

# HLA and Cytokine Gene Polymorphisms Are Independently Associated With Responses to Hepatitis B Vaccination

Chengbin Wang,<sup>1</sup> Jianming Tang,<sup>2</sup> Wei Song,<sup>1</sup> Elena Lobashevsky,<sup>1</sup> Craig M. Wilson,<sup>2,3</sup> and Richard A. Kaslow<sup>1,2</sup>

Variable immune responses to hepatitis B virus (HBV) infection and recombinant HBV vaccines have been associated with polymorphisms in several genes within the human leukocyte antigen (HLA) complex. Analyses of polymerase chain reaction (PCR)-based genotyping data from 164 North American adolescents vaccinated with recombinant HBV products confirmed that *HLA-DRB1\*07* (relative odds [RO] = 5.18,  $P < .0001$ ) and human immunodeficiency virus type 1 (HIV-1) infection (RO = 3.91,  $P < .001$ ) were both associated with nonresponse to full-dose vaccination. Further associations were observed with single nucleotide polymorphisms (SNPs) at the *IL2* and *IL4* loci along with insertion/deletion variants at the *IL12B* locus ( $P = .003-.01$ ). Host genetic associations were independent of one another as well as other *HLA* (*A*, *B*, *C*, and *DQB1*) and cytokine gene (*IL4R*, *IL6*, *IL10*, and *TNF*) variants. Statistical adjustments for nongenetic factors (gender, ethnicity, age, HIV-1 infection, and vaccination protocols) did not substantially alter the strengths of the genetic relationships. The overall distribution pattern of genetic variations was similar between the analyzed vaccinees and additional adolescents ( $n = 292$ ) from the same cohort. In conclusion, *DRB1\*07* (or a closely linked allele) and immunoregulatory cytokine gene polymorphisms correlate with variable immune response to recombinant HBV vaccines. (HEPATOLOGY 2004;39:978–988.)

Chronic hepatitis B virus (HBV) infection affects 350 million people worldwide and is the leading cause of liver cirrhosis and hepatocellular carcinoma.<sup>1,2</sup> Vaccination for HBV infection has been highly successful,<sup>3</sup> but immune responses to multidose HBV

vaccines vary from individual to individual. At least 5% to 10% of most healthy adult populations fail to produce protective levels of antibodies to recombinant HBV surface antigen (HBsAg) following standard vaccination protocols.<sup>4–6</sup> HLA diversity may account in part for selective suboptimal HBV antigen presentation,<sup>5,7,8</sup> while epidemiologic and experimental studies have also documented inter-individual differences in other measures of immunity, including magnitude of the HBsAg-specific T cell response,<sup>9</sup> breadth of the B cell repertoire,<sup>10</sup> and intensity of T-helper (T<sub>H</sub>) type 1 (T<sub>H1</sub>) and 2 (T<sub>H2</sub>) cytokine secretion.<sup>11–13</sup>

Several markers in the *HLA* region have been associated with responder and nonresponder phenotypes following full-dose HBV vaccination.<sup>5,7,8,14–17</sup> Other candidate genes, especially those encoding immunoregulatory cytokines, may further modulate these differential responses. Potential effects of cytokine genes encoding interleukin (IL)-2 and IL-12 deserve special attention, because recombinant forms of these molecules are increasingly used as immune adjuvants in animal and human vaccine studies. In a cohort of North American adolescents, we detected multiple independent associations of *HLA* class II as well as T<sub>H1</sub> and T<sub>H2</sub> cytokine gene variants with differential responses to full-dose HBV vaccination.

Abbreviations: HBV, hepatitis B virus; HLA, human leukocyte antigen; PCR, polymerase chain reaction; RO, relative odds; HIV-1, human immunodeficiency virus type 1; SNP, single nucleotide polymorphism; HBsAg, HBV surface antigen; T<sub>H</sub>, T-helper; IL, interleukin; SNP, single nucleotide polymorphism; REACH, Reaching for Excellence in Adolescent Care and Health study; anti-HBsAg, HBsAg serum antibody; SSP, sequence-specific primers; LD, linkage disequilibrium; HWE, Hardy-Weinberg equilibrium; HAART, highly active antiretroviral therapy.

From the Departments of<sup>1</sup>Epidemiology, <sup>2</sup>Medicine, and <sup>3</sup>Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama.

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Address reprint requests to: Jianming Tang or Richard A. Kaslow, Program in Epidemiology of Infection and Immunity, School of Public Health, University of Alabama at Birmingham, 1665 University Blvd., Birmingham, AL 35294-0022. E-mail: jtang@uab.edu or rkaslow@uab.edu; fax: 205-934-8665.

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**Table 1. General Characteristics of Vaccinated REACH Adolescents and a Reference Group Being Targeted in This Study**

Patient Characteristics	Entire REACH cohort			Fully vaccinated group only		
	Fully vaccinated (N = 164)	Reference group (N = 292)	P	Non-responders (N = 79)	Responders (N = 85)	P
Ethnicity			0.12			—
Black	104 (63.4)*	212 (72.6)*		52 (65.8)*	52 (61.2)*	
White	17 (10.4)	23 (7.9)		7 (8.9)	10 (11.8)	
Other	43 (26.2)	57 (19.5)		20 (25.3)	23 (27.1)	
Gender			—			—
Male	43 (26.2)	70 (24.0)		19 (24.1)	24 (28.2)	
Female	121 (73.8)	222 (76.0)		60 (75.9)	61 (71.8)	
Age at baseline			—†			—
<17	62 (37.8)	95 (32.7)		28 (35.4)	34 (40.0)	
>17	102 (62.2)	196 (67.3)		51 (64.6)	51 (60.0)	
HIV-1 status			<b>.002</b>			<b>.002</b>
Seropositive	114 (69.5)	160 (54.8)		66 (83.5)	48 (56.5)	
Seronegative	50 (30.5)	132 (45.2)		13 (16.5)	37 (43.5)	
CD4+ T cells			.08†			<b>.002</b>
>400/ $\mu$ L	44 (26.8)	57 (19.7)		28 (35.4)	16 (18.8)	
<400/ $\mu$ L	120 (73.2)	233 (80.3)		51 (64.6)	69 (81.2)	
CD8+ T cells			<b>.01†</b>			<b>&lt;.0001</b>
>40%	89 (54.3)	122 (42.1)		56 (70.9)	33 (38.8)	
<40%	75 (45.7)	168 (57.9)		23 (29.1)	52 (61.2)	
HAART‡			<b>.006†</b>			—
Yes	29 (25.4)	20 (12.5)		16 (24.2)	13 (27.1)	
No	85 (74.6)	140 (87.5)		50 (75.8)	35 (72.9)	
Asthma			<b>.04†</b>			—
Yes	34 (21.7)	35 (13.9)		18 (23.7)	16 (19.8)	
No	123 (78.3)	216 (86.1)		58 (76.3)	65 (80.3)	

NOTE: P values  $\leq .05$  are shown in **bold**; those  $> .20$  have been omitted (—).

