Association of HLA-DRB1–restricted CD4+ T cell responses with HIV immune control

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The contribution of HLA class II–restricted CD4+ T cell responses to HIV immune control is poorly defined. Here, we delineated previously uncharacterized peptide-DRB1 restrictions in functional assays and analyzed the host genetic effects of HLA-DRB1 alleles on HIV viremia in a large cohort of HIV controllers and progressors. We found distinct stratifications in the effect of HLA-DRB1 alleles on HIV viremia, with HLA-DRB1*15:02 significantly associated with low viremia and HLA-DRB1*03:01 significantly associated with high viremia. Notably, a subgroup of HLA-DRB1 variants linked with low viremia showed the ability to promiscuously present a larger breadth of peptides with lower functional avidity when compared to HLA-DRB1 variants linked with high viremia. Our data provide systematic evidence that HLA-DRB1 variant expression has a considerable impact on the control of HIV replication, an effect that seems to be mediated primarily by the protein specificity of CD4+ T cell responses to HIV Gag and Nef.

Control of viral infections is crucially dependent on CD4+ T cell responses1–3. CD4+ T cells provide help for the generation of high-affinity antibodies and the induction of memory in CD8+ T cells and B cells4 and may also directly exert antiviral activity2,3,5. Yet the role of virus-specific CD4+ T cell responses in HIV infection is less clear6 because HIV preferentially infects HIV-specific CD4+ T cells7. We have previously shown that the breadth and specificity of HIV-specific CD4+ T cell responses is significantly associated with low viremia8. However, little is known about the HLA class II restriction of these responses and how HLA class II alleles affect HIV control. The strongest known genetic association with HIV control is mediated by HLA class I alleles9–11 such as HLA-B*57 (refs. 9–11), yet at least two studies have suggested that HLA class II alleles12,13, particularly at the HLA-DRB1 locus, may also influence HIV control.

To define the role of DRB1-restricted HIV-specific CD4+ T cell responses on viral control, we tested 201 HIV-specific CD4+ T cell lines recognizing 67 peptide specificities from 42 HIV-infected individuals in functional HLA-DRB1 restriction assays. We observed a high level of promiscuity, a characteristic feature of antigen-specific CD4+ T cell recognition in which a single peptide can be presented in the context of multiple HLA class II variants expressed by different individuals14 (Fig. 1a). Notably, all peptides tested across the HIV proteome (Supplementary Table 1), 43% were restricted by two or more HLA-DR variants (Supplementary Table 2), OLP-41 in Gag p24, which is the most frequently targeted peptide recognized by HIV-specific CD4+ T cells, showed the highest level of promiscuity, with 12 distinct HLA-DRB1 restrictions. We also evaluated the peptide-binding capacity14 of five immunodominant peptides recognized by CD4+ T cells to 13 distinct HLA-DRB1 variants. These peptides showed comparable or even higher binding promiscuity (Supplementary Table 3), thus confirming our restriction data. Furthermore, detailed analysis of OLP-41 revealed only limited variability in the minimal stimulatory epitopes presented by different HLA-DRB1 variants (Supplementary Fig. 1), confirming that promiscuous HLA-DRB1 variants share largely overlapping epitope-binding registers15,16. This high degree of peptide-binding promiscuity is probably mediated by the open conformation of HLA class II, which allows long peptides recognized by CD4+ T cells to extend beyond the HLA binding groove.

Despite the high degree of HLA-DRB1 binding promiscuity, we observed marked differences in the number of peptides restricted by each DRB1 variant (Fig. 1b). Variants such as DRB1*01:01 and DRB1*04:01 had the lowest contribution to the total number of peptide restrictions, with only three confirmed peptides, whereas DRB1*13:01, DRB1*13:02 and DRB1*13:03 restricted a total of 18, 11 and 8 HIV-specific peptides recognized by CD4+ T cells, respectively. Notably, expression of DRB1*13 variants, particularly of DRB1*13:03, has been associated with lower HIV viral load12,13. We next examined the relationship between HLA-DRB1 allele expression and viral load in a large cohort of 1,085 treatment-naive HIV-infected individuals of European ancestry from the combined

