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ORIGINAL INVESTIGATION

Clear and independent associations of several *HLA-DRB1* alleles with differential antibody responses to hepatitis B vaccination in youth

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Abstract To confirm and refine associations of human leukocyte antigen (HLA) genotypes with variable antibody (Ab) responses to hepatitis B vaccination, we have analyzed 255 HIV-1 seropositive (HIV^+) youth and 80 HIV-1 seronegatives (HIV^-) enrolled into prospective studies. In univariate analyses that focused on *HLA-DRB1*, *-DQA1*, and *-DQB1* alleles and haplotypes, the DRB1*03 allele group and DRB1*0701 were negatively associated with the responder phenotype (serum Ab concentration ≥ 10 mIU/mL) ($P = 0.026$ and 0.043, respectively). Collectively, DRB1*03 and DRB1*0701 were found in 42 (53.8%) out of 78 non-responders (serum Ab <10 mIU/mL), 65 (40.6%) out of 160 medium responders (serum Ab 10–1,000 mIU/mL), and 27 (27.8%) out of 97 high responders (serum Ab >1,000 mIU/mL) ($P < 0.001$ for trend). Meanwhile, DRB1*08 was positively associated with the responder phenotype ($P = 0.010$), mostly due to DRB1*0804 ($P = 0.008$). These immunogenetic relationships were all independent of non-genetic factors, including HIV-1 infection status and immunodeficiency. Alternative analyses confined to HIV^+ youth or Hispanic youth led to similar findings. In contrast, analyses of more than 80 non-coding, single nucleotide polymorphisms within and beyond the three HLA class II genes revealed no clear associations. Overall, several *HLA-DRB1* alleles were major predictors of differential Ab responses to hepatitis B vaccination in youth, suggesting that T-helper cell-dependent pathways mediated through HLA class II antigen presentation are critical to effective immune response to recombinant vaccines.

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Introduction

Hepatitis B virus (HBV) infection affects 350–400 million people globally (Custer et al. 2004; Dienstag 2008; Fattovich et al. 2008; Lai and Yuen 2008; McGovern 2007). Vaccines based on recombinant HBV surface antigen (HBsAg) offer a safe and effective strategy for preventing HBV infection, as anti-HBsAg antibody (Ab) concentrations can exceed the minimum protective level of 10 mIU/mL for up to 95% of healthy recipients. Nonetheless, lack of

efficacy can be a problem to vaccine recipients with compromised or impaired immune system (Collier et al. 1988; Zuin et al. 1992), which has prompted the search for further improvements through (a) increasing the amount of antigen given in each injection (Cornejo-Juarez et al. 2006; Cruciani et al. 2009; de Vries-Sluijs et al. 2008; Fonseca et al. 2005; Pasricha et al. 2006), (b) adding an extra (boosting) dose when the standard three-dose regimen fails to induce protective Ab response (Cruciani et al. 2009; Rey et al. 2000), and (c) using immunoregulatory cytokine or unmethylated CpG oligonucleotide as an adjuvant in one of the priming doses (Cooper et al. 2008; Sasaki et al. 2003).

Suboptimal Ab response to full-dose HBV vaccination was common in adolescents and youth enrolled by the Reaching for Excellence in Adolescent Care and Health (REACH) study supported by the Adolescent Medicine Trials Network for HIV/AIDS Interventions (ATN) (Wang et al. 2004; Wilson et al. 2001). Key factors associated with this unfavorable outcome included: (1) immunodeficiency as a result of HIV-1 infection, (2) human leukocyte antigen (HLA) class II genotype, especially DRB1*07, and (3) promoter sequence variations in several cytokine genes (Wang et al. 2004). To confirm and refine some of these findings, new interventional studies nested within ATN have enrolled youth for prospective evaluation of HBV vaccination. These studies have facilitated the systematic analyses of HLA class II alleles and haplotypes in relation to differential Ab responses to HBV vaccination. Genotypes defined by non-coding, single nucleotide polymorphisms (SNPs) within and beyond the HLA class II region can also be tested for their potential associations.

Methods

Study populations

Between February 2006 and June 2008, 255 HIV-1 seropositive (HIV$^+$) youth (12–24 years of age) and 80 HIV-1 seronegatives (HIV$^-$) (12–17 years of age) were enrolled at 22 clinical sites (listed in Appendix) into prospective studies (http://www.clinicaltrials.gov/ct/show/NCT00142740) (Table 1). The HIV$^-$ subjects were recruited from the US and Puerto Rico, while the HIV$^+$ youth came from US, Puerto Rico, Brazil, and South Africa. The primary objective was to evaluate genetic influences on peak Ab responses in patients with three full doses of hepatitis B vaccination, based on analyses of eligible patients who were free of hepatitis before completing full vaccination regimens within the first 24 weeks (6 months) after enrollment. For HIV$^+$ youth, their baseline CD4+ T cell counts were also measured as potential covariates for immune responses. This study was first approved by the Institutional Review Board (IRB) at University of Alabama at Birmingham (UAB), as protocol F051212004. Additional IRB approval was obtained from each participating clinical site before patients were asked to provide written informed consent for participation. Import of patient specimens from international sites in Brazil and South Africa to US was approved by the Centers for Disease Control and Prevention (permit no. 2006-12-053 and 2007-12-015).