Abbreviation: HAART, highly active antiretroviral therapy.

\*Numbers correspond to n (%).

†Information is not available for all subjects in these analyses.

‡In HIV-1 seropositive individuals only.

## Materials and Methods

**HBV Vaccine Study Group and Control Population.** Covering 15 participating institutions in 13 United States cities, this HBV vaccine study was nested within the Reaching for Excellence in Adolescent Care and Health (REACH) project approved by local institutional review boards. The REACH study design, eligibility criteria, demographic characteristics, and methods for virologic and immunologic measurements have been reported elsewhere.<sup>18,19</sup> Informed consent was obtained from all adolescents (N = 545, age 13-18 years). The vaccinees did not carry HBsAg and were free of hepatitis B core antibody at baseline and subsequent quarterly visits.<sup>20</sup> Participants received HBV vaccine according to the standard schedule at 0, 1, and 6 months. Among the 15 REACH clinics, six used exclusively Energix-B (Smith Kline Beecham Pharmaceuticals, Philadelphia, PA), seven used exclusively Recombivax HB (Merk and Co., Inc., West Point, PA), and the remaining two used both. Although immunogenicity of these products apparently showed little difference, adjustment for vaccine product

use as a covariate could minimize possible confounding of differential responses. Participants who had already received at least three doses of vaccine before they entered the REACH study had their serum antibodies to HBsAg (anti-HBsAg) quantified retrospectively; those who received at least one more dose after enrollment had anti-HBsAg quantified prospectively.

**T Lymphocyte Phenotyping.** CD4<sup>+</sup> and CD8<sup>+</sup> subsets of T cells were quantified by flow cytometry at each clinical site using AIDS Clinical Trials Group standardized protocols as described previously, while CD8<sup>+</sup>/CD38<sup>+</sup> T cell immunophenotyping was done at the Immunology Core Laboratory of the Children's Hospital of Philadelphia.<sup>21</sup> For all defined T cell populations, the absolute counts at the time of enrollment were analyzed as continuous variables. Alternatively, T cell percentages were dichotomized along their median values for the entire cohort (Table 1).

**DNA Extraction and HLA Genotyping.** Genomic DNA for each individual was extracted from  $2 \times 10^6$  peripheral blood mononuclear cells using the QIAamp blood kit (QIAGEN Inc., Chatsworth, CA). Of the 545

REACH participants, 530 (97%) had suitable DNA specimens for immunogenetic analyses. All DNA samples were diluted to 200 ng/ $\mu$ L and stored at 4°C in TE buffer (10 mM Tris-HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid) before use.

*HLA* class I (*A*, *B*, and *C*) and class II (*DRB1* and *DQB1*) variants were resolved to their two- to five-digit specificities based on a combination of PCR-based techniques, including PCR with sequence-specific primers (SSP) (Pel-Freez Clinical Systems, Brown Deer, WI), automated reference-strand conformation analyses (Pel-Freez Clinical Systems), and automated solid-phase DNA sequencing of locus-specific PCR amplicons (Amersham Pharmacia Biotech, Piscataway, NJ).<sup>22-24</sup>

**Genotyping of Cytokine Gene Polymorphisms.** A panel of 14 common SNPs from seven genes (*IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, and *TNF- $\alpha$* ) representing both  $T_H1$  and  $T_H2$  pathways in the cytokine network were typed by PCR-SSP using procedures recommended by the manufacturer (Department of Transplantation Immunology, University of Heidelberg, Heidelberg, Germany) and the 13th International Histocompatibility Workshop.<sup>25</sup> Reliability of this genotyping strategy was verified in an initial analysis of 50 reference DNA samples distributed by the 13th International Histocompatibility Workshop. When multiple SNPs at a single locus were present, their *cis* and *trans* relationships were directly established by PCR for all except two at the *IL6* locus. The insertion/deletion variants in the *IL12B* promoter sequence were also targeted, with the two alleles (L and S) assigned according to apparent fragment sizes differing by 4-bp after PCR amplification and denaturing gel electrophoresis.<sup>26,27</sup>

**General Genetic Analyses.** Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE) were assessed using the statistical package PopGene v1.32 (University of Alberta, Edmonton, Alberta, Canada). Local haplotypes consisting of two or more adjacent SNP alleles were assigned on the basis of strong-calculated LD or directly revealed by PCR. Deviation ( $P < .05$ ) from HWE was deemed indicative of sample selection bias or evolutionary advantage for particular SNP genotypes in a given population.

**Analyses of Genetic Associations With Response to HBV Vaccines.** Associations of genetic variants with the nonresponder/responder phenotypes were determined by several statistical routines in Statistical Analysis Software, version 9.0 (SAS Institute, Cary, NC). First, allele and carriage (population) frequencies were established through direct counting, with the numbers of chromosomes ( $2N$ ) and of individuals ( $n$ ) serving as the respective denominators. Second, the overall allelic and/or geno-

typic heterogeneity between responder and nonresponder patients was assessed by  $\chi^2$  or exact tests, in which rare alleles (frequencies  $<0.01$ , applicable to *HLA* variants only) at each locus were treated as a single entry. To obviate the need for Bonferroni correction for multiple comparisons at each individual locus, only those loci showing overall genetic heterogeneity at  $P < .05$  were analyzed further for individual marker effects. Third, in those loci demonstrating overall heterogeneity, Mantel-Hanszel  $\chi^2$  or Fisher's exact tests were applied to compare population (marker) frequencies of individual genetic variants in vaccine nonresponders and responders with adjustment for ethnicity; a nominal  $P$  value of  $<.05$  was considered suggestive of a nonrandom association. Fourth, unconditional logistic regression was used to adjust for the effects of nongenetic factors including ethnicity, gender, age (at enrollment), HIV infection, and different vaccination regimens. Fifth, the population frequencies of major haplotypes and heterozygosity at all candidate loci and haplotypes were also compared as described in the third and fourth steps. Sixth, the strength and independence of all genetic and nongenetic associations with differential antibody responses was assessed with multivariable logistic regression analyses. Seventh, the small group ( $n = 51$ ) of HIV-1-seronegative vaccinees was examined separately in multivariable logistic models to assess potential confounding by HIV-1 infection and use of highly active antiretroviral therapy (HAART) in some HIV-1 seropositive patients.

