HLA Class I Supertype Associations With Clinical Outcome of Secondary Dengue Virus Infections in Ethnic Thais

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Background. Human leukocyte antigen (HLA) supertypes are groups of functionally related alleles that present structurally similar antigens to the immune system.

Objectives. To analyze HLA class I supertype associations with clinical outcome in hospitalized Thai children with acute dengue illness.

Methods. Seven hundred sixty-two patients and population-matched controls recruited predominantly in Bangkok were HLA-A and -B typed. HLA supertype frequencies were compared and tested for significant dengue disease associations using logistic regression analyses. Multivariable models were built by conducting forward stepwise selection procedures.

Results. In the final logistic regression model, the HLA-B44 supertype was protective against dengue hemorrhagic fever (DHF) in secondary infections (odds ratio [OR] = 0.46, 95% confidence interval [CI], .30–.72), while the HLA-A02 supertype (OR = 1.92, 95% CI, 1.30–2.83) and the HLA-A01/03 supertype (OR = 3.01, 95% CI, 1.01–8.92) were associated with susceptibility to secondary dengue fever. The B07 supertype was associated with susceptibility to secondary DHF in the univariate analysis (OR = 1.60, 95% CI, 1.05–2.46), whereas that was not retained in the final model.

Conclusions. As the HLA-B44 supertype is predicted to target conserved epitopes in dengue, our results suggest that B44 supertype-restricted immune responses to highly conserved regions of the dengue proteome may protect against secondary DHF.

Keywords. HLA; class I; B44; supertype; associations; secondary; dengue; infections; Thais.

Received 7 January 2014; accepted 11 February 2015; electronically published 4 March 2015.

Presented in part: 3rd International Conference on Dengue Fever and Dengue Hemorrhagic Fever, Bangkok, Thailand, 22 October 2013; the American Association of Histocompatibility and Immunogenetics 40th Annual Meeting, Denver, Colorado, 21 October 2014; and 12th International Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases, Bangkok, Thailand, 12 December 2014.

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The Journal of Infectious Diseases® 2015;212:939–47

DOI: 10.1093/infdis/jiv127

In mainland southeast (SE) Asia, all 4 dengue virus (DENV) serotypes are in seasonal circulation. Epidemiologic studies indicate DENV is amplifying in large population-dense and ethnically diverse urban centers, such as Bangkok [1]. Here, host immunologic pressure exerts positive selection on DENV [2], before the fittest serotypic strains spread from urban centers out in cyclical waves across the region into adjacent populations [1]. Clinical symptoms range from relatively benign dengue fever (DF) to the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3, 4],

the latter 2 conditions associating with a more intense yet transient viremia [4, 5]. Both primary and secondary DENV infections can induce clinical illness, but most patients with DHF are undergoing secondary infection with a virus serotype different from prior exposures [6]. Thus, immunologic priming to DENV influences disease expression, the level of viremia, and in turn, the selection and transmission of the fittest viral strains.

The immunologic function of class I human leukocyte antigens (HLA-A, -B, and -C) is to present degraded fragments of endogenously synthesized virus antigens to $CD8^+$ T cells of the adaptive immune response [7]. Some HLA class I molecules are also recognized by natural killer (NK) cells that have an innate ability to recognize and destroy infected cells [8]. HLA class I genes are extremely polymorphic and encode thousands of alleles, the majority of which are distinct structural and functional variants (see http://www.ebi.ac.uk/imgt/hla). Most polymorphism affects specific amino acids forming a series of pockets (A-F), which accommodate virus-derived peptides in the class I antigen-binding cleft, thus influencing the types of antigens presented to T cells [9]. Nevertheless, analysis of the protein sequence of antigens bound by different HLA class I molecules has identified groups of alleles or "supertypes," with a common preference to present short peptides or antigen "supermotifs" sharing a similar structure [10].

The frequency of HLA class I alleles and combinations of alleles in linkage disequilibrium or haplotypes varies considerably between different ethnic groups [11, 12, see http://www.allelefrequencies.net]. Analysis of HLA class I allele and haplotype profiles in ethnic Thais has shown that this population is highly representative of the genetic admixture present in mainland SE Asians [12, 13]. Case-control HLA association studies with dengue disease have been performed in various cohorts identified in mainland SE Asia [14]. Collectively, nearly 1600 dengue patients have been HLA typed in Thailand [15–17], Vietnam [18, 19] and Malaysia [20]. Studies in ethnic Thais have indicated that a variety of HLA class I allele associations occur with secondary DENV infections, in particular with the HLA-B locus [16, 17], which is the most polymorphic in the human genome (see http://www.ebi.ac.uk/imgt/hla) and is also consistent with previous observations that HLA-B dominates the immune response to DENV and other RNA viruses [21–23].