Table 1 Characteristics of 335 youth with defined peak antibody (Ab) response to full-dose hepatitis B vaccination

	HIV-1 seropositive (N = 255)	HIV-1 seronegative (N = 80)	P value[a]
Baseline age (mean ± SD)	20.9 ± 0.2	15.8 ± 0.2	<0.0001
Sex ratio (F/M)	1.60 (157/98)	0.57 (29/51)	<0.0001
Race/ethnicity combined			0.230
Black, non-Hispanic	76 (29.8)	17 (21.3%)	
White, non-Hispanic[b]	7 (2.8)	2 (2.5)	
Hispanic (Black or White)	169 (66.3)	60 (75.0)	
Others[b]	3 (1.2)	1 (1.3)	
Ab response[c]			<0.0001
Non-responder (NR)	74 (29.0%)	4 (5.0%)	
Medium responder (MR)	121 (47.5%)	39 (48.8%)	
High responders (HR)	60 (23.5%)	37 (46.3%)	

[a] By comparing the two patient groups using either t test (for age) or logistic regression (for other variables)

[b] Treated as one group in subsequent analyses (e.g., Table S2)

[c] Non-responders have serum Ab concentrations <10 mIU/mL at week 28 (4 weeks after last dose of vaccination); medium responders at 10–1,000 mIU/mL; high responders >1,000 mIU/mL

Quantification and categorization of serum Ab concentration

Using serum sample drawn at week 28, i.e., 4 weeks after the final recommended dose of vaccine, Ab specific for HBsAg was measured in a central laboratory (Quest Diagnostics Inc., Baltimore, MD), using two commercial assays that differ in their lower limits of detection (LLD) (3 and 5 mIU/mL). The vast majority (98.5%) of samples were tested using the second assay, which provided an upper limit of detection (ULD) at 1,000 mIU/mL. A small number of subjects had no remaining serum to allow re-measure and so these subjects only had Ab results reported using the first test (ULD at 150 mIU/mL). Patients with peak serum Ab concentration less than 10 mIU/mL (the minimum protection level) were classified as non-responders (NR). Those with Ab concentrations ranging from 10 to 1,000 mIU/mL were designated as medium responders (MR), while high responders (HR) were the remainder of patients with Ab concentrations over 1,000 mIU/mL.

PCR-based genotyping of three HLA class II genes (*HLA-DRB1*, *-DQA1*, and *-DQB1*) in the human major histocompatibility complex (MHC)

Using DNA extracted from whole blood or buffy coat (PureGen kits from Gentra/Qiagen, Valencia, CA, USA), *HLA-DQA1* and *-DQB1* alleles were resolved with automated hybridization of sequence-specific oligonucleotide (SSO) probes to gene-specific PCR amplicons (Innogenetics Inc., Alphartetta, GA). Tests were facilitated by the Auto LiPA 30 system, under conditions recommended by the manufacturer (Innogenetics). A total of 35 and 37 SSO probes were used for *HLA-DQA1* and *-DQB1* typing, respectively. Homozygosity was confirmed and ambiguities with SSO results were resolved by sequencing-based typing (SBT), using commercial kits from Abbott Molecular Inc. (Des Plaines, IL). *HLA-DRB1* genotyping began with SBT (Abbott Molecular Inc.), with ambiguities resolved by SSO assays (57 probes) (Innogenetics). The same or similar genotyping techniques had been applied extensively in earlier studies involving samples from African, European, and North American populations (Shao et al. 2004; Tang et al. 2004, 2008). The final allele assignments for the three HLA class II genes considered two major functional properties. First, allele groups defined by the first two-digit designations (e.g., DRB1*01) often correspond to serological specificities (as alloantigens) (Schreuder et al. 2005) or shared motifs and lineages (Agrawal et al. 2007; Bergstrom et al. 1999; Dorak et al. 2006; Hohjoh et al. 2003). Second, alleles with four-digit designations (e.g., DRB1*0101, *0102, and *0103) define functional units that differ in amino acid sequences and the protein structure. Higher (six-digit) resolution like DRB1*010201 was also achievable for various alleles in the study population, with the 5th and 6th digits capturing additional, synonymous DNA polymorphisms. However, only DRB1*1101 had multiple common alleles with different six-digit designations (i.e., *110101 and *110102) in the study population. Further intronic sequence polymorphisms reflected by the 7th and 8th digits were not considered because intronic sequences were not part of our HLA genotyping strategies and because only a small number of alleles have such known designations (Robinson et al. 2006).

Computational assignment of HLA class II haplotypes

Following resolution of individual HLA alleles at each locus, linkage disequilibrium (LD) between specific HLA allele groups (two-digit designations) and alleles (four-digit designations) at neighboring loci were determined by relative difference (D') between the observed and expected frequencies of co-existence (presence) on the same chromosome or by correlation coefficients (r). Two-locus haplotype assignments were based on the expectation-maximization (EM) algorithm in SAS Genetics (SAS Institute, Cary, NC), which was further applied to the evaluation of extended, three-locus haplotypes. All haplotypes assigned computationally were unambiguous, with statistical probability exceeding 99%.

Primary association analyses of HLA class II variants

Using SAS program package, version 9.2 (including SAS Genetics) (SAS Institute), the distribution of HLA class II alleles and haplotypes was first assessed for Hardy–Weinberg equilibrium (HWE) among patient groups defined by race, ethnicity, HIV-1 infection status, and Ab responses (NR, MR, and HR). Population (marker) frequencies of HLA allele groups, alleles, and their haplotypes were then compared across the three groups of patients defined by Ab responses. Individual variants with nominal $P < 0.050$ in univariate tests for trend (logit procedure) were further evaluated in multivariable logistic regression models. Different model selection procedures (backward, forward, stepwise) were applied until a reduced model could sufficiently capture all independent predictors of the responder phenotype. The summary statistical measures included effective sample sizes as well as strengths of associations, as indicated by beta estimates (mean ± standard error), proportional odds ratio (pOR) across three Ab response groups (HR, MR, and NR), OR for being responders (HR + MR vs. NR), 95% confidence interval (CI) for all pOR and OR estimates, along with P values (either nominal or adjusted).