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Massachusetts General Hospital (MGH) and SCOPE (Observational Study of the Consequences of the Protease Inhibitor Era) cohorts. We categorized subjects into two groups, HIV controllers (<2,000 HIV RNA copies per ml) and HIV progressors (>10,000 HIV RNA copies per ml), and we then evaluated the effect of individual HLA-DRB1 allele expression on mean viral load in a logistic regression model. We used an odds ratio to analyze the strength of the associations, with <1 indicative of greater HIV control and >1 indicative of relatively poor HIV control. We used the well-defined HLA class I protective alleles HLA-B*57 and HLA-B*27 and the risk allele HLA-B*35Px (refs. 9, 17) as covariates in this model to eliminate their confounding effects on HIV viremia. Notably, although we observed a wide range in the confidence intervals, there was a hierarchy of more protective to more hazardous HLA-DRB1 alleles (Fig. 2). Specifically, subjects positive for HLA-DRB1*15:02 had the lowest odds ratio (0.22), and expression of this allele was significantly associated with control of HIV viremia (P = 0.003, q = 0.04). In contrast, HLA-DRB1*03:01 had the highest odds ratio (1.68) and was significantly associated with high viremia (P = 0.004, q = 0.04). We found distinct stratifications in the odds ratio associated with other HLA-DRB1 alleles, but we were under-powered to determine statistical differences in their association with HIV control after correction for multiple comparisons in this cohort. Whereas some of the HLA class II alleles associated with low viremia are relatively rare at the population level, we found no evidence of a ‘rare allele’ effect as suggested for HLA class I (ref. 18).

We previously described marked differences in the protein specificity of HIV-specific CD4+ T cell responses between HIV controllers and high-viremia progressors8. Therefore, we next investigated whether the HLA-DRB1 variants linked with either low or high viral load in our genetic study (MGH and SCOPE cohorts) restricted different HIV proteins in our initial functional screen. The cohorts used for the genetic and functional analyses were independent but did not differ in HLA-DRB1 frequencies (Supplementary Fig. 2 and Supplementary Table 4). We grouped the HIV-specific CD4+ T cell responses on the basis of the upper quartile of HLA-DRB1 alleles linked with low viremia (HLA-DRB1*15:02, HLA-DRB1*10:01, HLA-DRB1*13:03, HLA-DRB1*09:01 and HLA-DRB1*13:02) and lower quartile of HLA-DRB1 alleles linked with high viremia (HLA-DRB1*03:01, HLA-DRB1*04:04, HLA-DRB1*01:01, HLA-DRB1*15:01 and HLA-DRB1*08:01). (HLA-DRB1*12:01 was not included owing to a lack of subjects expressing this allele in our functional cohort.) We assessed a total of 26 HIV-infected subjects (17 HIV controllers and 9 progressors) for the sum of promiscuous peptide-DRB1 restrictions per protein. Notably, we observed that HLA-DRB1 variants linked with low viremia showed a significantly greater number of peptide restrictions when compared to HLA-DRB1 variants linked with high viremia (Fig. 3a, P = 0.018, Fisher’s exact test). In particular, HLA-DRB1 variants linked with low viremia were able to promiscuously present a total of 21 Gag peptides, yet only 14 Gag peptides were restricted by HLA-DRB1 variants linked with high viremia. Moreover, Nef peptides were exclusively restricted by HLA-DRB1 variants linked with low viremia. A caveat to this approach is that odds ratio values can only be used as an approximate guide for stratifying alleles into the two groupings, given that many alleles had wide confidence intervals in the precision of their odds ratio estimates, and only two individual alleles (HLA-DRB1*15:01 and HLA-DRB1*03:01) were significantly associated with HIV control after correction for multiple comparisons. Nevertheless, the observed functional comparisons remained significant even after higher-stringency selection of HLA-DRB1 alleles on the basis of 10% significance before multiple comparisons (P = 0.0148) (Supplementary Table 5).