Rather than introducing the arbitrary and usually conservative Bonferroni correction for multiple statistical tests, we guarded against the possibility of type I error as a result of multiple comparisons in two ways. First, as noted above, only loci with globally significant differences in allelic distributions were analyzed in detail. Second, in testing differences at those loci, two levels of uncertainty were established in evaluating the findings. For previously known associations (e.g., with *HLA-B\*08*, *B\*44*, *DRB1\*03*, *DRB1\*07*, *DRB1\*13*, and the *B\*08-DRB1\*03* haplotype), the nominal  $P$  value in significance testing was accepted as closely approximating the probability of a valid null hypothesis. For any previously unstudied or unrecognized relationship (e.g., with cytokine gene polymorphisms), we have treated the findings as exploratory and the statistical analysis as potentially useful in future studies.

## Results

**Characteristics of HBV Vaccinees and Others in the REACH Cohort.** Of the 545 REACH study participants, 164 (30%) were fully vaccinated participants; the

remaining 381 (70%) could serve as the reference group for genetic comparisons, but only 292 (77%) of them had complete genotyping data (see Table 1). Among the vaccinees, 79 (48%) were nonresponders (anti-HBsAg < 10 mIU/mL)<sup>28</sup> for at least two follow-up intervals and 85 (52%) were responders (anti-HBsAg  $\geq$  10 mIU/mL).

The proportion of African Americans who were vaccinated was somewhat lower ( $P = .06$ ) than the proportions of the other ethnic groups, but the group of HBV vaccinees was similar to the reference group ( $P > .10$ ) in ethnic background, sex ratio, and age (see Table 1). The vaccinated and reference populations differed more ( $P = .002-.08$ ) in their proportion with HIV-1 infection, CD4<sup>+</sup> T cell counts < 400/ $\mu$ L, CD8<sup>+</sup> T cell percentage, and asthma. Exposure to HAART was more common in HBV vaccinees (25.4%) than others in the reference group (12.5%) ( $P = .006$ ). Nonresponse to HBV vaccines was 57.9% among HIV-1 seropositives and 26.0% among HIV-1 seronegatives (relative odds [RO] = 3.91, 95% confidence interval = 1.88–8.15). Accordingly, a higher proportion of nonresponders than responders had CD4<sup>+</sup> T cell counts of less than 400/ $\mu$ L ( $P = .002$ ), and nonresponders showed a higher percentage of CD8<sup>+</sup> T cells ( $P < .0001$ ), accompanied by a similar CD8<sup>+</sup>/CD38<sup>+</sup> cell percentage (data not shown). These factors, which could potentially confound the genetic relationships, warranted statistical adjustment in subsequent analyses.

**General Population Genetic Comparisons.** PCR-based genotyping at the target loci (*HLA*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, and *TNF*) was complete for 97% to 100% ( $n = 159-164$ ) of HBV vaccinees (Table 2) and for 97% to 100% ( $n = 284-292$ ) of the control population. Exclusive LD between *IL10*-819T and *IL10*-592A, noted here and elsewhere,<sup>25</sup> justified analysis of *IL10*-592A by itself.

Not surprisingly, the differences in distributions of genetic variations between African Americans and others were substantial ( $P = .001-.01$ ; data not shown). In analyses stratified by ethnic group, however, overall distributions of homozygous and heterozygous genotypes at individual target loci rarely deviated from HWE ( $P = .07-.84$ ). Common haplotypes detected at the *HLA*, *IL2*, *IL4*, *IL6*, *IL10*, and *TNF* loci (Table 3) also closely resembled those reported in the literature.<sup>25,29-32</sup> For the *IL12B* 3'UTR SNP and insertion/deletion in the promoter region, strong LD ( $\Delta = 0.06$ ,  $P < .0001$ ) also prompted analysis of distinctive *IL12B* haplotypes not recognized before (see Table 3). Further comparisons of HBV vaccinees with the REACH cohort revealed only marginal differences in the distributions of a few haplotypes (e.g.,

*HLA-B\*07-Cw\*07*, *B\*15-Cw\*02*, *B\*58-Cw\*06*, and *IL12B LA*) ( $P = .05-.08$ ; see Table 3).

**Associations of Individual HLA and Cytokine Gene Variants With Nonresponder and Responder Phenotypes Following HBV Vaccination.** Following trends revealed by tests of overall genetic heterogeneity between vaccine responders and nonresponders, *HLA* associations with the nonresponder phenotype could be attributed to several variants, including *HLA-Cw\*03* (RO = 2.83,  $P = 0.02$ ), *DRB1\*07* (RO = 5.18,  $P < .0001$ ), and *DQB1\*02* (RO = 2.55,  $P = .004$ ). Each of these relationships remained stable ( $P = .0002-.06$ ) after statistical adjustments for nongenetic factors, including ethnicity, gender, age, HBV vaccine product group, and HIV-1 serostatus. Conversely, *DRB1\*15* and *DQB1\*06* were both associated with the responder phenotype (adjusted RO = 0.71–0.72,  $P = .02-.05$ ) after accounting for the effects of nongenetic factors (see Table 2). *IL12B* promoter S allele (allele 2, having a 4-bp deletion) was the only cytokine gene variant associated with the nonresponder phenotype (adjusted RO = 2.82,  $P = .03$ ). The same univariate analyses did not yield evidence supporting the involvement of other *HLA* alleles (*HLA-B\*08*, *B\*44*, *DRB1\*03*, and *DRB1\*13*) previously associated with either HBV clearance or response to vaccination.<sup>33</sup>