Genotyping of SNPs within and beyond the HLA class II region

Single nucleotide polymorphism selection focused on three major genomic blocks within and between HLA class I and class II genes (Table S1). The first (HLA class I region) has three genes (*ZNRD1*, *HLA-C*, and *HCP5*) recently implicated as important to HIV-1 pathogenesis in several genome-wide association studies (Dalmasso et al. 2008; Fellay et al. 2007; Limou et al. 2009) and additional follow-up studies (Catano et al. 2008; Shrestha et al. 2009; van Manen et al. 2009). The second (central HLA region) has a cluster of genes (*TNF*, *LTA*, and *LTB*) encoding tumor necrosis factor alpha (TNF-α), lymphotoxin alpha (LT-α or TNF-β), and LT-β that regulate inflammatory responses. The third (HLA class II region) has multiple non-coding SNPs that can effectively tag HLA class II alleles associated with autoimmune and related diseases (de Bakker et al. 2006). Gene-specific SNPs in HLA class I and central regions were selected in five steps. First, SNPs assembled through SNPper (http://snpper.chip.org/bio/snpper-explain)

were filtered to allow an initial focus on "functional SNPs" with a potential phenotypic effect, including those located in exons, transcription factor binding sites, and intron/exon boundaries. Second, SNPs with minor allele frequencies (MAF) <0.02 in any of the three major populations (Africans, Asians, and Europeans) targeted by the International HapMap Project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap26_B36/) were excluded for lack of statistical power. Third, the Tagger program (http://www.broad.mit.edu/mpg/tagger/) was applied to select additional tagging SNPs (tagSNPs) that would capture multiple SNPs using a stringent tagging algorithm ($r^2 \geq 0.8$), with the r^2 values derived from the HapMap project or the Perlegen Genotype Browser (http://genome.perlegen.com/browser/index.html). Fourth, SNPs commonly analyzed in earlier population studies (de Bakker et al. 2006; Fellay et al. 2007) were added to serve as benchmarks that could facilitate inter-cohort comparisons. Fifth, SNPs chosen after the first four steps were evaluated for their respective suitability for bead array-based genotyping (Goldengate SNP typing assay) at Illumina (San Diego, CA). SNPs predicted to have low (<50%) success rates were replaced by new ones of equal tagging capabilities. The last (fifth) step was repeated twice to yield a total of 88 (3 coding and 85 non-coding) SNPs (Table S1) for high-throughput genotyping, with quality control facilitated by previously genotyped reference DNA and by random duplicates of DNA samples derived from the study population.

Statistical approach to analyzing SNP genotypes

For each of the 88 MHC SNPs analyzed here, homozygous and heterozygous genotypes were tested in 3×3 and 3×2 contingency tables, i.e., patients with (2 or 1 copy) and without (0 copy) the minor allele at each locus were stratified by the three levels of Ab response (NR, MR, and HR). A nominal P value < 0.050 in Chi-square test was considered indicative of possible associations and all neighboring SNPs were evaluated jointly for potential involvement of local haplotypes. Evaluation of the relative and independent contribution of SNP alleles and haplotypes required statistical adjustments for HLA and non-genetic factors revealed by primary association analyses. Given the multiple testing of 88 target SNPs, about four random (false positive) discoveries were anticipated when type I statistical error was accepted at the 0.050 level, while a truly probable association would require a P value $\leq 5.7 \times 10^{-4}$ to withstand correction for multiple comparisons. As a compromise between least and most stringent evaluations, formal multivariable statistical models were considered useful only for SNP variants with $P < 0.001$ in the initial univariate tests.

Results

Patient characteristics and assessment of non-genetic factors in Ab responses

Among the 255 HIV-1 seropositive (HIV^+) and 80 seronegative (HIV^-) youth enrolled into this study, HIV^- individuals had a mean age of 15.8 years and they were mostly male (63.8%) and Hispanic (75.0%). HIV^+ individuals were slightly older (average age = 20.9 years, $P < 0.0001$ when compared with HIV^- subjects) and they were mostly female (61.6%) and Hispanic (66.3%).

As seen in analyses of another youth population (Wang et al. 2004; Wilson et al. 2001), Ab response clearly differed between HIV^+ and HIV^- recipients (Table 1). More specifically, 5.0% of HIV^- subjects were classified as NR (Ab concentration <10 mIU/mL), 48.8% as MR (10–1,000 mIU/mL), and 46.3% as HR (>1,000 mIU/mL), while HIV^+ subjects had 29.0% NR, 47.5% MR, and 23.5% HR (overall $P < 0.0001$ by logistic regression). In univariate analysis, the negative impact of HIV-1 infection was clearly reflected by the mean parameter (β) estimate and standard error (SE) (-0.61 ± 0.13, $P < 0.0001$), along with the estimates of proportional odds ratio (pOR = 0.29) and 95% confidence interval (CI) (0.18–0.48) in test for trend across the three patient groups defined by high to low Ab responses (i.e., ordered as HR, MR, and NR) (Table 2). To minimize potential confounding by non-genetic factors, all subsequent association analyses considered age, sex, ethnicity, and HIV-1 infection status as cofactors (whenever possible).

