

A Novel Variant Marking HLA-DP Expression Levels Predicts Recovery from Hepatitis B Virus Infection

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Variants near the *HLA-DP* gene show the strongest genome-wide association with chronic hepatitis B virus (HBV) infection and HBV recovery/persistence in Asians. To test the effect of the HLA-DP region on outcomes to HBV infection, we sequenced the polymorphic *HLA-DPB1* and *DPA1* coding exons and the corresponding 3' untranslated regions (3' UTRs) in 662 individuals of European-American and African-American ancestry. The genome-wide association study (GWAS) variant (*rs9277535*; *550A/G*) in the 3' UTR of the *HLA-DPB1* gene that associated most significantly with chronic hepatitis B and outcomes to HBV infection in Asians had a marginal effect on HBV recovery in our European- and African-American samples (odds ratio [OR] = 0.39, *P* = 0.01, combined ethnic groups). However, we identified a novel variant in the *HLA-DPB1* 3' UTR region, *496A/G* (*rs9277534*), which associated very significantly with HBV recovery in both European and African-American populations (OR = 0.37, *P* = 0.0001, combined ethnic groups). The *496A/G* variant distinguishes the most protective *HLA-DPB1* allele (*DPB1*04:01*) from the most susceptible (*DPB1*01:01*), whereas *550A/G* does not. *496A/G* has a stronger effect than any individual *HLA-DPB1* or *DPA1* allele and any other *HLA* alleles that showed an association with HBV recovery in our European-American cohort. The *496GG* genotype, which confers recessive susceptibility to HBV persistence, also associates in a recessive manner with significantly higher levels of HLA-DP surface protein and transcript level expression in healthy donors, suggesting that differences in expression of HLA-DP may increase the risk of persistent HBV infection.

Hepatitis B virus (HBV) infection is a major global health concern that can result in liver cirrhosis and hepatocellular carcinoma. Upon HBV infection in adulthood, up to 95% of people have a robust immune response that leads to recovery from the infection and development of protective antibodies, but in 5 to 10% of the population, viral persistence occurs, leading to chronic infection. About 2 billion people worldwide have been infected with the virus and about 350 million live with chronic infection (11). Factors that determine viral recovery or persistence include age at infection, immunodeficiency states, gender, and host genetic variation (7, 29). Several reports have pointed to variation in the human leukocyte antigen (HLA) class I and II genes located within the major histocompatibility complex (MHC) as determinants of outcome after HBV infection and HBV disease pathogenesis in several global populations (23, 29). HLA genes encode molecules that are central to the host immune response, and variation in these genes likely affects outcome after infection with any given pathogen. Recently the importance of HLA genes was further highlighted by the first genome-wide association study (GWAS) in Asian patients with chronic hepatitis B, where *HLA-DP* gene variation showed the strongest association with chronic HBV infection (8), which was validated subsequently in another Asian study (4). This variant also associated with HBV vaccination response in Asians (18). HLA-DP is an HLA class II heterodimeric molecule consisting of alpha and beta chains that are encoded by the *HLA-DPA1* and *-DPB1* genes, respectively. The HLA-DP molecule is expressed on antigen-presenting cells (APC), including B cells, dendritic cells, and macrophages, where it functions by pre-

senting extracellular antigens to CD4⁺ T cells, initiating cellular and humoral immune responses.

In general, *HLA-DP* genes have been somewhat ignored in terms of their impact on human disease relative to *HLA-DR* and *-DQ*, in part because *HLA-DPA1* and *-DPB1* are less polymorphic and also because HLA-DP cell surface expression levels are likely to be lower than that of HLA-DR or *-DQ* (2). Indeed, *HLA-DPA1* and *-DPB1* had never been investigated for potential effects on HBV disease or outcomes prior to the GWAS findings in Asians. Here we describe the effects of *HLA-DP* loci on outcomes of HBV infection among individuals of European and African descent. We identified a novel 3' UTR variant termed *496A/G* (*rs9277534*) that has a stronger association with HBV recovery/persistence than the single nucleotide polymorphisms (SNPs) identified previously in the Asian studies and than any of the individual *HLA-DP* alleles. The *496A/G* variant correlates with levels of both HLA-DP surface

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protein and DPB1 transcript levels in peripheral blood cells from healthy donors, suggesting that HLA-DP expression levels may represent the functional basis for the association of this locus with outcome to HBV infection.