**Haplotypic Associations With Nonresponder and Responder Phenotypes Following HBV Vaccination.** We analyzed haplotypes involving either adjacent *HLA* loci including *B-C* and *DRB1-DQB1* or cytokine gene SNPs (see Table 3). The associations of *HLA* haplotypes *B\*15-Cw\*03* and *DRB1\*07-DQB1\*02* (see Table 3), respectively, with the responder and nonresponder phenotypes were consistent with the effects of the individual alleles (see Table 2). Although far less common than *DRB1\*07-DQB1\*02*, the closely related *DRB1\*07-DQB1\*03* haplotype was also more frequent in the nonresponder group. Thus, these associations were mostly driven by *DRB1\*07* and not by the haplotypes *per se*. In contrast, the *DRB1\*15-DQB1\*06* haplotype was not so strongly associated with responder phenotype as its component alleles. Subsequent analyses focused on those individual *HLA* alleles showing statistically significant relationships (e.g., *Cw\*03* and *DRB1\*07*). The *IL4* TTC haplotype (fully resolved by PCR-SSP) was associated with the responder phenotype (RO = 3.03,  $P = .007$ ), although its component alleles were more equally distributed between responders and nonresponders (see Table 2). This haplotypic effect was independent of nongenetic factors (adjusted  $P = .01$ ).

**Heterozygosity and HBV Vaccination.** Heterozygosity at several *HLA* and cytokine loci has been associated with altered cytokine production levels<sup>26,34</sup> and

**Table 2. Population Frequencies [n (%)] of Common Genetic Variations Detected Among Fully Vaccinated Participants and a Reference Group From the REACH Cohort of Adolescents**

Genetic Variants	Fully Vaccinated	Reference Group	P Value*	Nonresponders	Responders	P Value*	Adjusted P Value†
<i>HLA-A</i> ‡	N = 161	N = 290		N = 77	N = 84		
*01	15 (9.3)	29 (10.0)	—	5 (6.5)	10 (11.9)	—	—
*02	57 (35.4)	109 (37.5)	—	28 (36.4)	29 (34.5)	—	—
*03	28 (17.4)	54 (18.6)	—	15 (19.5)	13 (15.5)	—	—
*23	29 (18.0)	55 (18.9)	—	11 (14.3)	18 (21.4)	—	—
*30	41 (25.5)	61 (21.0)	—	20 (26.0)	21 (25.0)	—	—
*33	21 (13.0)	37 (12.7)	—	8 (10.4)	13 (15.5)	—	—
*68	33 (20.5)	53 (18.2)	—	17 (22.1)	16 (19.1)	—	—
Other A's	75 (46.6)	135 (46.4)	—	38 (49.4)	37 (44.1)	—	—
<i>HLA-B</i> ‡	N = 161	N = 290		N = 77	N = 84		
*07	30 (18.6)	40 (13.8)	—	42 (54.6)	50 (59.5)	—	—
*15	45 (28.0)	60 (20.6)	—	23 (29.9)	22 (26.2)	—	—
*35	26 (16.2)	50 (17.2)	—	11 (14.3)	15 (17.9)	—	—
*42	18 (11.2)	35 (12.0)	—	7 (9.1)	11 (13.1)	—	—
*44	32 (19.9)	39 (13.4)	.07	18 (23.4)	14 (16.7)	—	—
*53	36 (22.4)	66 (22.7)	—	20 (26.0)	16 (19.1)	—	—
*58	15 (9.3)	39 (13.4)	—	10 (13.0)	5 (6.0)	—	—
Other B's	92 (57.1)	179 (61.5)	—	42 (54.6)	50 (59.5)	—	—
<i>HLA-Cw</i> †	N = 160	N = 290		N = 77	N = 84		
*02	26 (16.2)	49 (16.9)	—	10 (13.0)	16 (19.1)	—	—
*03	23 (14.3)	52 (17.9)	—	16 (20.8)	7 (8.3)	.03	.06
*04	58 (36.0)	115 (39.7)	—	29 (37.7)	29 (34.5)	—	—
*06	19 (11.8)	45 (15.5)	—	9 (11.7)	10 (11.6)	—	—
*07	67 (41.6)	104 (35.9)	—	30 (39.0)	37 (44.1)	—	—
*08	14 (8.7)	35 (12.1)	—	7 (9.1)	7 (8.3)	—	—
*16	29 (18.0)	44 (15.2)	—	14 (18.2)	15 (17.9)	—	—
*17	14 (8.7)	26 (9.0)	—	5 (6.5)	9 (10.7)	—	—
Other C's	38 (23.6)	70 (24.1)	—	16 (20.8)	22 (26.2)	—	—
<i>DRB1</i> ‡	N = 157	N = 284		N = 78	N = 82		
*01	20 (12.7)	29 (10.2)	—	11 (14.1)	10 (12.2)	—	—
*03	47 (30.0)	74 (26.0)	—	19 (24.4)	29 (35.4)	.20	.11
*04	27 (17.2)	53 (18.6)	—	13 (16.7)	14 (17.1)	—	—
*07	36 (22.9)	57 (20.0)	—	28 (35.9)	8 (9.8)	<.0001	.0002
*08	19 (12.1)	32 (11.2)	—	9 (11.5)	10 (12.2)	—	—
*11	34 (21.7)	62 (21.8)	—	13 (16.7)	23 (28.1)	.03	.08
*13	38 (24.2)	92 (32.3)	—	22 (28.2)	18 (22.0)	—	—
*15	35 (22.3)	71 (24.9)	—	13 (16.7)	22 (26.8)	.14	.05
Others	37 (23.6)	57 (20.0)	—	18 (23.1)	19 (23.2)	—	—
<i>DQB1</i> ‡	N = 158	N = 285		N = 78	N = 83		
*02	70 (44.3)	112 (39.3)	—	43 (55.1)	27 (32.5)	.003	.002
*03	77 (48.7)	132 (46.3)	—	35 (44.9)	44 (53.0)	—	—
*04	33 (20.9)	46 (16.1)	—	12 (15.4)	22 (26.5)	.11	.07
*05	44 (27.9)	85 (29.8)	—	24 (30.8)	23 (27.7)	—	—
*06	59 (37.3)	124 (43.5)	—	23 (29.5)	36 (43.4)	.08	.02
<i>IL2</i>	N = 159	N = 290		N = 78	N = 81		
-330G	38 (23.9)	50 (17.2)	.09	14 (18.0)	24 (29.6)	.10	.15
-330T	150 (94.3)	283 (97.6)	.08	72 (92.3)	78 (96.3)	—	—
166G	149 (93.7)	281 (96.9)	.14	74 (94.9)	75 (92.6)	—	—
166T	42 (26.4)	64 (22.1)	—	19 (24.4)	23 (28.4)	—	—
<i>IL4</i>	N = 162	N = 292		N = 78	N = 84		
-1098G	45 (27.8)	87 (29.8)	—	21 (26.9)	24 (28.6)	—	—
-1098T	161 (99.4)	290 (99.3)	—	78 (100.0)	83 (98.8)	—	—
-590C	94 (58.0)	177 (60.6)	—	50 (64.1)	44 (52.4)	.08	.12
-590T	129 (79.6)	239 (81.9)	—	62 (79.5)	67 (79.8)	—	—
-33C	133 (82.1)	240 (82.2)	—	61 (78.2)	72 (85.7)	—	.10
-33T	105 (64.8)	186 (63.7)	—	53 (68.0)	52 (61.9)	—	—
<i>IL4R</i>	N = 162	N = 292		N = 78	N = 84		
1902A	100 (61.7)	175 (59.9)	—	46 (59.0)	54 (64.3)	—	—
1902G	125 (77.2)	227 (77.7)	—	61 (78.2)	64 (76.2)	—	—
<i>IL6</i>	N = 162	N = 292		N = 78	N = 84		
-174C	30 (18.5)	47 (16.1)	—	13 (16.7)	17 (20.2)	—	—
-174G	160 (98.8)	284 (97.3)	—	77 (98.7)	83 (98.8)	—	—