Distribution and LD of HLA class II variants

Before any stratification, common allele groups found in at least ten individuals ranged from five at *HLA-DQA1* and *-DQB1* loci to 12 at the *HLA-DRB1* locus. These allele groups contributed to 18 common DRB1–DQB1 haplotypes. Distribution of homozygous and heterozygous genotypes at each locus did not deviate from HWE, even for subsets of HLA typing results restricted to either racial groups or HIV-1 infection status (data not shown). Similarly, distribution of four-digit alleles also conformed to HWE, and the common alleles detected at each locus ranged from 12 at the *HLA-DQA1* locus to 25 at the *HLA-DRB1* locus. Twenty DRB1–DQB1 haplotypes were also common in tabulations based on the four-digit allelic designations.

LD was quite strong for many four-digit alleles resolved at the three HLA class II loci, leading to the unambiguous (probability >99%) assignment of three-locus haplotypes in all 335 individuals. However, within the three main racial

Table 2 Univariate and multivariable models to compare relative impact of HIV-1 infection and HLA allelic variants on antibody responses among 335 youth

Factors (covariates)	*n*	Univariate analyses[a]				Multivariable model 1[b]				Multivariable model 2[b]			
		$\beta \pm$ SE	*P*	pOR	95% CI	$\beta \pm$ SE	*P*	pOR	95% CI	$\beta \pm$ SE	*P*	pOR	95% CI
HIV-1 infection	255	−0.61 ± 0.13	<0.0001	0.29	0.18–0.48	−0.60 ± 0.16	0.002	0.30	0.16–0.56	−0.59 ± 0.16	<0.001	0.31	0.16–0.57
DRB1*03[c]	71	−0.56 ± 0.25	0.026	0.57	0.35–0.93	−0.55 ± 0.27	0.040	0.58	0.34–0.98	NA[c]		NA[c]	
DRB1*0701[c]	71	−0.56 ± 0.25	0.043	0.60	0.37–0.98	−0.58 ± 0.26	0.025	0.56	0.34–0.93	NA[c]		NA[c]	
DRB1*0804	21	1.17 ± 0.44	0.008	3.22	1.35–7.63	1.31 ± 0.47	0.005	3.69	1.48–9.21	1.37 ± 0.46	0.003	3.92	1.59–9.69
DQB1*02	116	−0.50 ± 0.22	0.020	0.60	0.40–0.93	NA[c]				−0.37 ± 0.22	0.100	0.69	0.45–1.08

[a] In analyses of three categories of antibody responses (Table 1), summary statistical measures include beta estimate (mean ± standard error), proportional odds ratio (pOR), and 95% confidence interval (CI), as described in the text

[b] Age, ethnicity, and sex are treated as additional covariates in these models

[c] Strong linkage disequilibrium between these DRB1 variants and DQB1*02 (*0201 and *0202) precludes their presence in a single model (*NA* not applicable)

groups (Table 1), pairs of common alleles with strong enough LD ($r^2 \geq 0.80$) to be mutually tagging were limited to (a) DRB1*0301 versus DQB1*0201 ($r = 0.82$–0.95), (b) DRB1*0301 versus DQA1*0501 ($r = 0.92$–1.00), (c) DRB1*0701 versus DQA1*0201 ($r = 0.86$–0.98), (d) DR B1*1101 versus DQA1*0105 ($r = 0.72$–0.94), (e) DRB1 *1301 versus DQB1*0603 ($r = 0.81$–0.92), (f) DRB1* 1401 versus DQA1*0104 ($r = 0.92$–1.00), (g) DRB1* 1401 versus DQB1*0503 ($r = 0.92$–1.00), (h) DRB1*1402 versus DQB1*0503 ($r = 0.89$–0.92), (i) DRB1*1502 versus DQB1*0601 ($r = 0.71$–1.00), (j) DQA1*0501 versus DQB1* 0201 ($r = 0.83$–0.96), and (k) DRB1*0301–DQB1*0201 haplotype versus DQA1*0501 ($r = 0.92$–1.00). These allele combinations and their two-digit allele groups were treated as joint entities in order to minimize the number of tests for genetic associations with variable Ab responses.

As HIV-1 infection status alone was a strong predictor of suboptimal Ab response, HLA class II alleles and haplotypes that were differentially ($P < 0.050$) distributed among HIV^+ and HIV^- subjects were also sorted (Table S2). Alleles enriched in HIV^- subjects were DRB1*0407, DRB1*0802, and DQA1*0301. Alleles enriched in HIV^+ subjects were DRB1*0804, DQA1*0103, DQB1*0503, and DQB1*0603 (Table S2).