We next investigated whether subjects with HLA-DRB1 alleles linked to lower viremia have generally broader HIV-specific CD4+ T cell responses when compared to subjects that express HLA-DRB1 alleles linked with higher viremia. For this analysis, we evaluated the breadth of HIV-specific CD4+ T cell responses in a subcohort of 43 treatment-naive, chronically HIV-infected subjects, with the exception of subjects with HLA-DRB1*15:02, for whom sample
availability limited us to Gag, Nef and Env gp120 only (which probably underestimates the total breadth). We detected no significant differences between the two groups in their mean HIV viral load or CD4+ T cell count (Supplementary Fig. 3). Notably, individuals with HLA-DRB1 alleles linked with low viremia showed significantly greater breadth of overall HIV-specific CD4+ T cell responses directly ex vivo than individuals with HLA-DRB1 alleles linked with higher viremia (Fig. 3b, \( P = 0.0395 \), Mann-Whitney \( U \)-test). The wide variability in HIV-specific CD4+ T cell responses within each group exists because each individual expresses a variety of HLA class II alleles, and thus some peptide-specific responses within the total breadth are probably restricted by an allele that is neither protective nor non-protective. We also assessed the functional binding avidity of peptide-specific CD4+ T cell lines restricted in the context of HLA-DRB1 alleles linked with lower as compared with higher viremia. Unexpectedly, we observed that CD4+ T cells specific for HLA-DRB1 variants linked with low viremia in general bind HIV epitopes with lower functional avidity compared to CD4+ T cells specific for those variants linked with high viremia (Fig. 3c, \( P < 0.05 \), two-way analysis of variance (ANOVA) with Bonferroni correction). These data suggest a hypothesis in which HLA-DRB1 variants linked with low viremia may bind at lower avidity and may show greater promiscuity in their peptide interactions as compared to those associated with high viremia. This difference in binding modality might give HIV-specific CD4+ T cell responses restricted by potentially protective HLA-DRB1 variants a survival advantage, in which CD4+ T cells with lower binding avidity are less activated and thus less susceptible to infection or activation-induced cell death19,20.

The contribution of HLA class II peptide presentation to the control of viral infection in humans has been poorly defined. Here, we provide, to our knowledge, the first systematic approach to the analysis of

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**Figure 2** Association of HLA-DRB1 allele expression with HIV viral load at the population level. Association of HLA-DRB1 alleles with differential odds ratios in a large cohort of antiretroviral therapy-naive, chronically HIV-infected individuals of European ancestry (\( n = 1,085 \)). The odds ratio per DRB1 allele (filled circle) is shown with whiskers spanning 95% confidence intervals. The vertical line indicates an odds ratio of 1, with <1 indicative that DRB1 allele expression is associated with HIV control and >1 indicative that DRB1 allele expression is associated with HIV progression. The analysis was adjusted for subjects expressing HLA class I alleles HLA-B*57, HLA-B*27 and HLA-B*35x to eliminate their confounding effects on HIV viremia.