MATERIALS AND METHODS

Study participants. A total of 662 subjects were included, consisting primarily of European-American and African-American participants from one of the following cohort studies: (i) Multicenter AIDS Cohort Study (MACS) (17), (ii) Multicenter Hemophilia Cohort Study (MHCS) (3), (iii) Hemophilia Growth and Development Study (HGDS) (5), and (iv) AIDS Linked to Intravenous Experience (ALIVE) (31). Details of the subjects used in this study have been described previously (26, 27).

A nested case-control design was used in which all individuals with persistent HBV infection ($n = 241$) were matched to one or two persons from the same cohort who recovered from HBV infection ($n = 421$) but were otherwise similar with regard to nongenetic factors. Matching criteria included geographic location and factors that have been associated with HBV recovery, including age within 10 years, gender, and HIV-1 status. Subjects were considered persistently infected with HBV if their serum or plasma tested positive for hepatitis B surface antigen (HBsAg) at two visits separated by a minimum of 6 months. Testing for antibodies against hepatitis B core antigen (anti-HBc) and against HBsAg (anti-HBs) was performed as needed to exclude primary HBV infection. HBV status of HIV-positive subjects was determined before antiretroviral therapy was available. The majority of subjects were European-American ($n = 505$; 185 with persistent infection and 320 who cleared the virus) and a smaller subset was African-American ($n = 131$; 46 with persistent infection and 85 who recovered). This study was approved by the institutional review boards at all participating institutions.

Peripheral blood from 75 healthy donors of European-American ancestry was obtained. This study was approved by the protocol review office of the U.S. National Cancer Institute institutional review board. A cohort of 112 healthy African-American donors was recruited at the Duke Human Vaccine Institute as part of the CHAVI 008A study. Written informed consent was obtained from all subjects at all study sites.

HLA-DP genotyping/sequencing. *HLA-DPA1* and *-DPB1* alleles were characterized using sequence-based typing protocols recommended by the 13th International Histocompatibility Workshop (21, 32). Exons 2 of *HLA-DPA1* and *-DPB1* were selectively amplified with locus-specific primers. The amplicons were sequenced using the ABI BigDye Terminator cycle sequencing kit version 1.1 and ABI3730xl DNA analyzer (Applied Biosystems). *HLA-DPA1* and *-DPB1* alleles were assigned on the basis of known alleles using the ASSIGN software (Conexio Genomics).

The 3'UTR region of the *HLA-DPB1* was amplified using *HLA-DPB1*-specific primers (forward, 5'-TTCAACGAGGATCTGCATAA-3'; reverse, 5'-GGTTAAAGTGTATTAGATTA-3'). This amplified product was then sequenced using the same primers on an ABI BigDye Terminator cycle sequencing kit version 1.1 and ABI3730xl DNA analyzer (Applied Biosystems). Data for 33 SNPs in the *HLA-DPB1* 3'UTR region was available for 638 patients.

Statistical analysis. SAS 9.1 (SAS Institute) was used for data management and statistical analyses. PROC FREQ was used to compute frequencies on individual variables. Statistical significance refers to two-sided P values of <0.05 . Odds ratios (OR) for persistent infection and P values were calculated by using conditional logistic regression with PROC LOGISTIC. A conditional logistic regression analysis with stepwise selection was performed in 662 patients, which included all *HLA-DPB1* alleles with a frequency of greater than 5%, 496A/G, 550A/G, and other class I and II loci that were reported previously to associate with HBV recovery in our study cohort (26, 27). These variables were tested in a model using PROC LOGISTIC to determine which had independent effects. The significance level for selecting variables in the model was P values of <0.05 .

