast, both statistical models failed to detect differences in CD8 counts that could be attributed to the three HIV-1 VL groups (adjusted P-value range, 0.251 to 0.795).

**Supporting evidence from sensitivity analyses.** When analyses were restricted to the 3- to 24-month period after EDI, the multivariable model for repeated outcome measurements (3,440 person visits) also supported the independent associations between CD8 counts and A*03 (adjusted Δ = 0.06 ± 0.03, P = 0.019), B*15:10 (adjusted Δ = 0.07 ± 0.02, P = 0.003), and B*58:02 (adjusted Δ = 0.07 ± 0.02, P < 0.001) (Table 3), as did the alternative model for mean CD8 counts (adjusted P-value range, <0.001 to 0.199 as well) (Table 3). Again, the variance in mean CD8 counts was not attributable to distinct VL groups (adjusted P-value range, 0.340 to 0.527).

**No clear additive effects of three HLA factors.** In the study cohort, 26 SCs had a combination of A*03, B*15:10, and B*58:02. The mean CD8 counts over the 3- to 36-month intervals were found to be the highest in this small subgroup when the counts were compared with those in SCs with a single HLA factor and the reference group (all others) without any HLA variants of interest (Fig. 4) (P < 0.0001 by ANOVA), but the difference between the first two subgroups was modest (P = 0.281 by t test). The mean CD8 counts over the 3- to 24-month intervals yielded similar results (P = 0.272 for multiple alleles versus a single allele).

**Findings based on bioinformatics.** In populations of African ancestry (35), HLA-A*03:01 is known to be tagged by rs2524024, a SNP that is distant (30 kb away) from the 5’ end of HLA-A, while B*15:10 is tagged by two SNPs, rs3819294 (an HLA-B intrinsic SNP) and rs2523638 (a SNP between DMRF2 and MICA). These SNPs are also in strong LD with multiple neighboring variants, including eQTLs associated with gene expression profiles in Afri-
cans, but none of them have been associated with outcomes related to HIV-1 infection (41–43). On the other hand, B*58:02 is a somewhat unfavorable allele in HIV-1 infection (44) and has no strong LD with any neighboring SNP variants. Thus, high-throughput SNP genotyping platforms are not expected to provide sufficient coverage of all three HLA alleles being highlighted here.

**Genetic evidence from other studies.** At least two studies have examined the genetic impact on CD8 T-cell counts in human populations (45, 46). In study cohorts from Australia and the UK (45), a SNP (rs2524054) located in an intergenic region between HLA-B and HLA-C was associated with absolute CD8 T-cell counts in the general population. However, rs2524054 (close to HLA-B) is not known to tag specific HLA alleles in Africans (35). Instead, it is part of a sequence motif that has potential regulatory function, as reflected by its association with eight quantitative (gene expression) traits. Strong LD between rs2524054 and two downstream SNPs (rs2524143 and rs2853928) precludes a definitive mechanism, but HLA-B gene expression might be a possible connection (45). On the other hand, the relationship between an HLA-A*03-related MHC haplotype and CD8 T-cell counts was inconclusive for highly selected patients with hereditary hemochromatosis (iron overload) from three geographically distant regions (46).

**DISCUSSION**

Our analyses of longitudinal data from HIV-1-infected Africans suggest that CD8 T-cell counts have characteristics that differ .
starkly from those of two other commonly studied outcomes, i.e., the HIV-1 VL and CD4 T-cell counts. First, unlike the VL and CD4 counts, which often differ by sex and geography (a proxy for viral subtypes) (32, 33, 47), CD8 counts and their trajectories during primary HIV-1 infection are similar between men and women and between eastern and southern Africans, which can substantially simplify the search for generalizable and biological correlates using aggregated (instead of stratified) data (47). Second, despite their narrow ranges, log_{10}-transformed CD8 counts are informative quantitative traits for various statistical modeling, as multiple factors associated with CD8 counts can be established. Third, HLA variants (A*03:01, B*15:10, and B*58:02) associated with CD8 counts have little or no overlap with those (e.g., B*18, B*45, B*53, B*57, and B*81) previously reported for VL and CD4 counts in the same study cohort (32, 33, 47), suggesting that the underlying mechanisms should be distinct and may even precede HIV-1 infection (i.e., through intrinsic functions). Analyses of similar data from other cohorts should facilitate a better understanding of CD8 T-cell function in HIV-1 infection and in general populations (45).