To date, there have been no reports of any HLA class I supertype associations with dengue disease. Nevertheless, bioinformatic modeling of the dengue proteome for predictive HLA class I binding antigen supermotifs has been performed [23]. In this study, we have HLA typed just over 750 ethnic Thai dengue patients and controls, and stratified the detected HLA class I alleles into their respective supertype groups. Using logistic regression analyses, we have identified a variety of HLA class I supertype associations with dengue disease severity and susceptibility, and discuss our observations in the context of previous case-control-association studies and computational analyses of HLA supermotifs in the DENV proteome.

MATERIALS AND METHODS

Patients and Controls

Unrelated hospitalized ethnic Thai children with serologically and virologically confirmed DENV infections between the ages of 3 and 14 years and weighing >20 kg were enrolled in Bangkok with fully informed consent between 1994 and 2007, as previously described [16]. Clinical diagnoses of DF and DHF were assigned by an expert physician reviewer guided by the 1997 World Health Organization (WHO) criteria [3]. Two hundred seventy-seven DF and 163 DHF patients were available for HLA supertype analysis. One hundred fifty-two of these patients had been included in a previous HLA allele association study [16]. DHF was further classified into increasing grades of clinical severity, namely DHF-1, -2, -3, and -4 according to WHO criteria [3]. Serological responses to DENV were measured by both enzyme immunoassay and hemagglutination inhibition test. DENV serotypes (DENV-1, -2, -3, and -4) were identified by isolation in *Toxorhynchites splendens* mosquitoes or reverse transcription polymerase chain reaction (RT-PCR), as previously described [4, 5]. The infecting DENV serotype was determined in all but 7 patients. Primary or secondary infections were defined on the basis of DENV-specific immunoglobulin M:immunoglobulin G serum antibody ratios and the titer of hemagglutination inhibition antibodies, according to WHO guidelines [3]. On this basis, 77/277 (27.8%) DF patients had primary infections, 200/277 (72.2%) DF patients had secondary infections, 13/163 (8.0%) DHF patients had primary infections, and 150/163 (92.0%) DHF patients had secondary infections. Two hundred twenty-seven unrelated, ethnically and geographically matched, normal healthy Thai blood donors with no clinical history of autoimmune or malignant disease, whose families were known to have resided in the Bangkok metropolitan area for many generations, were used as population controls. However, the previous exposure to DENV in this control group was unknown. A further 95 unrelated Thai patients (aged 3–14 years), composed of 51 secondary DF and 44 secondary DHF patients enrolled in Kamphaeng Phet (located 320 km northwest of Bangkok) [4], were used to test and confirm the major HLA-B44 supertype association identified in the Bangkok patients. All of the Kamphaeng Phet patients were included in a previous HLA allele association study [16]. No geographically matched blood donor controls for the Kamphaeng Phet population were available.

HLA-A and -B Typing and Supertype Determination

Both serological phenotyping of fresh peripheral blood lymphocytes and molecular genotyping of full length genomic DNA, using PCR with sequence-specific primers and direct sequencing, were performed to HLA type patients and controls, as previously described [12, 16, 17, 24, 25]. This enabled confirmation of HLA class I allele expression and the exclusion of null or unexpressed alleles, before HLA-A and -B alleles were assigned according to

Marsh et al [26] and supertypes according to Sidney et al [10]. HLA supertype frequencies (SFs%) were determined using the following formula; SF (%) = n/N × 100, where n = number of individuals with a given supertype and N = the total number of individuals supertyped in each of the patient and control groups.