Measurement of HLA-DP surface protein expression. Specificities of purified murine monoclonal antibodies (MAbs) B7/21 (AbCam), BrafB6 (Santa Cruz), Tu39, and SK10 (both Becton Dickinson) for HLA class II allotypes were tested by screening a panel of 86 HLA class II allotypes (One Lambda) using a Luminex 100 cytometer as previously described (1). For each antibody, binding was compared to purified IgG1 or IgG2a isotype controls (Sigma). HLA staining of 112 African-American donors was performed by isolation of peripheral blood lymphocytes (PBL) using Lymphoprep (Lonza) followed by incubation with human IgG (Lampire Biological) to block FcγR before staining with the unconjugated murine MAbs. Purified sheep IgG (Lampire Biological) was then added to block the nonspecific binding from the subsequent antibody, which was a sheep polyclonal phycoerythrin (PE)-conjugated anti-mouse antibody (Sigma). Free secondary-antibody-binding sites were blocked with murine IgG (Lampire Biological) before B cells were identified by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD19 antibody compared to an isotype control (both Beckman Coulter). All African-American samples were acquired using identical settings on a single FACSCalibur cytometer and analyzed using Cellquest (both Becton Dickinson). HLA staining of 75 European-American donors was performed using 0.5 ml of freshly drawn blood directly with the same antibody incubations as described above for African-Americans. Red blood cells were then removed by incubation for 30 min at 4°C in an isotonic lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM Na₂EDTA, pH 7.4). Staining European-American whole blood gave greater discrimination of HLA class II binding from control MAb binding than using purified PBL for the African-American staining. Both isotype control and HLA class II staining could not be acquired using the same instrument settings for all European-Americans, as the fluorescence range exceeded that of the cytometer for some donors. We confirmed that HLA class II MAbs were staining CD19⁺ cells compared to isotype controls (see Fig. S1 in the supplemental material). We then used one set of cytometer settings for acquiring HLA class II MAbs and a second cytometer setting with higher PE amplification for acquiring the isotype control in all 75 European-American donors. European-American samples were acquired using a single FACScan cytometer and analyzed using CellQuest. The European- and African-American populations were acquired within separate periods of less than 15 consecutive days and showed no trends with time (data not shown). Pellets of 5×10^6 PBL or 0.5 ml of whole blood mixed with 1 ml of DNAzol (Molecular Research Center) were stored frozen from each donor for genotyping, with DNA prepared using DNeasy columns (Qiagen).

Measurement of HLA-DPB1 mRNA transcript levels. RNA was prepared from cell suspensions of freshly isolated PBL (1×10^7 cells) using a combination of TRIzol reagent (Invitrogen) and the RNeasy microkit (Qiagen). All 15 RNA samples from European-American donors were treated with RNase-free DNase I (Qiagen) prior to cDNA synthesis. Quality and quantity of RNA was checked on an HT RNA LabChip (Caliper, Life Sciences). Each sample had two distinct peaks representing 18S and 28S rRNAs with, on average, a ratio greater than 2.0 and an RNA quality score of >8.5 . Reverse transcription was performed using 1 μg of total RNA, oligo(dT), and SuperScript III reverse transcriptase (RT) (Invitrogen) in a volume of 20 μl. The gene of interest (GOI), *HLA-DPB1*, was then quantified using SYBR green quantitative PCR (qPCR) using the threshold cycle (C_T) method (12) in an ABI 7900HT PCR machine (Applied Biosystems). Each PCR included 5 μl of power SYBR green PCR master mix (Applied Biosystems), 200 nM primers (see Table S1 in the supplemental material), and 2 μl of cDNA (5× dilution) in a total volume of 10 μl using the recommended ABI qPCR protocol. The specificity of the reaction was confirmed by melt curve analysis using the dissociation step following the qPCR protocol. Sequencing and melt curve analysis of *HLA-DPB1* PCR amplicons established that the primers (see Table S1) were specific for the cDNA prepared (data not shown) and did not cross-react with other genes. All reactions were standardized to the expression of two different reference genes, RPLP0 and GAPDH (see Table S1). The amplification efficiencies of the different genes were tested by making standard

TABLE 1 Significant *HLA-DPB1* associations in the study cohorts^a

Sample ^a	Clearance		Persistence		Association	P value	OR	95% CI
	No. of samples	% of samples	No. of samples	% of samples				
All samples	421		241					
01:01	60	14.3	51	21.2	01:01 vs others	0.01	1.86	1.16–2.99
Others	361	85.8	190	78.8				
04:01	249	59.1	117	48.6	04:01 vs others	0.003	0.56	0.38–0.82
Others	172	40.9	124	51.5				
European-Americans	320		185					
01:01	26	8.1	22	11.9	01:01 vs others	0.18	1.55	0.81–2.96
Others	294	91.9	163	88.1				
04:01	225	70.3	108	58.4	04:01 vs others	0.01	0.55	0.36–0.84
Others	95	29.7	77	41.6				
African-Americans	85		46					
01:01	32	37.7	28	60.9	01:01 vs others	0.01	2.7	1.24–5.75
Others	53	62.4	18	39.1				
04:01	19	22.4	4	8.7	04:01 vs others	0.05	0.27	0.08–0.97
Others	66	77.7	42	91.3				

^a Data were adjusted by race and cohort.

curves using serial dilutions of cDNA from a sample that was also designated a calibrator. Additionally, quantitative PCR using a relative quantification method (16) was confirmed by using two primer pairs that targeted different regions of the *HLA-DPB1* transcript. The quantity of *HLA-DPB1* in the 15 samples and calibrator were normalized to the quantity of three different reference genes (the *ACTB*, *RPLP0*, and *GAPDH* genes).