Although they were statistically significant in the overall analyses and robust in sensitivity models, the effects of three HLA variants on CD8 counts were all relatively modest during the study andys and robust in sensitivity models, the effects of three HLA variants on CD8 counts were all relatively modest during the study and analyses (as indicated by the magnitude of a 15 to 17% (0.06- to 0.07-log_{10}) difference). The biological consequences may depend on the longevity of these seemingly minor differences and the subsets of CD8 T cells that are mostly affected. Earlier research has suggested that steady CD8 T-cell counts during chronic HIV-1 infection may reflect a prolonged differentiation rather than elevated activation (9). This long-lasting phenomenon may indirectly impair other arms of immune responses, at least in individuals with HLA-A*03 (exclusively A*03:01 in the study cohort) because this allele is enriched in subjects who did not develop HIV-1-specific, broadly neutralizing antibody responses (48). Assuming that antagonism and competition do exist between the cellular and humoral arms of adaptive immunity, especially in lymphoid tissues, where both space and resources are limited (49, 50), one can also envision that HLA alleles B*15:10 and B*58:02 may operate in a similar fashion. Meta-analyses of data from different studies should offer new insights into this new hypothesis. Indeed, a recently reported association between HLA-A*02 and enhanced humoral (IgG) responses to HIV-1 vaccination (the RV144 trial in Thailand) (51) may be viewed as anecdotal evidence for this hypothesis, although it is still not clear if such conclusions can apply to various populations that differ in HLA-I allele frequencies and/or allele frequencies.

Previously, a genome-wide association study (45) identified a single SNP (rs2524054) to be a major correlate of CD8 counts in healthy adolescent twins from Australia (effect size = −0.31 ± 0.03 log_{10}). Located between HLA-C and HLA-B, rs2524054 has some functional attributes (gene expression patterns), but there is no indication that rs2524054 tags specific HLA-I alleles (35) or SNPs (rs2524024, rs3819294, and rs2525638) that are in strong LD with A*03:01 and B*15:10. Recent fine-mapping data do suggest that LD between rs2524054 and a functional (causal) SNP variant (rs2247056-T) can account for the association of rs2524054 with serum triglycerides in healthy subjects (40). Although fine mapping can be influenced by ethnic backgrounds, a focus on gene expression and lipid metabolism is expected to expedite future research on immunogenetic control of the CD8 T-cell function in health and diseases.

On the other hand, the positive impact of B*58:02 on CD8 counts is not complicated by neighboring SNPs (35). In several studies of HIV-1-infected Africans (52–54), B*58:02 has been recognized to be unfavorable (associated with a high viral load and low CD4 counts), being functionally and epidemiologically distinct from another closely related allele, B*58:01 (52–54). By our analysis, B*58:01 and B*58:02 do seem to have opposing impacts on CD8 counts, but the statistical power in our study favors the analysis of B*58:02 rather than B*58:01 (which were found in 72 versus 55 subjects, respectively, in our cohort). A more definitive conclusion will obviously require a larger sample size to strengthen the analysis of B*58:01.

One major limitation in this study is the lack of CD8 count data before HIV-1 infection and after ART initiation. As our study cohort was designed for the evaluation of primary HIV-1 infection, preinfection and post-ART data from other study populations will help assess the relationships between HLA-I alleles and the dynamics of CD8 counts in Africans. For example, a hematology reference panel has included CD8 counts in 2,105 healthy subjects from eastern and southern Africa (55). Preparation for vaccine trials may justify HLA-I genotyping in this large study population. Meanwhile, assembling a prospective post-ART data set will likely require years of concerted efforts, as the implementation of new guidelines for early HIV-1 therapy has been a slow process.

The frequencies of HLA-I alleles being highlighted in this study ranged from 9% to 14% in our study cohort (Table 1). Collectively, they were found in over 29% of subjects (Fig. 4). The distribution of these alleles in other ethnic groups can vary, but A*03:01 is a globally common allele and should be readily analyzed in other cohorts, including general populations where CD8 T-cell counts are measured (45, 55). Overall, our findings should broaden the attention to immunogenetic factors, since variability in CD8 counts before antiretroviral therapy may relate to the function of multiple HLA-I variants. This concept can be equally pertinent to studies of CD8 T-cell function after antiretroviral therapy (56).

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