Study Design and Statistical Analysis

HLA-A and -B SFs were compared between each patient group (stratified for primary or secondary infections, disease severity, or the infecting DENV serotype) and the controls, and tested for associations using the Wald χ^2 test for heterogeneity (2 × 2 contingency tables). P values < .05 were considered significant. Correction of P values (P_c) for multiple comparisons (Bonferroni's) and random supertype associations was dependent on the number and type of comparisons made; namely the number of HLA-A (n = 7) or -B (n = 7) supertypes identified, combined with the number of patient and control group comparisons (n = 7, primary DF vs controls, secondary DF vs controls, secondary DHF vs controls, all secondary infections vs controls, primary DF vs secondary DF, primary DF vs secondary DHF, secondary DF vs secondary DHF), which equates to 7 + 7 or 14 comparisons. P_c values < 0.05 were considered highly significant. Odds ratio (OR) with 95% confidence interval (CI) was used to assess the risk of either disease severity (OR with 95% CI > 1.0) or protection (OR with 95% CI < 1.0), associated with specific supertypes in either primary or secondary DENV infections. Previous (a priori) power calculations demonstrated that all patient and control groups were of sufficient size to achieve at least 80% power to detect HLA class I supertype differences of 11%–21% (P < .05) [17], except for the primary DHF group (n = 13), which was underpowered and thus deemed too small for inclusion in the analysis.

Multivariable regression models were also created to address the adjusted relationship between the clinical outcome of primary or secondary DENV and HLA class I supertype. Dengue infection status was classified into 3 clinical types (primary DF, secondary DF, and secondary DHF), and the relationship between risk of each clinical phenotype versus no disease (control) and HLA supertype was quantified using 3 separate logistic regressions. A forward stepwise selection procedure was performed to select each final multivariable model. All HLA class I supertypes were evaluated for inclusion in the multivariable model and retained if the P value for an HLA supertype was <.05. In order to assess whether the presence of 1 HLA supertype modified the relationship between another HLA supertype and risk of specific dengue clinical outcomes, 2-way interaction terms were tested for supertypes retained in the final model.

RESULTS

HLA-A Supertypes

Our analysis of a Bangkok cohort of hospitalized pediatric dengue patients revealed a moderately significant association between the A02 supertype and susceptibility to secondary DF and DHF, compared to the ethnically and geographically matched controls (Table 1). The preferred supermotif amino acids held in the B and F pockets of the HLA class I antigen binding clefts of A02 supertype alleles, together with the A02 supertype alleles identified in our Thai cohort, are also given in Table 1.

HLA-B Supertypes

In the equivalent analysis of HLA-B supertypes, we detected another modest association between the B07 supertype and susceptibility to DHF in patients with secondary infections in Bangkok (Table 2). By contrast, the most significant association detected was with the B44 supertype and protection against developing severe DHF in secondary DENV infections, when compared to secondary DF patients and the controls (P_c < 0.05, Table 2). This association was tested in a second cohort of hospitalized Thai children with DENV infections identified in a geographically distinct region (Kamphaeng Phet, Thailand). The frequency of the B44 supertype in Kamphaeng Phet secondary DHF (12/44, 27.3%) was also significantly reduced compared to secondary DF (24/51, 47.1%) in the same location (Wald χ^2 = 3.9, P = .0497, OR = 0.422, 95% CI, .178–.999).

Logistic Regression Model

To investigate whether the evaluated HLA class I supertypes were related to either increased or decreased risk of primary/secondary DF or secondary DHF versus controls, logistic regression models were built (Table 3). The primary DF model did not retain any significant supertype associations. However, the secondary DF model showed that A02 (OR = 1.92; P = .0011) and A01/A03 (OR = 3.01; P = .0474) supertypes were related to increased risk of secondary DF. The secondary DHF model demonstrated that the B44 supertype was related to decreased risk of secondary DHF (OR = 0.46; P = .0007). Separate models predicting secondary DF that included 2-way interactions between A02 with A01/A03, A02 with B44, B07 with B44, and A01/A03 with B44, did not demonstrate significant interactions between these HLA class I supertype combinations, suggesting the presence of one did not significantly modify or change the relationship of the other supertype to secondary DF and DHF.

B44 Supertype Protects Against Secondary DHF With All Four DENV Serotypes

Stratification for the known infecting DENV serotype indicated that the B44 supertype was reduced in frequency in secondary DHF patients infected with any of the 4 serotypes (Table 4). This trend reached significance only in the DENV-1 secondary infection group, which was the largest of the stratified groups (Table 4).