RESULTS

HLA-DPB1 alleles associate with HBV recovery. Given that variants near *HLA-DP* showed association with chronic HBV infection and outcome after infection in Asian cohorts (4, 8), we genotyped the *HLA-DPA1* and *HLA-DPB1* genes in 662 individuals, primarily of European and African ancestry, to determine potential associations with HBV recovery/persistence. While no *HLA-DPA1* alleles associated significantly ($P \leq 0.01$) with HBV outcome, *HLA-DPB1*04:01* and *HLA-DPB1*01:01* associated significantly with recovery and persistence of HBV, respectively, in all ethnic groups combined (Table 1). The association with these alleles was consistent across the two ethnic groups (Table 1), and no other *HLA-DPB1* alleles reached significance in the combined or the stratified groups.

A novel SNP in the 3'UTR region of *HLA-DPB1* has the strongest association with HBV recovery. The variant that showed the strongest association with chronic HBV infection in Asians, which we term *550A/G* (*rs9277535*), is located in the 3'UTR of the *HLA-DPB1* gene (8). To determine whether this or another variant showed a similar effect in European- and African-Americans, we sequenced the entire 3'UTR region of *HLA-DPB1* in all subjects, identifying 33 variants in this region. The *550A/G* variant associated weakly with HBV outcome in our cohort (OR = 0.39, $P = 0.01$; see Table S2 in the supplemental material) relative to another variant in the 3'UTR, *rs9277534* (*496A/G*; OR = 0.37, $P = 0.0001$; see Table S2). The *496GG* genotype variant had a significantly higher frequency in the persistence group (19.83%) than in the recovery group (9.85%), whereas the frequency distribution of *496AA* and *496AG* genotypes (these combined genotypes are termed *496AX*) did not differ significantly

between outcome groups. The recessive susceptible association of *496GG* was consistent across the stratified European- and African-American cohorts (Fig. 1). This association was also consistent in HIV-positive and HIV-negative subjects (data not shown). The *496A/G* SNP is in perfect linkage disequilibrium (LD) with four other SNPs (*rs9277530*, *rs9277531*, *rs9277533*, and *rs9277536*) in the 3'UTR region of *HLA-DPB1* (see Table S2), any of which could potentially be causal. None of the other 28 SNPs in the 3'UTR region of *HLA-DPB1* had as strong an association as position 496, suggesting that they are not as likely to be directly involved in the mechanism underlying viral recovery.

We showed previously that *HLA-A*03:01*, *-B*08*, *-B*44*, and *-DRB1*13:02* were associated with HBV recovery/persistence in the same European-American samples used in the present study (27). In order to determine whether *496A/G* in the 3'UTR of *HLA-DPB1* has an effect on HBV recovery that is independent of these

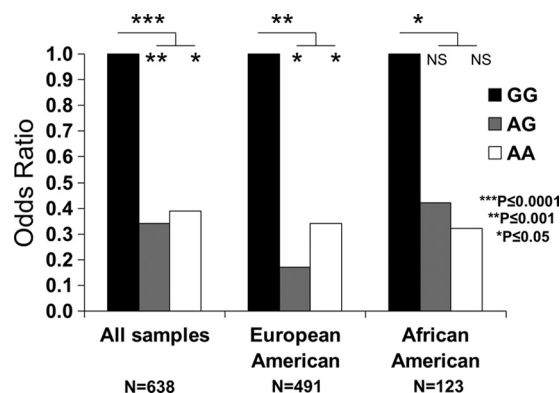


FIG 1 The *496A* variant in the 3'UTR region of *HLA-DPB1* confers dominant protection against HBV. The odds ratios are based on the frequency distribution of the three genotypes (*GG*, *AG*, and *AA*) of the *496A/G* SNP in the persistence group versus the clearance group in the study cohort with all samples, European-Americans and African-Americans. *496GG* is the reference genotype set at an OR of 1.0.