Table 2. Continued

Genetic Variants	Fully Vaccinated	Reference Group	P Value*	Nonresponders	Responders	P Value*	Adjusted P Value†
565A	31 (19.1)	46 (15.8)	–	14 (18.0)	17 (20.2)	–	–
565G	160 (98.8)	285 (97.6)	–	77 (98.7)	83 (98.8)	–	–
<i>IL10</i>	<i>N</i> = 162	<i>N</i> = 292		<i>N</i> = 78	<i>N</i> = 84		
–1082A	143 (88.3)	264 (90.4)	–	69 (88.5)	74 (88.1)	–	–
–1082G	89 (54.9)	153 (52.4)	–	41 (52.6)	48 (57.1)	–	–
–592A§	96 (59.3)	176 (60.3)	–	46 (59.0)	50 (59.5)	–	–
–592C§	136 (84.0)	245 (83.9)	–	63 (80.8)	73 (86.9)	–	.16
<i>IL12B</i>	<i>N</i> = 162	<i>N</i> = 292		<i>N</i> = 78	<i>N</i> = 84		
Promoter L	100 (61.7)	174 (59.6)	–	53 (68.0)	47 (56.0)	.06	.07
Promoter S	140 (86.4)	243 (83.2)	–	72 (92.3)	68 (81.0)	<b>.05</b>	<b>.03</b>
3' UTR A	148 (91.4)	254 (87.0)	.19	70 (89.7)	78 (92.9)	–	–
3' UTR C	86 (53.1)	176 (60.3)	.14	45 (57.7)	41 (48.8)	–	–
<i>TNF</i>	<i>N</i> = 162	<i>N</i> = 292		<i>N</i> = 78	<i>N</i> = 84		
–308A	39 (24.1)	62 (21.2)	–	78 (100)	84 (100)	–	–
–308G	160 (98.8)	290 (99.3)	–	76 (97.4)	84 (100)	–	–
–238A	11 (6.8)	23 (7.9)	–	5 (6.4)	6 (7.1)	–	–
–238G	162 (100)	292 (100)	Ref.	78 (100.0)	84 (100.0)	–	–

\*Based on Mantel-Haenszel  $\chi^2$  tests or Fisher exact tests, with adjustments for ethnicity. P values  $\leq .05$  are shown in **bold**; those  $> .20$  have been omitted (–).

†Adjusted for other factors including HIV-1 status, gender, ethnicity, and vaccination protocols.

‡Individual HLA variants shown have a frequency above 5%; minor alleles are grouped as others.

§*IL10* -592A and -592G are found in exclusive linkage disequilibrium with -819T and -819C, respectively, as also noted elsewhere<sup>25,59</sup>.

difference in the severity/persistence of several diseases including hepatitis B.<sup>35–38</sup> Our analyses revealed that *IL2*-330 G/T was associated with favorable response to HBV vaccination (RO = 0.33,  $P = .01$ ), while *IL12B* L/S was associated with nonresponsiveness (RO = 2.59,  $P = .003$ ) (Table 4). Adjustments for nongenetic factors did not alter these relationships (adjusted  $P = .002$ –.05).

**Multivariable Models.** The independence of host genetic associations with HBV vaccination outcomes was evaluated in multivariable models, with adjustments for nongenetic factors including ethnicity, gender, HIV-1 infection, CD4<sup>+</sup> T cell counts, and CD8<sup>+</sup> T cell counts whenever necessary (Table 5). Collectively, *HLA-DRB1\*07* and the *IL12B* promoter heterozygosity (L/S) remained as major contributors to the nonresponder phenotype (adjusted RO = 2.74–6.33,  $P = .0003$ –.01), while the *IL4* TTC haplotype still showed the opposite association (adjusted RO = 0.36–0.43,  $P = .04$ –.06). Among the nongenetic factors tested here, HIV-1 infection closely correlated with the variability in CD8<sup>+</sup> T cell levels (Spearman  $r = 0.69$ ,  $P < .0001$ ); an alternative model (Model II) retained CD8<sup>+</sup> T cell percentage as another major factor contributing to the lack of antibody response to vaccination (adjusted OR = 7.24,  $P < .0001$ ). Further correlation between CD8<sup>+</sup> and CD4<sup>+</sup> T cell counts (Spearman  $r = 0.46$ ,  $P < .0001$ ) led to the dismissal of CD4<sup>+</sup> T cell counts as an independent cofactor. In addition, age was the only factor that could not be assessed reliably in any of these multivariable models because of its clear correlation with at least two major factors (HIV-1 status and CD8<sup>+</sup> T cell percentage;  $r = -0.36$  to