Table 1. HLA-A Supertype Frequencies in Bangkok Thai Dengue Patients and Controls

HLA Class I Supertype	HLA-A Locus Alleles in Thais	Recognized Supermotif in Target Antigens	Supertype Frequency (SF%)			
			Primary Infection	Secondary Infection		Control
			DF N = 77 n = (SF)	DF N = 200 n = (SF)	DHF N = 150 n = (SF)	N = 227 n = (SF)
A01	*A*01* *A*26* *A*32*	Pos 2: Small aliphatic (Ala, Thr, Ser, Val, Leu, Iso, Met, Gln) Pos 9/10: Aromatic and large hydrophobic (Phe, Trp, Tyr, Leu, Iso, Met)	7 (9%)	11 (6%)	14 (9%)	23 (10%)
A02	*A*02*	Pos 2: Small aliphatic (Ala, Thr, Ser, Val, Leu, Iso, Met, Gln) Pos 9/10: Aliphatic and small hydrophobic (Leu, Iso, Val, Met, Gln, Ala)	36 (47%)	**105 (53%) *A,*C**	**75 (50%) *B,*C**	**85 (37%) *A, *B, *C**
A03	*A*03* *A*11* *A*31* *A*33*	Pos 2: : Small aliphatic (Ala, Thr, Ser, Val, Leu, Iso, Met, Gln) Pos 9/10: Basic (Arg, His, Lys)	57 (74%)	149 (75%)	99 (66%)	170 (75%)
A24	*A*24*	Pos 2: Aromatic and aliphatic (Phe, Trp, Tyr, Leu, Iso, Val, Met, Gln) Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Try, Tyr, Leu, Ile, Met, Val, Ala)	32 (42%)	65 (33%)	63 (42%)	82 (36%)
A01/A03	*A*30*	Pos 2: Small and aliphatic (Ala, Thr, Ser, Val, Leu, Iso, Met, Gln) Pos 9/10: Aromatic and basic (Tyr, Arg, Lys)	1 (1%)	11 (6%)	4 (3%)	5 (2%)
A01/A24	*A*29*	Pos 2: Small, aliphatic and aromatic (Ala, Ser, Thr, Val, Leu, Ile, Met, Gln, Phe, Trp, Tyr) Pos 9/10: Aromatic and large hydrophobic (Phe, Trp, Tyr, Leu, Iso, Met)	. . .	3 (2%)	3 (2%)	3 (1%)
UC	*A*34*	Unknown	2 (3%)	4 (2%)	2 (1%)	3 (1%)
Wald χ^2			***P* =**	P_c **=**	**OR**	**95% CI**
*A A2 SF% in secondary DF infections vs controls, Wχ^2 = 9.7			**.0019**	**0.0266**	**1.85**	**1.26–2.72**
*B A2 SF% in secondary DHF infections vs controls, Wχ^2 = 5.8			**.0161**	0.2254	**1.67**	**1.10–2.54**
*C A2 SF% in all secondary infections (DF and DHF) vs controls, Wχ^2 = 10.8			**.0010**	**0.0140**	**1.77**	**1.26–2.49**

HLA-A supertypes were designated according to Sidney et al [10] and are given together with their known target antigen supermotifs. HLA-A alleles detected in ethnic Thais (patients and controls) and known to be in defined supertype groups [10], are as given. The 2-digit HLA allele designations indicate that all related class I alleles detected in the Thai population [28] fall within the defined supertype group. Within the respective supermotifs the amino acid at position 2 (pos 2) binds to the B pocket, and amino acids at pos 9 or 10 bind specifically to the F pocket of the HLA class I antigen-binding groove [9, 10]. N = total number of individuals in either patient or control groups, n = number of patients or controls with given HLA-A supertype. HLA-A class I supertype frequencies (SF) are given in parentheses and expressed as a relative percentage of the respective patient and control groups. SF showing significant differences between patient and control groups are indicated by an asterisk. Wald χ^2, *P* values, Bonferroni's corrected *P* values (P_c), odds ratio (OR) and 95% confidence interval (CI) are given in the last 4 rows. Significant *P* and P_c values, OR, and 95% CI are given in bold. P_c for *A, *B, *C = P × 14 (7 supertypes +7 patient group and control comparisons). Thirteen patients were identified with primary DHF but were excluded from the supertype analysis due to the relatively low numbers and lack of statistical power in testing for associations in this group (see "Materials and Methods" section).

Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever; HLA, human leukocyte antigen.