TABLE 2 The effect of the 3'UTR variant at position 496 is independent of previously reported HLA associations with HBV recovery^a in European- and African-Americans

Significant independent variable	P value	OR	95% CI
European-Americans (n = 505)			
496AX vs GG	0.01	0.36	0.16–0.80
HLA-A*03:01 vs others	0.02	0.54	0.33–0.90
HLA-B*08 vs others	0.04	1.67	1.02–2.73
HLA-B*44 vs others	0.003	1.95	1.25–3.03
African-Americans (n = 131)			
496AX vs GG	0.01	0.36	0.16–0.81
HLA-DQA*0501 vs others	0.005	3.28	1.44–7.45

^a Data were adjusted by cohorts and analyzed using a conditional logistic regression model with stepwise selection.

HLA alleles and the closely located *HLA-DPB1* coding region, we used a stepwise logistic regression model that included all *HLA-DPB1* alleles with a frequency of greater than 5%, the four alleles associated with HBV previously, and the 496A/G and 550A/G variants. The analysis indicates that the 496AX genotypes associate with viral recovery independently of the other variables (Table 2) in the European-Americans. A similar analysis was performed in African-American samples that included *HLA-DQA1*0501* and *-DQB1*0301*, which were previously shown to associate with HBV persistence in these same African-Americans (26), along with all *HLA-DPB1* alleles with a frequency of greater than 5%, and the 496A/G and 550A/G variants. In this subset of patients, the 496AX genotypes consistently associated with viral recovery independent of other variables. Notably, none of the *HLA-DPB1* alleles remained in the model involving either ethnic group, suggesting that the mechanism underlying *HLA-DPB1* association with HBV recovery does not involve presentation of a specific viral peptide by a specific HLA-DP allotype. Likewise, the 550A/G variant that was identified in the Asians to associate with outcomes of HBV infection and disease persistence did not remain in the model (neither ethnic group), showing that the association of this SNP in Asians was likely due to its strong LD with the 496A/G variant. The

rs3077 SNP located in the *HLA-DPA1* 3'UTR region showed the second strongest association with chronic HBV infection in the Asian GWAS (8). However, this SNP associated only weakly with HBV recovery in our African-American cohorts (OR = 0.32 and $P = 0.02$) and did not remain significant after correcting for the effect of the 496A/G variant (data not shown). Moreover, this variant did not associate significantly with outcomes of HBV infection in European-Americans. We thus focused on the variation in the 3'UTR of *HLA-DPB1* rather than that of *HLA-DPA1* for the remainder of the study.

HLA-DP surface expression levels correlate with 496A/G genotypes. Previous work has shown that cell surface levels of HLA-C expression in healthy donors correlate with outcome after HIV infection (28), and a variant in the 3'UTR of *HLA-C*, which differentially binds a microRNA, accounts in part for the varied expression across HLA-C allotypes (10). We therefore hypothesized that the 496A/G variant in *HLA-DPB1* may correlate with various levels of cell surface expression across HLA-DP allotypes, which in turn could contribute to the association of this SNP with HBV control. To test this possibility, monoclonal antibodies with reported HLA class II reactivity were subjected to extensive characterization using a panel of 86 common HLA class II allotypes (see Fig. S2 in the supplemental material). The MAbs B7/21 and BrafB6, which were previously shown to specifically immunoprecipitate HLA-DP from lymphoblastoid cell lines (6, 33), reacted strongly with all 24 HLA-DP allotypes but not with any allotypes encoded by other HLA loci tested in the panel. The MAbs Tu39 and SK10 are known to detect a range of HLA class II antigens (9, 20), and we found that a pool of these MAbs together bound all 86 HLA-DR, -DP, and -DQ allotypes tested. B7/21 and BrafB6 have a similar affinity for different HLA-DP alleles, because they show the same binding to each HLA-DP allotype relative to the level of HLA present on each bead (see Fig. S2).

We then compared staining of CD19⁺ cells with these MAbs across 75 European-American donors that were randomly selected from a healthy donor pool. Fluorescence intensity of HLA-DP staining correlated significantly with the genotype of 496A/G ($P < 0.0001$) (Fig. 2A), whereas that for the pool of MAbs