–0.22,  $P = .0001$ –.005). Further alternative models (not shown) did not confirm significant involvement of other previously reported HLA markers (*HLA-B\*08*, *B\*44*, and *DRB1\*03*).<sup>33</sup>

**Multivariable Models Restricted to HIV-1 Seronegative Subjects.** To eliminate HAART and progressive immunodeficiency (CD4<sup>+</sup> T cell depletion) as possible explanations for the genetic associations with differential response to HBV vaccination, healthy HIV-1 seronegative subjects ( $n = 51$ ) were evaluated separately in another multivariable model (not shown). The association between *HLA-DRB1\*07* and nonresponsiveness to HBV vaccination was particularly strong in this group (adjusted RO = 43.7,  $P = .02$ ). Trends for opposing associations involving the *IL4* haplotype TTC (RO = 0.03,  $P = .04$ ) and *IL12B* promoter L/S (RO = 6.73,  $P = .09$ ) were also confirmed.

## Discussion

Our analyses of HIV-1–infected and –uninfected adolescents reconfirmed the association of *DRB1\*07* with suboptimal immune response to HBV vaccination, as reflected by the unusually low levels of anti-HBsAg antibodies in *DRB1\*07*-positive individuals. With adjustment for other genetic and nongenetic effects, *DRB1\*07* was associated with a two- to sixfold higher proportion of nonresponders. Further associations of two other genetic variations—namely, the *IL4* TTC haplotype and *IL12B* promoter L/S heterozygosity—were more modest in magnitude and have not been observed before.

**Table 3. Population Frequencies [n (%)] of Major HLA and Cytokine Gene Haplotypes Detected Among Fully Vaccinated Participants and a Reference Group From the REACH Cohort of Adolescents**

Haplotypes	Fully Vaccinated	Reference Group	P*	Nonresponders	Responders	P*	Adjusted P Value†
<i>HLA-B-Cw</i>	N = 161	N = 290		N = 77	N = 84		
B*07-Cw*07	28 (17.4)	28 (9.6)	.06	12 (15.6)	16 (19.1)	—	—
B*14-Cw*08	12 (7.5)	16 (5.5)	—	7 (9.1)	5 (6.0)	—	—
B*15-Cw*02	16 (9.9)	15 (5.1)	—	6 (7.8)	10 (11.9)	—	—
B*15-Cw*03	15 (9.3)	21 (7.2)	—	4 (4.8)	11 (14.3)	<b>.04</b>	.10
B*35-Cw*04	21 (13.0)	36 (12.3)	—	11 (6.7)	13 (7.9)	—	—
B*42-Cw*17	14 (8.7)	27 (8.2)	—	9 (11.7)	12 (14.3)	—	—
B*44-Cw*04	10 (6.2)	20 (6.9)	—	5 (6.5)	5 (6.0)	—	—
B*53-Cw*04	31 (19.3)	57 (19.5)	—	18 (11.0)	13 (7.9)	—	—
B*58-Cw*06	4 (2.5)	19 (6.5)	.08	2 (2.4)	2 (2.6)	—	—
B*58-Cw*07	7 (4.4)	8 (2.7)	—	4 (5.2)	3 (3.6)	—	—
<i>DRB1-DQB1</i>	N = 161	N = 290		N = 77	N = 84		
*01.*05	20 (12.7)	29 (10.2)	—	11 (14.1)	10 (12.2)	—	—
*03.*02	23 (14.7)	44 (15.4)	—	12 (15.4)	11 (13.4)	—	—
*03.*04	27 (17.2)	36 (12.6)	.19	10 (12.8)	18 (22.0)	.11	.07
*04.*03	23 (14.7)	40 (14.0)	—	10 (12.8)	13 (15.9)	—	—
*07.*02	33 (20.1)	51 (17.9)	—	25 (32.1)	8 (9.8)	<b>.0007</b>	<b>.0002</b>
*07.*03	4 (2.6)	6 (2.1)	—	3 (3.9)	1 (1.2)	—	—
*11.*03	26 (16.6)	41 (14.4)	—	10 (12.8)	18 (22.0)	.13	—
*15.*06	35 (22.3)	71 (24.9)	—	13 (16.7)	22 (26.8)	.13	.08
<i>IL2</i>	N = 159	N = 290		N = 78	N = 81		
GG	121 (76.1)	240 (82.8)	—	14 (18.0)	24 (29.6)	.10	.15
TG	131 (82.4)	259 (89.3)	.17	65 (83.3)	66 (81.5)	—	—
TT	42 (26.4)	64 (22.1)	—	19 (24.4)	23 (28.4)	—	—
<i>IL4‡</i>	N = 162	N = 292		N = 78	N = 84		
GCC	26 (16.1)	52 (17.8)	—	12 (15.4)	14 (16.7)	—	—
TCC	76 (46.9)	146 (50.0)	—	41 (52.6)	35 (41.7)	.10	.08
TTC	45 (27.8)	82 (28.1)	—	14 (18.0)	31 (36.9)	<b>.004</b>	<b>.01</b>
TTT	103 (63.6)	176 (60.3)	—	53 (68.0)	50 (59.5)	—	—
Others	19 (11.7)	36 (12.3)	—	9 (11.5)	10 (11.9)	—	—
<i>IL6</i>	N = 162	N = 292		N = 78	N = 84		
CA	30 (18.5)	45 (15.4)	—	13 (16.7)	17 (20.2)	—	—
GG	160 (98.8)	284 (97.3)	—	77 (98.7)	83 (98.8)	—	—
<i>IL10</i>	N = 162	N = 292		N = 78	N = 84		
GCC	89 (54.9)	153 (52.4)	—	41 (52.6)	48 (57.1)	—	—
ACC	79 (48.8)	148 (50.7)	—	37 (47.4)	42 (50.0)	—	—
ATA	96 (59.3)	176 (60.3)	—	46 (59.0)	50 (59.5)	—	—
<i>IL12B</i>	N = 162	N = 292		N = 78	N = 84		
LA	40 (24.7)	51 (17.5)	<b>.05</b>	17 (21.8)	23 (27.4)	—	—
LC	70 (43.2)	146 (50.0)	—	37 (47.4)	33 (39.3)	—	—
SA	133 (82.1)	224 (76.7)	—	67 (85.9)	66 (78.6)	—	—
SC	22 (13.6)	45 (15.4)	—	12 (15.8)	10 (11.9)	—	—

\*Based on Mantel-Haenszel  $\chi^2$  tests or Fisher exact tests, with adjustments for ethnicity. P values  $\leq .05$  are shown in **bold**; those  $> .02$  have been omitted (—).