B44 Supertype Protects Against the Most Severe Forms of DHF

Our analysis of Bangkok DHF patients stratified for increasing disease severity according to WHO classification of DHF grades 1, 2, 3, and 4 [3] indicated that the B44 supertype associates with protection against the more severe forms of DHF-2 and DHF-3/4 (Table 5). The most frequent B44 supertype-related alleles in our Thai cohort were *HLA-B*40*, *B*18*, and *B*44*, which were all relatively reduced in frequency in the secondary DHF patients (13.0%, 6.5%, and 5.2%, respectively) compared to the equivalent antigen or phenotype frequencies in the controls (21.1%, 11.5%, and 11.9%, respectively). This indicates that the B44 supertype associations described in Tables 2–5 are not due to relatively increased or decreased frequencies of any particular B44-related allele.

DISCUSSION

The largest HLA gene association studies with dengue have been performed in mainland SE Asian populations [reviewed in 14]. A variety of HLA-A and -B locus alleles have been reported to

Table 2. HLA-B Supertype Frequencies in Bangkok Thai Dengue Patients and Controls

			Supertype Frequency (SF%)			
			Primary Infection	Secondary Infection		Control
HLA Class I Supertype	HLA-B Locus Alleles in Thais	Recognized Supermotif in Target Antigens	DF N = 77 n = (SF)	DF N = 200 n = (SF)	DHF N = 150 n = (SF)	N = 227 n = (SF)
B07	*B*07 B*35 B*51 B*54 B*55 B*56 B*67*	Pos 2: Proline Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Try, Tyr, Leu, Ile, Met, Val, Ala)	27 (35%)	**76 (38%) *B**	**64 (43%) *A,*B**	**72 (32%) *A,*B**
B08	*B*08*	Pos 2: undefined Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Try, Tyr, Leu, Ile, Met, Val, Ala)	. . .	2 (1%)	. . .	3 (1%)
B27	*B*27 B*38 B*39 B*48*	Pos 2: Basic (Arg, His, Lys) Pos 9/10: Aromatic, aliphatic, basic and hydrophobic (Phe, Try, Trp, Leu, Ile, Met, Arg, His, Lys, Val, Ala)	13 (17%)	50 (25%)	35 (23%)	48 (21%)
B44	*B*18 B*37 B*40 B*44 B*50*	Pos 2: Acidic (Asp, Glu) Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Trp, Tyr, Leu, Ile, Val, Met, Gln, Ala)	29 (38%)	**80 (40%) *C,*E**	**40 (27%) *C,*D, *E**	**100 (44%) *D,*E**
B58	*B*15:17 B*57 B*58*	Pos 2: Small (Ala, Ser, Thr) Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Trp, Tyr, Leu, Ile, Val, Met, Gln, Ala)	12 (16%)	24 (12%)	28 (19%)	40 (18%)
B62	*B*15:01 B*15:02 B*15:12 B*15:13 B*15:25 B*46 B*52*	Pos 2: Aliphatic (Leu, Iso, Val, Met, Gln) Pos 9/10: Aromatic, aliphatic and hydrophobic (Phe, Trp, Tyr, Leu, Ile, Val, Met, Gln, Ala)	43 (56%)	95 (48%)	77 (51%)	121 (53%)
UC	*B*13 B*15:21 B*15:27 B*15:32*	Unconfirmed	13 (17%)	36 (18%)	24 (16%)	31 (14%)
Wald χ^2			***P* =**	**P_c =**	**OR**	**95% CI**
*A B07 SF% in secondary DHF infections vs controls, **Wχ^2 = 4.7**			**.0307**	0.4298	**1.60**	**1.05–2.46**
*B B07 SF% in all secondary infections (DF and DHF) vs controls, **Wχ^2 = 4.0**			**.0442**	0.6188	**1.44**	**1.01–2.04**
*C B44 SF% in secondary DHF vs secondary DF infections, **Wχ^2 = 6.7**			**.0097**	0.1358	**0.55**	**.35–.86**
*D B44 SF% in secondary DHF vs controls, **Wχ^2 = 11.5**			**.0007**	**0.0098**	**0.46**	**.30–.72**
*E B44 SF% in all secondary infections (DF and DHF) vs controls, **Wχ^2 = 5.5**			**.0185**	0.2590	**0.66**	**.47–.93**