Individual HLA class II variants in relation to differential Ab responses in the entire study population

Primary association analyses tested the key hypothesis that HLA class II alleles and/or haplotypes, especially those observed in another youth population (Wang et al. 2004), correlate with variable Ab response to hepatitis B vaccination, irrespective of other characteristics of the study population. After accounting for sample size (statistical power) and patterns of LD (genetic confounding), four allele groups stood out to be the most likely predictors of differential Ab responses (Table 2). First, DRB1*03 (mostly *0301 and *0302) and DRB1*07 (exclusively *0701) were negatively associated with the responder phenotype, with similar effective sample size ($n = 71$ for both), β estimates (-0.56 for both) and P values (≤ 0.043), as well as similar pOR (≤ 0.60). Second, DRB1*08 seen in 51 patients had a positive association ($\beta = 0.75$, $P = 0.010$; pOR = 2.11, 95% CI = 1.20–3.72), mostly due to DRB1*0804 ($n = 21$, $\beta = 1.17 \pm 0.44$, $P = 0.008$; pOR = 3.22, 95% CI = 1.35–7.63). In contrast, DRB1*0802 (Table S2) as another common allele in the DRB1*08 group did not show a clear trend for positive or negative association ($n = 16$, $\beta = 0.18 \pm 0.48$, $P > 0.70$), neither did the less frequent allele DRB1*0801 ($n = 9$, $\beta = 0.74 \pm 0.64$, $P = 0.249$). Third, DQB1*02, which was found in 116 patients and in LD with DRB1*03 ($r = 0.32$–0.43, $P \leq 0.0001$) and

DRB1*07 ($r = 0.55$–0.72, $P \leq 8.0 \times 10^{-10}$), paralleled the associations of the two *DRB1* allele, with similar parameter estimates ($\beta = -0.50 \pm 0.22$, $P = 0.020$; pOR = 0.60, 95% CI = 0.40–0.93).

In multivariable models, the impact of three *HLA-DRB1* variants on Ab responses was clearly independent of one another and these associations were insensitive to statistical adjustments for the effects of HIV-1 infection and other potential confounders (adjusted $P = 0.005$–0.040) (Table 2). Alternating DRB1*03 and DRB1*0701 with DQB1*02 did not change the parameter estimates for non-genetic factors and DRB1*0804, but it was evident that DQB1*02 itself was not a statistically significant factor (adjusted $P = 0.100$) (Table 2). Collectively, DRB1*03 and DRB1*0701 were found in 42 (53.8%) out of 78 NR, 65 (40.6%) out of 160 MR, and 27 (27.8%) out of 97 HR ($P < 0.001$ for trend).

Evaluation of HLA haplotypes in the entire study population

Associations attributed to individual DRB1 alleles or allele groups were partially reflected by several DRB1-DQB1 haplotypes (Table S2). For example, the negative impact of DRB1*03 and DRB1*07 on Ab response was mostly due to the DRB1*03–DQB1*04 (DRB1*0302–DQB1*0402) haplotype ($\beta = -0.75 \pm 0.41$, $P = 0.067$) and the DRB1*07–DQB1*02 (DRB1*0701–DQB1*0202) haplotype ($\beta = -0.48 \pm 0.26$, $P = 0.066$), respectively. The positive association of DRB1*08 was mainly because of DRB1*08–DQB1*03 ($n = 22$, $\beta = 1.17 \pm 0.43$, $P = 0.007$) or DRB1*0804–DQB1*0301 ($n = 18$, $\beta = 1.26 \pm 0.48$, $P = 0.009$), but not DQB1*0301 itself ($n = 108$, $\beta = 0.23 \pm 0.22$, $P = 0.290$). The parameter estimates for the major haplotypes involving DRB1*08 were highly comparable with those for DRB1*0804 alone (Table 2). Further analyses of extended (three-locus) haplotypes did not yield any definitive relationships.

Two alternative models to allow inter-study comparisons

To facilitate direct comparison with earlier findings based on vaccine recipients without apparent immunodeficiency, 27 HIV$^+$ subjects with severe immunodeficiency (<200 CD4+ T cells/μL) were excluded from the testing of an alternative model (Table 3). DRB1*03, DRB1*0701, and DRB1*0804 remained as cofactors associated with differential Ab responses ($P = 0.002$–0.006), even after statistical adjustment for residual effects of HIV-1 infection and other non-genetic factors. A second alternative model used the strategy tested in another cohort of youth (Wang et al. 2004), when responders were not further differentiated into HR and MR groups. Again, DRB1*03 and *0701 were independently associated with differential Ab response status (Table 3), with similar parameter estimates ($\beta = -0.88 \pm 0.35$ and -1.03 ± 0.36, $P = 0.013$ and 0.005, respectively). The effect of DRB1*0804 could not be reliably established, as the allele was only present in the aggregated responder group (HR + MR).

Analyses restricted to 255 HIV$^+$ subjects

Stepwise analyses were further applied to the 255 HIV$^+$ individuals alone (Table 4). By univariate analyses, severe immunodeficiency (<200 CD4+ T cells/μL) and moderate immunodeficiency (200–450 CD4+ T cells/μL) had a strong negative impact on Ab responses ($P < 0.001$). DRB1*0701 became a minor factor ($n = 56$, $P = 0.159$) when compared with DRB1*03 ($n = 58$, $P = 0.039$) and DRB1*0804 ($n = 18$, $P = 0.003$). Two additional *DRB1* variants, DRB1*04 ($n = 44$, $P = 0.054$) and DRB1*15 ($n = 55$, $P = 0.092$), were recognized as marginally unfavorable and favorable, respectively, with the latter found exclusively on the DRB1*15–DQB1*06 haplotype. Only DRB1*03, DRB1*0701, and DRB1*0804 could be retained as independent factors (adjusted $P = 0.005$–0.029) in the reduced multivariable model, after statistical adjustments

Table 3 Additional multivariable models to facilitate direct comparison with some of the earlier findings