†Adjusted for other factors including HIV-1 status, gender, ethnicity, and vaccination protocols.

‡Minor haplotypes (defined by SNPs at positions -1098, -590, and -33) with a frequency above 5% (in all patient groups) are grouped as others.

Weak associations with additional HLA and cytokine gene variants were due mostly to LD or to reciprocity of positive and negative associations necessarily exhibited by the frequencies of alleles at any locus. In particular, effects attributable to the *DQB1\*02* and DR53 group (which consists of the *DRB1\*04*, *\*07*, and *\*09* alleles) were due to *DRB1\*07* because the *DRB1\*07* haplotype containing *DQB1\*03* showed a comparable association, while neither *DRB1\*04* nor *DRB1\*09* showed any trend. Thus, most of the effects attributed to class II haplotypes and lineages in univariate analyses were heavily driven by

*DRB1\*07*. Collectively, these findings are highly consistent with the essential roles that HLA and cytokine molecules play in the process of immune activation and regulation.<sup>9,39</sup>

The highly polymorphic DR $\beta$  chain component of HLA-DR molecules has been shown to bind a major immunodominant peptide (p39-146) in HBsAg.<sup>14</sup> Earlier epidemiologic studies have repeatedly associated HLA-*DRB1\*07* and its most common haplotype *DRB1\*07-DQB1\*02* with nonresponse to HBV vaccination;<sup>5,7,8,40</sup> these relationships were unequivocal in our analysis. In

**Table 4. Heterozygosity of HLA and Cytokine Genes Among Fully Vaccinated Participants and a Reference Group From the REACH Cohort of Adolescents**

	Fully Vaccinated	Reference Group	P*	Nonresponders [n (%)]	Responders [n (%)]	P*	Adjusted P Value*
HLA-A†	140 (85.4)	308 (82.1)	—	66 (83.5)	74 (87.1)	—	—
HLA-B†	136 (82.9)	282 (75.2)	<b>.05</b>	69 (87.3)	67 (78.8)	.15	—
HLA-Cw†	132 (80.5)	323 (86.4)	.08	62 (78.5)	70 (82.4)	—	—
HLA-DRB1‡	151 (94.4)	339 (93.1)	—	74 (94.9)	77 (93.9)	—	—
HLA-DQB1‡	139 (86.3)	300 (82.0)	—	66 (84.6)	73 (88.0)	—	—
IL2							
-330G/T	29 (18.2)	43 (14.8)	—	8 (10.3)	21 (25.9)	<b>.01</b>	<b>.05</b>
-166G/T	32 (20.1)	55 (19.0)	—	15 (19.2)	17 (21.0)	—	—
IL4							
-1098G/T	44 (27.2)	85 (29.1)	—	21 (26.9)	23 (27.4)	—	—
-590C/T	61 (37.7)	124 (42.5)	—	34 (43.6)	27 (32.1)	.13	—
-33C/T	76 (46.9)	134 (45.9)	—	36 (46.2)	40 (47.6)	—	—
IL4R 1092G/A	63 (38.9)	110 (37.7)	—	29 (37.2)	34 (40.5)	—	—
IL6							
-174C/G	28 (17.3)	39 (13.4)	—	12 (15.4)	16 (19.1)	—	—
-565A/G	29 (17.9)	39 (13.4)	.19	13 (16.7)	16 (19.1)	—	—
IL10							
-1082A/G	70 (43.2)	125 (42.8)	—	32 (41.0)	38 (45.2)	—	—
-592A/C	61 (37.7)	124 (42.5)	—	34 (43.6)	27 (32.1)	.13	—
IL12B							
L/S	78 (48.2)	125 (42.8)	—	47 (60.3)	31 (36.9)	<b>.003</b>	<b>.002</b>
3' UTR C/A	72 (44.4)	138 (47.3)	—	37 (47.4)	35 (41.7)	—	—
TNF							
-308G/A	37 (22.8)	60 (20.6)	—	18 (23.1)	19 (22.6)	—	—
-238G/A	11 (6.8)	23 (7.9)	—	5 (6.4)	6 (7.1)	—	—

\*Based on Mantel-Haenszel  $\chi^2$  tests or Fisher exact tests. P values  $\leq .05$  are shown in **bold**; those  $> .20$  have been omitted (—). Factors used for adjustments included HIV status, gender, ethnicity, and vaccination protocols.

†Heterozygosity at HLA class I loci (-A, -B, -C) is based on two-digit specificities.

‡Heterozygosity at HLA class II loci (DRB1 and DQB1) is based on four-digit alleles.

contrast, the *DRB1\*03* effect seen elsewhere<sup>41</sup> was far less impressive here (see Table 2). Likewise, the involvement of other HLA alleles (*HLA-B\*08*, *B\*44*, and *DRB1\*13*) previously associated with either HBV clearance or response to vaccination<sup>33</sup> could not be confirmed. However, type II error (false negative finding) could not be dismissed, because the vaccinated group in our study was rather small.