HLA-B supertypes were designated according to Sidney et al [10] and are given together with their known target antigen supermotifs. HLA-B alleles detected in ethnic Thais and known to be in defined supertype groups [10] are as given. The 2-digit HLA allele designations indicate that all related class I alleles detected in the Thai population [28] fall within the defined supertype group. Within the respective supermotifs, the amino acid at pos 2 binds the B pocket, and amino acids at pos 9 or 10 bind specifically to the F pocket of the HLA class I antigen-binding groove [9, 10]. N = total number of individuals in either patient or control groups, n = number of patients or controls with given HLA-B supertype. HLA-B class I supertype frequencies (SF) are given in parentheses and expressed as a relative percentage of the respective patient and control groups. SF showing significant differences between patient and control groups are indicated by an asterisk. Wald χ^2, *P* values, Bonferroni's corrected *P* values (P_c), odds ratio (OR), and 95% confidence interval (CI) are given in the last 6 rows. Significant *P* and P_c values, OR, and 95% CI are given in bold. P_c for *A, *B, *C, *D and *E = P × 14 (7 supertypes + 7 patient group and control comparisons). Thirteen patients were identified with primary DHF but were excluded from the supertype analysis due to the relatively low numbers and lack of statistical power in testing for associations in this group (see "Materials and Methods" section).

Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever; HLA, human leukocyte antigen.

associate with protection or susceptibility to various clinical manifestations of DENV infection. Some of these associations have been consistent across Thai [15–17], Vietnamese [18, 19], and Malaysian populations [20], while other associations have been either target population- or DENV serotype-specific. Such intraregional differences in HLA associations with dengue may reflect the cyclical dynamics of distinct epidemic foci in the most population-dense urban centers such as Bangkok and Ho Chi Minh

Table 3. Logistic Regression Models for HLA Class I Supertype Associations With Dengue Virus Infection

Secondary DENV Infection	HLA Class I Supertype	OR vs Controls	95% CI	*P* Value
DF	A02	1.92	1.30–2.83	.0011
	A01/A03	3.01	1.01–8.92	.0474
DHF	B44	0.46	.30–.72	.0007

Abbreviations: CI, confidence interval; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; HLA, human leukocyte antigen; OR, odds ratio.

City that are not necessarily in the same or synchronized DENV transmission zones. Moreover, given the genetic similarity of ethnic Thais and Vietnamese in terms of their HLA allele profiles [12, 13, see http://www.allelefrequencies.net], any apparent regional differences of HLA associations with dengue may also reflect variations in historic immunologic priming to disparate DENV serotypes. Nevertheless, HLA class I allele associations have been reported in a variety of other populations in south Asia and the Americas [reviewed in 14], which indicates that the clinical outcome of secondary exposure to DENV is at least in part mediated by T- and NK-cell-driven immune responses restricted by some HLA alleles.

Bioinformatic scanning of the DENV proteome for HLA binding antigen supermotifs has predicted that most class I supertypes target predominantly nonconserved regions of the virus [23]. Cross-reactive partial immunity to previously unseen DENV serotypes is a recognized feature of the T-cell response to DENV in secondary infections [27], and may relate to HLA class I presentation of DENV epitopes that are not conserved across all 4 DENV serotypes. By contrast, the B44 supertype is predicted to target conserved regions of the DENV proteome [23]. In particular, HLA-B*40:01 and B*18:01, which are relatively common B44 supertype alleles in ethnic Thais [28, see http://www.allelefrequencies.net], are considered most efficient at targeting conserved regions across the proteomes of all 4 DENV serotypes [23]. HLA-B*40 has also been associated with relatively high-magnitude T-cell responses to DENV in Sri Lankans (22). Thus, the protective associations we describe in Tables 2–5 may reflect HLA-B44 supertype-restricted T-cell responses in immunologically primed patients targeting highly conserved DENV proteins and effectively reducing the chance of severe complications of secondary DENV infections arising. Reduced frequencies of HLA-B44 supertype-related alleles have also been reported in Malaysian DHF patients [20]. Moreover, given that the HLA class I supertypes in our patient groups were compared against the equivalent supertype frequencies in the ethnically and geographically matched urban population controls that are most likely to have had historic exposure to DENV, the relative protective effect of the B44 supertype may even be stronger if compared against subclinical or asymptomatic controls.