Factors (covariates)	*n*	Alternative multivariable model 1[a]				Alternative multivariable model 2[a]			
		$\beta \pm$ SE	*P*	pOR	95% CI	$\beta \pm$ SE	*P*	OR	95% CI
HIV-1 infection	228	-0.44 ± 0.17	0.010	0.42	0.22–0.81	-0.73 ± 0.31	0.021	0.23	0.07–0.80
HLA variants									
DRB1*03	67	-0.78 ± 0.28	0.006	0.46	0.26–0.80	-0.88 ± 0.35	0.013	0.42	0.21–0.83
DRB1*0701	67	-0.84 ± 0.28	0.002	0.43	0.25–0.74	-1.03 ± 0.36	0.005	0.36	0.18–0.73
DRB1*0804[b]	20	1.37 ± 0.49	0.006	3.93	1.50–10.31	NA		NA	

[a] These analyses are restricted to 308 patients, when 27 HIV$^+$ patients with severe immunodeficiency (<200 CD4+ T cells/μL) are excluded. First model tests three ordered groups of patients (HR, MR, and NR), while the second tests two patient groups, i.e. (HR + MR) versus NR. In both models, age, ethnicity, and sex are treated as additional covariates for statistical adjustments

[b] DRB1*0804 is not suited for model 2 as it is only found in responders (HR + MR); *NA* not applicable

Table 4 Analyses confined to 255 HIV-1 seropositive (HIV$^+$) youth

Individual factors (covariates)	*n*	Parameters from univariate analyses[a]				Reduced multivariable model[a]			
		$\beta \pm$ SE	*P*	pOR	95% CI	$\beta \pm$ SE	*P*	pOR	95% CI
<200 CD4+ T cells/μL[b]	27	−2.69 ± 0.48	<0.0001	0.07	0.03–0.18	−2.95 ± 0.50	<0.0001	0.06	0.02–0.14
200–450 CD4+ T cells/μL[b]	96	−1.01 ± 0.26	<0.001	0.36	0.22–0.61	−1.03 ± 0.27	<0.001	0.36	0.21–0.61
Individual HLA variants[c]									
DRB1*03	58	−0.58 ± 0.28	0.039	0.56	0.32–0.97	−0.67 ± 0.31	0.029	0.51	0.28–0.93
DRB1*04	44	−0.61 ± 0.31	0.054	0.55	0.35–1.01	NA		NA	
DRB1*0701	56	−0.40 ± 0.28	0.159	0.67	0.38–1.17	−0.68 ± 0.31	0.026	0.50	0.28–0.92
DRB1*0804	18	1.40 ± 0.48	0.003	4.06	1.59–10.34	1.44 ± 0.51	0.005	4.23	1.55–11.58
DRB1*15[d]	55	0.49 ± 0.29	0.092	1.62	0.93–2.85	NA		NA	

[a] Univariate analyses correspond to tests for trend across three patient groups (HR, MR, and NR), while multivariable model highlights all independent factors (with further statistical adjustments for potential confounding by age, ethnicity, and sex). *NA* not applicable (adjusted $P > 0.050$)

[b] As measured at baseline (time of enrollment). Patients with >450 CD4+ T cell counts/μL serve as the reference group

[c] Other variants are dismissed by univariate and multivariable analyses

[d] In these 55 patients, DRB1*15–DQB1*06 is the only haplotype involving DRB1*15

for the clear, negative impact of severe and moderate immunodeficiency ($P < 0.001$). Thus, replacing HIV-1 infection status (Table 2) with CD4+ T cell count as a key non-genetic factor did not alter the independent associations of three *HLA-DRB1* variants with Ab responses.

Analyses confined to 229 Hispanic subjects

In further analyses confined to 229 Hispanics (Table 5), a reduced multivariable model continued to confirm the negative impact of HIV-1 infection on Ab responses ($P < 0.0001$). DRB1*0804 ($n = 11$) and DRB1*15 ($n = 47$) were the only HLA class II variants independently associated with Ab responses (adjusted $P = 0.045$ and 0.019, respectively). Analyses of other alleles (including DRB1*03 and DRB1*0701) and their haplotypes were inconclusive because of reduced statistical power.

Secondary analyses of SNPs

Secondary analyses tested the hypothesis that non-coding SNPs in and around HLA class II genes might also be contributory, because of their potential influence on gene expression and/or mRNA splicing, as implied by genome-wide association studies (Dalmasso et al. 2008; Fellay et al. 2007; Hafler et al. 2007; Kuniholm 2007; Limou et al. 2009). A panel of 88 SNPs within three blocks of human MHC was successfully genotyped in 280 patients with at least 2 μg of high-quality DNA at the completion of HLA genotyping. One SNP (rs2523685) mapped to MHC class I region had a nominal *P* value at 0.021 in test of SNP genotypes among the three Ab response groups (Fig. 1). This weak association was driven by the heterozygous genotype (rs2523685CG), which was present in 31 (37.8%) out of 82 HR, 70 (49.3%) out of 142 MR, and 33 (58.9%) out of 56 NR patients. Homozygosity for the minor allele (genotype GG) had no trend (24.4% HR, 14.1% MR, and 21.4% NR). The putative association of rs2523685 genotypes with differential Ab responses could be dismissed for three reasons. First, the rs2523685 SNP is mapped to the 5′ flanking region (distant from the promoter sequences) of *HCP5* (Table S2), without any predicted or known functional relevance for either allele. Second, likelihood of chance association was high—about two random associations were

Table 5 Multivariable analyses confined to 229 Hispanic youth

Individual factors (covariates)	*n*	Reduced multivariable model[a]			
		$\beta \pm$ SE	*P*	pOR	95% CI
HIV-1 infection	169	−0.83 ± 0.21	<0.0001	0.19	0.08–0.43
Individual HLA variants[b]					
DRB1*0804	11	1.26 ± 0.63	0.045	3.53	1.03–12.06
DRB1*15	47	0.77 ± 0.33	0.019	2.16	1.13–4.10