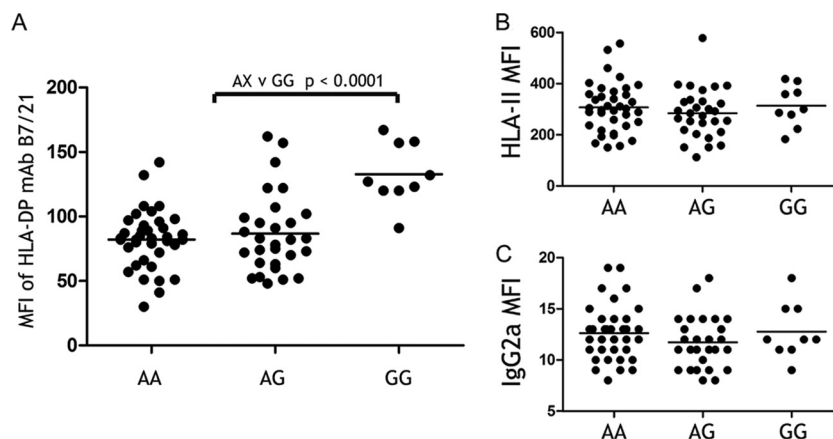


FIG 2 HLA-DP surface protein levels correlate with the 496A/G genotype in the 3'UTR region of *HLA-DPB1*. HLA-DP molecules were detected on peripheral blood CD19⁺ cells by flow cytometry using the HLA-DP-specific MAb B7/21. Median fluorescence intensity (MFI) of B7/21 staining was compared across 75 normal European-American donors, as grouped by their *HLA-DPB1* genotype (A). MFI of staining to the same cells by a pool of Tu39 and SK10 (HLA-II) MAbs that together bind all HLA-DR/DQ/DP allotypes (B) or an isotype-negative control (C) showed no correlation with the 496A/G genotype. P values were generated by unpaired Mann-Whitney t test.

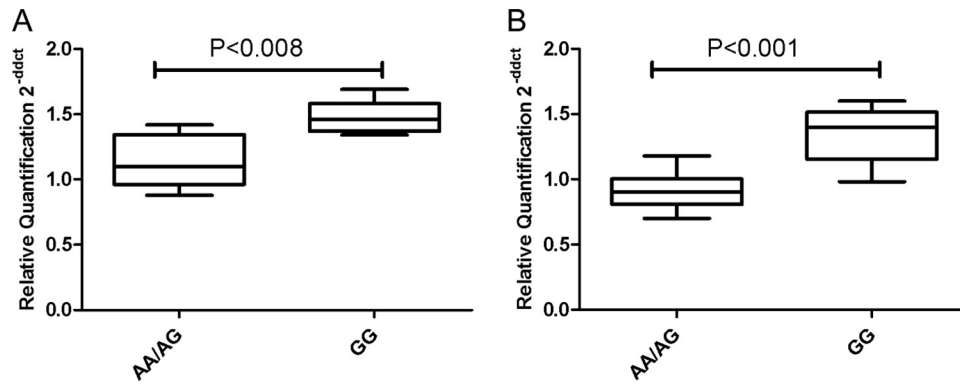


FIG 3 HLA-DPB1 mRNA levels correlate significantly with the 496A/G variant in the 3'UTR region of HLA-DPB1. HLA-DPB1 mRNA levels were detected by qPCR amplification of cDNA derived from fresh PBL of 15 healthy European-American donors. HLA-DPB1 mRNA levels were detected using a SYBR green method with primers specific for exon 3 of HLA-DPB1. Levels of HLA-DPB1 mRNA are shown relative to a calibrator using two different reference genes, *RPLP0* (A) and *GAPDH* (B). HLA-DPB1 mRNA levels in the group of individuals with 496AX ($n = 10$) were significantly lower than that from 496GG ($n = 5$) donors. These data are representative of three independent experiments. P values were generated by unpaired Mann-Whitney t test.

recognizing all HLA class II allotypes or an isotype control showed no correlation (Fig. 2B and C). Surface HLA-DP protein expression of donors with the GG genotype was significantly higher than that of either AG or AA, but expression on cells from individuals with the AG and AA genotypes did not differ from one another. Thus, consistent with the recessive susceptible effect of 496GG, this variant also correlated recessively with high expression of HLA-DP on B cell surfaces. B7/21 fluorescence intensity varied by up to 5.6-fold across the donor pool, with a difference of 1.6-fold on average between donors with the 496GG versus AX genotypes (see Fig. S3A and B in the supplemental material). This pattern of HLA-DP staining on CD19⁺ cells was replicated in a group of 112 healthy African-American donors. Once again, both of the HLA-DP-specific MAbs, B7/21 and BrafB6, bound to CD19⁺ cells from donors with the GG genotype at significantly higher levels than to cells from donors with the AX genotypes (see Fig. S4A and B in the supplemental material). CD19 staining on the same cells showed no difference between donors of GG versus AX genotypes (see Fig. S4C in the supplemental material). HLA-DP surface protein expression levels in the European- and African-American groups correlated only weakly with the genotype that associated with HBV recovery in Asians (i.e., 550A/G) (see Fig. S5A and B in the supplemental material).