The concept of HLA supertypes is based on extensive information derived from either peptide-binding studies to different HLA class I allelic products [10, 29] or from sequencing natural HLA class I ligands by mass spectrometry [30]. Some discrepancies exist between these 2 approaches in defining the precise specificities of the preferred peptides recognized by different class I supertypes. Nevertheless, both methods have effectively confirmed that all B44 supertype alleles share a common preference for antigenic supermotifs with a negatively charged amino acid (mostly Glu) in the first anchor position (pos 2) which binds the B-pocket, and a variety of hydrophobic residues at the C-terminus of the antigenic peptides (pos 9/10, see Table 2) which bind the F-pocket of the class I antigen-binding groove [9, 10, 29, 30]. Bioinformatic algorithms utilizing this information together with other computational tools that predict intracellular peptide cleavage and antigen transport efficiency have been used to screen conserved DENV protein sequences and identify putative B44 supermotifs [23, 31]. For example, the DENV NS3 protein, which has protease and helicase functions, is relatively rich in acidic or negatively charged amino acids [32]. Bioinformatic screening of NS3 for HLA class I supermotifs has revealed a 13-mer peptide (256-EIVDLMCHATFT-267) that is highly conserved across DENV-1, -2, -3, and -4 serotypes [31]. This pan-serotype DENV peptide (NS3 256–267) contains at least 2 potential B44 supermotifs (Asp256 with Ala264, and Glu259 with Phe266). DENV NS3 256–267 is also predicted to contain other HLA class I and II supermotifs [31], and was originally shown to be recognized by a serotype cross-reactive HLA-DP-restricted CD4 T-cell clone [33], making it an attractive potential candidate for inclusion in future vaccines.

Our observation that the A02 supertype is significantly increased in both secondary DF and DHF patients compared to geographically and ethnically matched controls (Table 1) is consistent with other studies conducted at different time points in Bangkok [15], which has the largest urban population exposed to DENV in mainland SE Asia. The A02 supertype is largely composed of *HLA-A*02*-related alleles [10], of which 456 variants have been identified worldwide (see http://www.ebi.ac.uk/imgt/hla and http://www.allelefrequencies.net). Most of the polymorphism that defines HLA-A*02 alleles is focused on the pockets of the class I peptide-binding groove. Such variation is likely to restrict which peptides are preferentially bound and affect the subsequent antigen-specific immune responses invoked, as different HLA*02 variants have been shown to correlate with either secondary DF or DHF [16]. Bioinformatic modeling of the DENV proteome has also predicted the A02 supertype preferentially binds variable sequences of the proteome that are not conserved between DENV serotypes [23], which would suggest that in immunologically primed individuals, A02-restricted

Table 4. Logistic Regression Analysis of HLA-B44 Supertype Frequencies in Secondary DENV-1, DENV-2, DENV-3, or DENV-4 Infections in Bangkok Thais

	DENV Serotype Identified in Secondary Infections								
	DENV-1		DENV-2		DENV-3		DENV-4		Control
	DF N = 74 n = (SF)	DHF N = 50 n = (SF)	DF N = 40 n = (SF)	DHF N = 39 n = (SF)	DF N = 42 n = (SF)	DHF N = 37 n = (SF)	DF N = 42 n = (SF)	DHF N = 21 n = (SF)	N = 227 n = (SF)
HLA B44 Supertype	32 (43%)	12 (24%)	14 (35%)	11 (28%)	18 (43%)	11 (30%)	15 (36%)	5 (24%)	100 (44%)
OR (95% CI) Patient Groups vs Controls (ref)	0.97 (.57–1.64)	**0.40 (.20–0.81)**[a]	0.68 (.34–1.38)	0.50 (.24–1.05)	0.95 (.49–1.85)	0.54 (.25–1.14)	0.71 (.36–1.40)	0.40 (.14–1.12)	Ref
Wald χ^2–derived *P* Value	.9031	**.0105**	.2877	.0675	.8862	.1056	.3172	.0809	
OR (95% CI) DHF vs DF (ref) within given DENV serotype	Ref	**0.41 (.19–.92)**[a]	Ref	0.73 (.28–1.89)	Ref	0.56 (.22–1.43)	Ref	0.56 (.17–1.84)	
Wald χ^2–derived *P* Value		**.0300**		.5168		.2291		.3419	

The HLA B44 supertype was designated according to Sidney et al [10] and the known target antigen supermotif is as given in Table 2. Secondary DF and DHF patients were stratified for infecting dengue virus serotype as described in "Materials and Methods" section. N = total number of individuals in each patient and control group, n = number of patients with HLA-B44 supertype. HLA-B44 supertype frequencies (SF) are given in parentheses and expressed as a relative percentage of the respective patient and control groups in row 1. The infecting virus serotype was not determined in 2 patients with secondary DF and 3 patients with secondary DHF (as given in Table 2). Of these patients with an undetermined dengue serotype 2 had the B44 supertype, 1 with secondary DF and the other with secondary DHF. Odds ratios (OR) with 95% confidence interval (95% CI) were generated using multivariable logistic regression. OR (95% CI) generated for comparisons of SF in patient groups versus controls (ref) are given in row 2. OR (95% CI) generated for comparisons of SF in DHF versus DF (ref) in patients infected with the same DENV subtype are given in row 4.