[a] Following similar strategies shown in Table 4, except that (i) status of HIV-1 infection replaces CD4+ T cell count as the primary non-genetic factor; (ii) only age and sex are treated as additional covariates

[b] Other HLA variants, including DRB1*03 and DRB1*0701, are dismissed (adjusted $P > 0.050$)

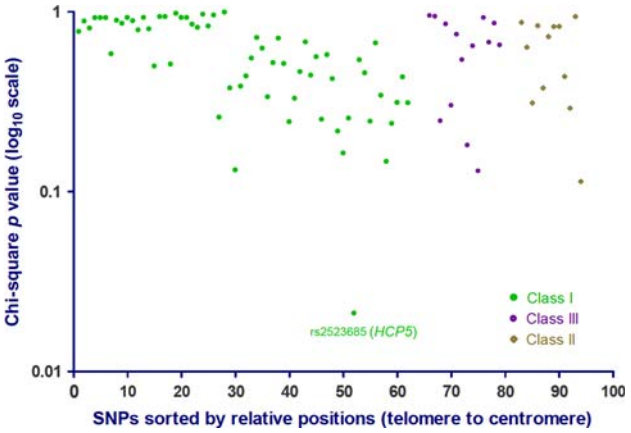

Fig. 1 Distribution of nominal *P* values in the analyses of 88 single nucleotide polymorphisms (SNPs) within three regions of the human major histocompatibility complex (MHC) (Table S2). MHC class I SNPs ($n = 62$) are mapped to HLA class I and neighboring genes, including *HCP5*, *HLA-C*, and *ZNRD1* previously associated with HIV-1-related outcomes (see text). MHC class III (central MHC) SNPs ($n = 14$) are mapped to *TNF*, *LTA*, and *LTB*, which encode TNF-α, lymphotoxic alpha, and lymphotoxic beta, respectively. MHC class II SNPs ($n = 12$) are in non-coding sequences around *HLA-DRB1*, *-DQA1*, and *-DQB1*. The nominal *P* values are based on Chi-square tests of non-responders (serum antibody concentrations <10 mIU/mL at week 28), responders (10–1,000 mIU/mL), and high responders (>1,000 mIU/mL). All SNPs are sorted by their relative order (from telomere to centromere) instead of actual distance on the short arm of chromosome 6. For clarity, three spaces are introduced between MHC blocks. Only a single SNP (rs2523685) in the 5′ flanking region of *HCP5* has trend for association ($P = 0.021$)

expected from the tests of 88 SNPs when *P* value was accepted at 0.021. Alternatively, 1.3 random associations were expected from the tests of 62 MHC class I SNPs alone. Third, the SNP is remote from the HLA class II genes that produced the strongest signals in all analyses.

Discussion

Our analyses of three HLA class II genes in 335 youth confirmed the negative impact of DRB1*03 and DRB1*0701 on peak Ab responses to HBV vaccination, as reported in prior studies of infant, adolescent, and adult populations (Belloni et al. 1993; Caillat-Zucman et al. 1998; Craven et al. 1986; del Canho et al. 1993; Hatae et al. 1992; Hohler et al. 1998, 2002b; Kramer et al. 1988; Martinetti et al. 1995; McDermott et al. 1997; Peces et al. 1997; Qian et al. 2002; Wang et al. 2004; Watanabe et al. 1988; Weissman et al. 1988). Although our study relied heavily on Hispanics and HIV$^+$ youth, the main findings most closely resembled those reported earlier for adults of European ancestry (Hohler et al. 1998). Studies on patients with natural HBV infection lend further support for these immunogenetic findings, as HLA haplotypes having either DRB1*03 or DRB1*07 were enriched in patients who were unable to clear HBV after infection (Thio et al. 2003). In addition, as both DRB1*03 and DRB1*0701 are in strong LD with DQB1*02 (DQ2 by serology), their relationships to Ab responses were partially reflected by DQB1*02 (Belloni et al. 1993; Hohler et al. 1998; Martinetti et al. 1995, 2000; McDermott et al. 1997; Peces et al. 1997; Stachowski et al. 1995).

In population-based analyses of diverse HLA alleles, statistical power (sample size and allele frequency), false discoveries, and population heterogeneity are some of the major concerns. The *HLA-DRB1* variants negatively associated with Ab responses were relatively common in our study population, but statistical power alone could not account for these key findings because statistical power actually favored the analyses of *HLA-DQA1* and *-DQB1* variants, which are much less diverse than *HLA-DRB1* alleles. False discovery was also unlikely because various studies have led to similar conclusions. Indeed, even the minor finding on DRB1*15 (DR2 by serology), which appeared to be a favorable factor in HIV$^+$ youth (Table 4), was also consistent with evidence from studies of infants and adults (Caillat-Zucman et al. 1993; Hohler et al. 1998). Population heterogeneity, on the other hand, could obscure causal relationships when analyses can only survey tagging variants instead of the functional units. However, heterogeneity in ethnic backgrounds could not compromise our major findings at least for three reasons. First, individual HLA alleles encode structurally and functionally distinct protein products that facilitate immune surveillance regardless of racial or ethnic background (Jones et al. 2006). Second, parameter estimates for DRB1*03 and DRB1*0701 were mostly insensitive to statistical adjustments for non-genetic factors, including race/ethnicity (Table 2). Third, our analyses dismissed the potential confounding by other genetic variants, especially those (e.g., DQB1*0201 and DQB1*0202) that are known to be in strong LD with DRB1*03 and DRB1*0701 (Table 2).