**Figure 3** HLA-DRB1 variants linked with low viremia present a greater breadth of peptides recognized by HIV-specific CD4+ T cells at low functional avidity. (a) Number of HIV-specific CD4+ T cell peptides restricted by HLA-DRB1 variants linked with high or low odds ratios. Peptide restrictions were grouped according to protein (Gag, Nef, Env, Pol and Acc (Accessory)). \( P = 0.018 \), Fisher’s exact test, 33/93 low odds ratio, 22/108 high odds ratio. (b) Breadth of total HIV-specific CD4+ T cell responses in HIV-infected individuals expressing HLA-DRB1 variants linked with high or low odds ratios (\( P = 0.0395 \), Mann-Whitney \( U \)-test). Subjects expressing HLA-DRB1 alleles linked with a low odds ratio are depicted by circles (\( n = 15 \)), subjects expressing HLA-DRB1 alleles linked with a high odds ratio are depicted by squares (\( n = 28 \)) and subjects expressing HLA-DRB1*15:02 tested only for Gag, Nef and Env gp120 peptides are depicted by triangles (\( n = 3 \)). Median is depicted with interquartile range. Individuals expressing more than one protective or nonprotective allele were only counted once, and none of the subjects dually expressed both alleles. (c) Functional avidity of CD4+ T cell lines restricted by HLA-DRB1 variants linked with high or low odds ratios. Significant \( P \) values are denoted by an asterisk (*) (\( P > 0.05 \), two-way ANOVA after Bonferroni correction). Subjects expressing low odds ratio–linked HLA-DRB1 alleles are depicted by squares (\( n = 8 \)). Data are expressed as means ± s.e.m. Interferon-γ (IFN-γ) spot-forming units at 20 μM were normalized to 100%.
HLA-DRB1 restrictions and show that HLA-DRB1 allele diversity may affect immune containment of HIV infection at the population level. In particular, the alleles HLA-DRB1*15:02 and HLA-DRB1*03:01 were significantly associated with HIV control and progression, respectively, and this association remained significant even after correction for multiple comparisons. The strength of these HLA-DRB1-mediated effects was independent of HLA-B*57, HLA-B*27 and HLA-B*35Px, yet it was markedly less than has been observed for these and other HLA class I alleles. Notably, the HLA class II alleles HLA-DRB1*03:01 and HLA-DRB1*15:02 were also listed among the many HLA class I alleles implicated in influencing viral control in a genome-wide association study analysis, yet only HLA-B*57 was independently associated with a protective effect. In sum, these data provide, to our knowledge, the first evidence linking HLA class II genetic associations with the functional responses of CD4+ T cells and point to an important role for HIV-specific CD4+ T cells in the control of HIV infection.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.R. designed the study, performed experiments, analyzed data, and wrote the manuscript; S.C., I.D., R.L., D.Z.S., G.K., M.D.F., M.L., B.K. and G.A. contributed to in vitro studies and experimental design; J.S. and A.S. generated the HLA-DR-transfected L cells and conducted peptide-HLA-DRB1 binding assays; Y.Q., X.G. and M.C. performed high-resolution HLA typing and analyzed HLA-DRB1 associations with viral control; S.G.D. and B.D.W. provided clinical samples from HIV-infected subjects enrolled in the SCOPE and MGH cohorts; M.C. and B.D.W. gave intellectual input; H.S. conceived of and designed the study, analyzed data, wrote the manuscript and was responsible for the overall study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. A total of 1,085 treatment-naive subjects with chronic HIV-1 infection were recruited at Massachusetts General Hospital (MGH) and at the San Francisco General Hospital and the San Francisco Veterans Affairs Medical Center (the two field sites of the UCSF SCOPE cohort). All MGH and SCOPE subjects used in this study were of European ancestry, and the analysis was statistically adjusted for those subjects expressing HLA class I alleles HLA-B*57, HLA-B*27 and HLA-B*35px. Overall, we classified 594 subjects as HIV controllers (<2,000 HIV RNA copies per ml mean viral load) and 491 subjects as HIV Progressors (>10,000 HIV RNA copies per ml mean viral load). We used viral load measurements from three or more time points during chronic infection to calculate the mean viral load per subject. The overall mean viral load in this cohort was 40,472 HIV RNA copies per ml. In addition, we analyzed an independent cohort of 42 treatment-naive, HIV-infected subjects of European ancestry at MGH for HLA-DRB1 restriction. These subjects were selected on the basis of prior delineation of their HIV-specific CD4+ T cell responses at the peptide level, availability of frozen peripheral blood mononuclear cell (PBMC) samples for further HLA class II restriction assays and a similar HLA-DRB1 allele distribution in this independent cohort when compared to the MGH and SCOPE cohorts. All subjects provided informed consent, and each study was approved by the respective institutional review boards: Partners Human Research Committee (PHRC) at MGH and Committee on Human Research (CHR) at UCSD.

Human leukocyte antigen typing. We performed high-resolution four-digit HLA class I and II genotyping by sequence-specific PCR in accordance with standard protocols. Briefly, we amplified HLA class I–encoding genes by PCR with primers spanning exons 2 and 3, and we identified HLA class II DRB1–encoding genes by PCR amplification and sequencing of exon 2. ASSIGN 3.5 software developed by Conexio Genomics was used to interpret the sequencing results.

HIV-specific CD4+ T cell lines. We used frozen CD8-depleted PBMC samples from a subset of 42 subjects with known CD4+ T cell responses to successfully generate a total of 201 peptide-specific CD4+ T cell lines spanning 67 peptide specificities. The targeted peptides were evenly distributed among all expressed HIV proteins (Supplementary Table 1). In brief, we thawed CD8-depleted PBMCs and simulated the cells with 10 µg/mL of peptide at a concentration of 2 million cells on a 24-well plate in the H10 medium (RPMI 1640 containing 10% heat-inactivated FCS, 2 mM l-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 10 mM HEPES) supplemented with 5 ng/mL recombinant interleukin-7 and 1 µg/mL of nevirapine. The cells were incubated at 37 °C and 5% CO2. After 2 d, we washed the cells and added fresh H10 medium supplemented with 100 U/mL recombinant interleukin-2. The CD4+ T cell lines were fed twice weekly with regular medium exchanges.