HLA-DPB1 mRNA transcript levels correlate with 496A/G genotypes. In order to validate the differential cell surface expression levels between 496GG and AX genotypes, we determined the differences in levels of HLA-DPB1 mRNA by quantitative PCR (qPCR) using the comparative C_T method (12). HLA-DPB1 transcripts were amplified using primers specific for exon 3 of the *HLA-DPB1* gene. Levels of HLA-DPB1 transcripts for each sample were compared to that of a calibrator (donor with 496AA genotype) and were measured by comparing the C_T values of the gene of interest (GOI) (i.e., HLA-DPB1) normalized to C_T values of a reference gene. The *RPLP0* and *GAPDH* reference genes had amplification efficiencies that matched the GOI. Significant differences in HLA-DPB1 mRNA expression in cells from 15 European-American donors correlated with the 496A/G genotypes in a manner that mirrored the cell surface expression (Fig. 3). Ten samples with 496AX genotypes had significantly lower HLA-DPB1 mRNA expression than did 5 samples with the 496GG ge-

notype after normalization to the *RPLP0* (Fig. 3A) and *GAPDH* (Fig. 3B) reference genes. Quantitative PCR using the relative quantification method (16) produced results very similar to those of the comparative qPCR method (see Fig. S6A to F in the supplemental material). We conclude that the 3'UTR variant 496A/G associates with variation in HLA-DPB1 mRNA expression levels in a manner that reflects our surface protein flow cytometry results.

Taken together with the observation that a novel 3'UTR variant, 496A/G, has the most significant overall *HLA* effect on HBV recovery in our European-American cohorts and the second most significant effect in our African-American cohorts, we propose that differential HLA-DP expression is one of the key determinants to outcome after HBV infection.

DISCUSSION

A number of host genes contain polymorphisms that associate with outcome to HBV infection, including *CCR5*, *RANTES*, and, most notably, *HLA* (24–27). A recent GWAS confirmed the primary involvement of *HLA* class II with chronic HBV infection, but surprisingly, the most significant genetic marker correlating with HBV outcome resided in the *HLA-DP* region (8) rather than in the commonly investigated *HLA-DR* and *-DQ* genes. Based on a GWAS, SNPs in the 3'UTR of *HLA-DPB1* (*rs9277535*) and *-DPA1* (*rs3077*) had the strongest and second strongest associations with chronic HBV infection, respectively, in two independent Asian populations. They also associated with HBV recovery/persistence in another Asian study (4). However, these SNPs associated weakly with HBV recovery/persistence in our cohorts consisting of African-American and European-American patients. Through sequence analysis of the entire 665 bp of the 3'UTR region of *HLA-DPB1*, we identified five variants, which are in perfect two-way LD with one another, that associate significantly with HBV recovery in both of the ethnic groups examined in our study. Since we do not know which of these variants might be causal, we have used the SNP at position 496 (*rs9277534*) as the representative for *rs9277530*, *rs9277531*, *rs9277533*, and *rs9277536* SNPs in all our analyses. The 496G allele has a recessive susceptibility effect compared to that of the 496A genotype; this association is stronger and more significant than any individual *HLA-DPB1* allele or any

other *HLA* allele previously associated with HBV recovery in our European-American cohort. Importantly, the most significant SNP (550A/G) in the GWAS report (8) did not remain significant in a stepwise multivariate model that considered 496A/G, *HLA-DPB1* alleles, and other *HLA* class I and II alleles that had been previously associated with HBV recovery in these same cohorts. In the smaller African-American cohort, the 496A variant also confers independent protection, though to a slightly lesser extent than *DQA1*0501*. In fact, a recent study in a Japanese population attributes both *HLA-DP* and *HLA-DQ* loci with risk of persistent HBV infection (14). The *HLA-DPA1* SNP *rs3077* had only a weak association in African-Americans that was no longer significant after correcting for the association with 496A/G (data not shown). We thus concentrated our efforts on deciphering the effect of SNPs in the *HLA-DPB1* region.