HLA class II alleles have been associated with differential Ab responses to other vaccines, including measles–mumps–rubella (MMR) vaccine (Ovsyannikova et al. 2004, 2006). In US schoolchildren (mostly European Americans) who received MMR vaccination, DRB1*08 was considered unfavorable—being negatively associated with desirable immune response (Ovsyannikova et al. 2004), which contrasted with its positive association with Ab response to HBV vaccination here. Another study reported a disadvantage for DRB1*0801 in primary biliary cirrhosis patients of European descent (Donaldson et al. 2006). While predominance of Hispanics and HIV$^+$ subjects in our study population might be one of the reasons for seemingly inconsistent findings between studies, MMR vaccine is quite different from HBV vaccine in that it uses attenuated viruses instead

of recombinant antigens. Moreover, DRB1*08 in our study population was represented by at least three common alleles (DRB1*0801, *0802, and *0804) and only DRB1*0804 had the clearest impact on Ab response to HBV vaccine (Table S2). In the absence of highly comparable data from other studies, the relevance of DRB1*08 to HBV vaccination deserves further evaluation in larger populations.

In contrast to HLA alleles with well-defined function, the MHC SNPs are mostly within non-coding sequences and are suitable for testing secondary hypotheses about relevance of gene expression and tagging of other HLA alleles (de Bakker et al. 2006; Listgarten et al. 2008; Miretti et al. 2005) not specifically captured in routine HLA genotyping. Our selection of SNPs in MHC class I region was dictated by recent studies, which have demonstrated the potential influence of MHC SNPs on HIV-1 pathogenesis and disease progression (Catano et al. 2008; Dalmasso et al. 2008; Fellay et al. 2007; Limou et al. 2009; Shrestha et al. 2009; van Manen et al. 2009). Although multiple SNPs can tag a few HLA class I alleles (e.g., B*5701) and serology groups (e.g., A10), these SNPs had little overlap with the panel of SNPs that can be used to reliably infer HLA class I alleles (Listgarten et al. 2008). The critical role of protective HLA class I alleles (e.g., B*5701 and B*5703) in immune control of HIV-1 infection (O'Brien and Nelson 2004; Tang and Kaslow 2003) was expected to indirectly benefit Ab response to HBV vaccination if patients are not receiving antiretroviral therapy. In analyses of MHC class I SNPs (Fig. 1), the absence of any apparent association was somewhat consistent with the earlier observation that HLA class I alleles and haplotypes were not associated with Ab responses in the REACH cohort (also consisted mostly of HIV-1-infected youth) (Wang et al. 2004). The new study population assembled here was not suitable for testing the indirect effects produced by HLA class I genotypes, because few HIV^+ youth (less than 10%) had severe immunodeficiency at the time of vaccination. The weak signal produced by a single, non-coding SNP within the *HCP5* gene is probably worth pursuing in larger, homogeneous cohorts, as analyses of SNPs without well-established functional attributes typically require stratification by race.

In summary, both primary and secondary analyses of HLA and related genotypes have reiterated multifactorial influences on Ab responses to HBV vaccination in youth. Recognition of immunodeficiency (the loss of CD4+ T cells) and several *HLA-DRB1* alleles as independent predictors of Ab response highlights the importance of T-helper cell-dependent pathways and HLA class II antigen presentation to effective vaccination using recombinant antigens. This work has also paved the way for further high-throughput SNP typing that may reveal additional factors, including regulatory (non-coding) sequence variations commonly found in immune response genes beyond the HLA region, as well as coding sequence variations that are also common in genes encoding killer immunoglobulin receptors (Bashirova et al. 2006; Parham 2005). Systematic evaluation of non-HLA genes can be informative, as studies of European twins have already indicated that almost 59% of heritability in Ab responses to HBsAg can be attributed to genetic variations beyond the *HLA-DRB1* locus (Hohler et al. 2002a).

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Appendix

This study (designated as ATN 052) also involved additional investigators and staff who enrolled patients at 22 clinical sites, including University of Maryland (Ligia Peralta, MD, Esther Collinetti, BA, Leonel Flores, MD, Reshma S. Gorle); Hospital dos Servidores do Estado, Rio de Janeiro (Esau C. Joao, MD, Leticia S. Cruz, MD, Eduarda Gusmao, MD, Angela B. N. Carvalho, MD); Universidade Federale de Minas Gerais, Minas Gerais (Jorge Pinto, MD, Flavia G. F. Ferreira, MD); St. Jude Research Hospital (Sarah Stender, MD, Kristen Branum, BS, Tina Culley, BS, RHIA, CCRP, Carla McKinley, RN, MSN, Thomas Wride, MS); Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto-Universidade de Sao Paulo, Sao Paulo (Geraldo Duarte, MD, Marisa M. M. Pinhata, MD, Carolina S. Vieira, MD); Instituto de Puericultura e Pediatria Martagao Geseteira, Rio de Janeiro (Ricardo H. S. Oliveira, MD, Maria C. C. Sapia, MD, Thalita F. Abreu, MD); Instituteo de Infectologia Emilio Ribas Sao Paulo (Marinella D. Negra, MD, Wladimir Queiroz, MD); Montefiore Medical Center (Donna Futterman, MD, Elizabeth Enriquez-Bruce,

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