Failure of HBV vaccination has been tied to dysfunction of both T<sub>H</sub>1 cells<sup>9,12,13</sup> and specific B cells.<sup>10</sup> Peripheral blood mononuclear cells from high responders to HBV vaccines show elevated production of IL-2, IL-12, and interferon- $\gamma$ .<sup>11,13</sup> The observed associations of variable vaccine responses with *IL2* and *IL12B* polymorphisms appeared to reflect functional mechanisms. For example, the *IL2*-330G variant has been linked to increased IL-2 production *in vitro*<sup>34</sup> and showed a univariate trend toward association with the responder phenotype, although it did not remain a significant, independent marker in multivariable analysis. In clear contrast to homozygous *IL12B* promoter genotypes *L/L* (1.1) and *S/S* (2.2), heterozygosity (*L/S* or 1.2) leads to reduced production of bioactive IL-12 p70 as well as IL-12 p40

messenger RNA,<sup>27</sup> and only *L/S* remained significantly associated with the nonresponder phenotype here irrespective of other cofactors. If these effects prove reproducible, the use of exogenous IL-2 and IL-12 as adjuvants to augment immune responses to HBV vaccines<sup>11,42–46</sup> may help overcome some of the genetically inheritable immunologic deficit.

IL-4 as a T<sub>H</sub>2 cytokine is a critical modulator of B cell function, especially immunoglobulin isotype switching.<sup>39,47</sup> The *IL4*-590T (also known as -589T) variant, which is already associated with several inflammatory and infectious diseases,<sup>48,49</sup> has been shown to modulate *IL4* promoter function and serum immunoglobulin E levels.<sup>49</sup> Among HIV-1-infected REACH adolescents, those with -590T/T homozygosity had higher CD4<sup>+</sup> T cell counts (C. Wang et al., submitted for publication) and more vigorous HIV-1-specific T cell responses (J. Tang et al., submitted for publication) than carriers of the other genotypes. However, it was not homozygosity for -590T *per se* but rather the *IL4*-590T-carrying TTC haplotype that showed the strong impact on responsiveness to HBV vaccines. The exact mechanisms for clear contrast between allelic and haplotypic associations are not immedi-

**Table 5. Multivariable Logistic Regression Analyses of Host Factors in Relation to the Nonresponder Phenotype Following Full-Dose HBV Vaccination in REACH Adolescents**

Individual Host Factors	Model I: RO (95% CI)	P*	Model II: RO (95% CI)	P*
Race/ethnicity				
African American	1.93 (0.70-5.30)	—	NA	NA
European American	1.23 (0.30-5.04)	—	NA	NA
Gender (female vs. male)	1.46 (0.58-3.67)	—	NA	NA
HIV infection (+ vs. -)	5.26 (1.96-14.29)	<b>.001</b>	Not included†	
CD4 <sup>+</sup> count (<400 vs. ≥400/μL)	1.82 (0.67-4.91)	—	NA	NA
CD8 <sup>+</sup> T cells (≥40% vs. <40%)	Not included†		5.83 (2.67-12.72)	<b>&lt;.0001</b>
HBV vaccine				
Energix-B vs. others	0.80 (0.24-2.65)	—	NA	NA
Recombivax HB vs. others	0.91 (0.29-2.82)	—	NA	NA
HLA-Cw*03	2.02 (0.59-6.86)	—	NA	NA
HLA-DRB1*07	4.89 (1.50-15.93)	<b>.008</b>	6.33 (2.36-17.01)	<b>.0003</b>
HLA-DQB1*02	1.30 (0.49-3.41)	—	NA	NA
HLA-DQB1*06	0.59 (0.25-1.40)	—	NA	NA
IL4 TTC haplotype	0.37 (0.14-0.94)	<b>.04</b>	0.43 (0.18-1.02)	.06
IL2-330T/G	0.54 (0.17-1.67)	—	NA	NA
IL12B L/S	2.87 (1.24-6.63)	<b>.01</b>	2.74 (1.28-5.86)	<b>.01</b>

Abbreviation: NA, not applicable (i.e., excluded from the final model when  $P > .05$  in stepwise testing).

\*Tests include all factors as shown in each model. Multivariable (adjusted)  $P$  values  $\leq .05$  and the associated factors are shown in **bold**;  $P$  values  $> .20$  have been omitted (—).

†HIV-1 infection and CD8<sup>+</sup> T cell percentage showed close correlation (Spearman  $r = 0.69$ ,  $P < .0001$ ) and could not be treated as separate/independent variables in the same model. For similar reasons, age was not treated as an independent covariate in these models (see text).

ately clear, but additional *IL4* SNPs and haplotypes identified more recently in several populations<sup>50,51</sup> may require reassessment of findings here and elsewhere. Ongoing examination of *IL4* promoter and exon 1 sequences have continued to uncover new SNPs and haplotypes (J. Tang et al., unpublished observations), suggesting that more definitive insights will emerge from systematic analyses in larger cohorts.

The vaccine response rate (74%) in HIV-1 seronegative adolescents studied here was somewhat lower than expected ( $\approx 90\%$ ) in adult populations but comparable to those seen in healthy adolescents following two doses of immunization.<sup>52,53</sup> Differences of our study population from others in the proportion with high risk sexual behaviors, relatively older age at the initial dose of vaccination, and frequent hyperimmune activation status (as measured by T cells with the CD8<sup>+</sup>/CD38<sup>+</sup> phenotype)<sup>20</sup> could have contributed to the increase in suboptimal HBV vaccine responses. Furthermore, as implied in earlier studies of HIV-infected adult and adolescent populations,<sup>54-56</sup> antibody levels in our HIV-infected adolescents may have declined with the progressive loss of CD4<sup>+</sup> T cells and concomitant increase in CD8<sup>+</sup> T cell proportions. We did observe a clear association between increasing levels of CD4<sup>+</sup> T cell counts and responder phenotype in HIV-infected individuals (see Table 1). Thus, if response to HBV vaccines is modulated by multiple immunogenetic factors in HIV-1-induced or other populations with numerically and functionally inadequate CD4<sup>+</sup> cells, it may

prove especially important to characterize the genetic influence.

For the evaluation of cytokine gene polymorphisms, as noted earlier, we elected not to correct  $P$  values for multiple comparisons even though they had not been studied previously in the context of vaccine responses. However, the *IL4* and *IL12B* variants highlighted here have been associated with functional consequences and clinical manifestations in certain infectious diseases.<sup>49-51,57,58</sup> The cytokine gene effects reported here must be confirmed and/or refined in other populations, but that effort should be straightforward because the *IL4* and *IL12B* variants can be readily defined using several routine techniques.

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