HLA-DRB1 restriction assay. After 14 d, we assessed the T cell lines simultaneously for their specificity and HLA-DRB1 restriction using a large panel of L cell line fibroblasts, each stably transfected with a plasmid encoding a single HLA-DRB1 variant, as previously described.14 We systematically tested each peptide-specific CD4+ T cell line against two HLA-DRB1–expressing L cell lines that matched the heterozygous HLA-DRB1 typing of the subject from whom the T cell lines were generated. In addition, we tested some peptide-specific CD4+ T cell lines against HLA-DRB3/4/5–expressing L cell lines when in linkage disequilibrium. Each L cell line was pulsed with 10 µg/mL peptide for 3 h at 37 °C and 5% CO2 and washed four times to remove free peptide. We used clade B 2001 consensus-sequence overlapping peptides spanning the whole HIV proteome in our assay. We then cultured 10,000 peptide-pulsed L cell lines in triplicate with each respective CD4+ T cell line at a ratio of 1:5 cells per well on a precoated IFN-γ plate. As a negative control, each CD4+ T cell line was cultured in triplicate with the appropriate L cell line in the absence of peptide. As a positive control, phytohemagglutinin (Sigma) was added at 1.8 µg/mL. The plates were incubated overnight at 37 °C and 5% CO2 and processed as previously described.21,22 We used the AID ELISPOT reader (Autoimmun Diagnostic GmbH, Strasbourg, Germany) to determine the number of spot-forming cells per 50,000 of the CD4+ T cell line. An HLA-DRB1 restriction was considered positive only if it was at least 2±3 times the mean background and also 2±3 times the s.d. of the negative control wells. If two overlapping peptide sets were experimentally restricted by the same HLA-DRB1 variant, they were counted as two independent responses. This is consistent with fine-mapping data demonstrating that the minimal epitope recognized by a single HLA-DRB1 may differ between overlapping peptides. However, a caveat to this approach is that overlapping peptides restricted by the same HLA-DRB1 could also correspond to a single response.

OLP-41 fine mapping. For fine-mapping analysis, we evaluated the IFN-γ responses of OLP-41–specific CD4+ T cell lines against serial truncations of OLP-41 in the context of five HLA-DRB1 restrictions. We tested each OLP-41–specific CD4+ T cell line against 20 µM of the original 18-mer OLP-41 (YVDRFYKTLRAEQASQEV) and 14 serial truncations from the N and C termini presented by the restricting HLA-DRB1–expressing L cell lines. Concordant with other CD4+ T cell studies,15,16 the ‘minimal stimulatory epitope’ was defined as the shortest peptide sequence triggering an IFN-γ response ≥50% of the original 18-mer.

HLA-DRB1 peptide binding assay. We tested immunodominant HIV-specific CD4+ T cell peptides for in vitro binding to a panel of 13 purified HLA-DRB1 molecules, as previously described.14 In brief, purified HLA-DRB1 molecules (5–500 nM) were incubated for 48 h with different concentrations of unlabeled HIV peptide and 1–10 nM 125I-radionabeled probe peptides. We calculated the concentration of peptide yielding 50% inhibition of the binding of the radionabeled probe peptide (IC50 nM), with a threshold of <1,000 IC50 nM for binding.

Statistical analyses. We categorized the 1,085 subjects from the MGH and SCOPE cohorts into two groups: HIV controllers (mean viral load <2,000 HIV RNA copies per ml plasma) and HIV Progressors (mean viral load >10,000 HIV RNA copies per ml plasma). We evaluated the effect of individual HLA-DRB1 allele expression (with frequency ≥5) on mean viral load with a logistic regression model. SAS 9.1 (SAS Institute) was used for statistical analyses. PROC FREQ was used to compute frequencies on each allele. PROC LOGISTIC was used to obtain odds ratios, 95% confidence intervals and two-sided P values per HLA-DRB1 allele. PROC GLM was used for analysis of variance, and Bonferroni correction was performed for multiple comparisons. To eliminate the confounding effects of HLA-B*57, HLA-B*27 and HLA-B*35px, these factors were used as covariates in the logistical regression model. A Fisher’s exact test was used to assess significant differences in peptide restriction between DRB1 alleles associated with high and low viremia. A Mann-Whitney U-test was used to evaluate the breadth of ex vivo HIV-specific CD4+ T cell responses in individuals expressing these two groups of HLA-DRB1 alleles. A two-way ANOVA with Bonferroni correction was used to analyze differences in functional avidity of peptide-specific CD4+ T cell lines generated from subjects expressing HLA-DRB1 alleles associated with low as compared with high viremia. Graphical presentation was performed using GraphPad Prism 5.0.

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