We surmise that the *rs927753* SNP (550A/G) does not have a strong effect with HBV recovery in our cohorts, because the protective 550A allele, which marks the *DPB1*04:01* protective allele, also marks the *DPB1*01:01* risk allele, diminishing the association of position 550 with HBV outcome (see Tables S3 and S4 in the supplemental material). This was not a confounding factor in the Asian population, because *DPB1*01:01* is uncommon in Asians and thus the 550A allele proved to be a suitable marker for protection. In contrast, the 496A/G variant distinguishes *HLA-DPB1*01:01* (496G) from **04:01* (496A) and therefore shows a much stronger association in subjects of African and European descent. 496A/G is likely to be a better marker in Asians, as well, since 496G is in LD with the risk *HLA-DPB1* alleles **05:01* and **03:01*, whereas 496A is in LD with the protective *HLA-DPB1* alleles **04:01* and **04:02* in Asians.

Evidence reported herein suggests that the influence of the *HLA-DP* region on HBV recovery is due to levels of *HLA-DPB1* expression and less likely to differences in the peptides presented by different *HLA-DPB1* alleles. All five of the common *HLA-DP* alleles, including *HLA-DPB1*04:01*, **04:02*, **02:01*, **01:01*, and **05:01*, share largely overlapping peptide binding repertoires (22) and comprise both the protective as well as the susceptible *HLA-DPB1* alleles. Thus, the effect of *HLA-DP* on HBV may be due to differences in levels of expression rather than differences in peptide presentation. Differences in expression levels of *HLA-C* class I molecules have previously been implicated in HIV disease (28), and this appears to be due in part to variation in the 3'UTR of the *HLA-C* locus, which determines binding of a microRNA (10). We show here that *HLA-DP* is expressed at differential levels on freshly isolated B lymphocytes from normal donors of African and European descent, and this cell surface expression correlates with 496A/G genotypes. Individuals with the GG genotype have higher levels of *HLA-DP* expression than do individuals with the AA/AG genotypes. This recessive effect of 496G (alternatively the dominant effect of 496A) suggests that the mechanism regulating *HLA-DP* expression probably has to do with some sort of event that is sensitive to a threshold. Thus, the 496GG genotype associates with both HBV persistence and higher *HLA-DP* expression levels, suggesting that high *HLA-DP* expression may promote HBV persistence.

In contrast to the 496A/G genotypes, *HLA-DP* surface expression correlated relatively poorly with 550A/G genotypes. The residual correlation of position 550 with *HLA-DP* expression is most likely due to the significant but incomplete LD with position 496. Based on an association between 550A/G genotype and

mRNA levels obtained from microarray data from liver cells and qPCR of mRNA from macrophages, O'Brien et al. (15) concluded that lower *HLA-DP* mRNA expression correlates with HBV disease pathogenesis. We examined *HLA-DPB1* transcript levels using *HLA-DPB1*-specific qPCRs that targeted two different regions of *HLA-DPB1* and normalized these results with three independent reference genes. The primers were specifically designed to recognize all alleles of the *HLA-DPB1* locus but no alleles of any other *HLA* locus. Our results show that *HLA-DPB1* mRNA levels were higher in total PBL from donors with the risk 496GG genotype than in donors with 496AA/AG genotypes. Thus, our findings are in complete contrast to the previous study (15) in that higher levels of *HLA-DP* at both the cell surface protein level and mRNA level associate with the 496GG genotype, which in turn associates with HBV persistence.

Clinical studies find that Th1 responses are more often observed in patients that recover from an HBV infection, while Th2 responses are a hallmark of chronically infected patients (13). Perhaps high *HLA-DP* expression favors a Th2 response characterized by vigorous antibody production along with poor CTL activity leading to HBV persistence. A recent multicenter genetic study implicates the *HLA-DPB1* locus in risk of the autoimmune disease rheumatoid arthritis (RA) (19). *HLA-DPB1* alleles that associated with risk of RA are the same alleles that are protective against HBV and are expressed at low levels. Taken together, this is consistent with our model of lower *HLA-DP* expression favoring Th1 responses that are effective in HBV recovery but are known to be disadvantageous in Th1-mediated RA (30).

Our results strongly implicate the 3'UTR region of *HLA-DP* with *HLA-DP* expression and outcomes of HBV infection. It is now necessary to determine the mechanism for varied expression of *HLA-DP*, which may involve regulation through differential microRNA activity, similar to *HLA-C* (10). If *HLA-DP* expression levels do indeed explain the association between *HLA-DP* and HBV disease pathogenesis, further work will be required to determine how varied expression of this molecule at the site of infection in the liver is affecting the immune response to HBV.

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