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; HLA, human leukocyte antigen.

[a] OR showing significant B44 SF differences between patient and control groups are indicated in bold and with an asterisk. Significant Wald χ^2–derived *P* values (<.05) are given in bold.

Table 5. Logistic Regression Analysis of HLA B44 Supertype Associations With Severe Secondary Dengue Infections in Bangkok Thais

	HLA-B44 Supertype Frequency (SF%) in Patients With Secondary Dengue and Controls				
	Secondary DF	Secondary DHF-1	Secondary DHF-2	Secondary DHF-3/4	Control
	N = 200 n = (SF)	N = 22 n = (SF)	N = 96 n = (SF)	N = 32 n = (SF)	N = 227 n = (SF)
HLA B44 Supertype	80 (40%)	7 (32%)	28 (29%)	5 (16%)	100 (44%)
OR (95% CI) Patient Groups vs controls (ref)	0.85 (.58–1.25)	0.59 (.23–1.51)	**0.52 (.31–.87)**[a]	**0.24 (.09–.63)**[a]	Ref
Wald χ^2–derived *P* Value	.3975	.2727	**.0131**	**.0041**	
OR (95% CI) DHF vs DF (ref)	Ref	0.70 (.27–1.79)	0.62 (.37–1.04)	**0.28 (.10–.75)**[a]	
Wald χ^2–derived *P* Value		.4574	.0711	**.0117**	

HLA-B44 supertypes were designated according to Sidney et al [10] and the known target antigen supermotif is as given in Table 2. Secondary DHF patients were stratified for disease severity into DHF-1, -2, and -3/4 groups as described in "Materials and Methods" section. Only 1 patient in the DHF-3/4 group was defined as DHF-4. N = total number of individuals in each patient and control group, n = number of patients with HLA-B44 supertype. HLA-B44 supertype frequencies (SF) are given in parentheses and expressed as a relative percentage of the respective patient and control groups in row 1. Odds ratios (OR) with 95% confidence interval (95% CI) were generated using multivariable logistic regression. OR (95% CI) generated for comparisons of SF in patient groups versus controls (ref) are given in row 2. OR (95% CI) generated for comparisons of SF in DHF versus DF (ref) are given in row 4.

Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever; HLA, human leukocyte antigen.

[a] OR showing significant B44 SF differences between patient and control groups are indicated in bold and with an asterisk. Significant Wald χ^2–derived *P* values (<.05) are given in bold.

immune responses to disparate DENV serotypes are less able to control viral replication.

The A24 supertype is composed of just HLA-A*24-related alleles [10], some of which have been associated with DHF and DSS in Vietnamese populations identified within or near to Ho Chi Minh City [18, 19], the second largest urban epicenter of DENV transmission in mainland SE Asia. The A24 supertype is also reputed to target variable sequences of the DENV proteome that are not conserved between DENV serotypes [23]. In ethnic Thais, HLA-A*24 alleles and the A24 supertype are increased in frequency in secondary DHF patients relative to secondary DF and controls, although these are trends rather than replicated associations reaching statistical significance [16] (Table 1). Similarly, the B07 supertype is significantly associated with susceptibility to secondary DHF (Table 2). However, this effect may be due to the presence of HLA-B*51 alleles in the B07 supertype, which we have previously shown to associate with DHF in ethnic Thais [16], but did not retain significance with the final model (Table 3). By contrast, our logistic regression model did reveal an association between the relatively rare A01/A03 supertype and protection against secondary DHF (Table 3), which was not identified in our univariate analysis (Table 1). The A01/A03 supertype does have subtle differences in its preferred antigenic supermotifs presented, and is represented by just the HLA-A*30 allele in ethnic Thais (Table 1).

A complex picture is emerging of the association of immune-response genes and the role of their products in determining disease outcome of DENV infection, particularly after secondary exposures in immunologically primed individuals. Specific HLA class I alleles [15–20], extended haplotypes encoding specific combinations HLA class I and II alleles [17], cytokine gene polymorphisms [17], major histocompatibility complex (MHC) class I chain-related (MIC) gene deletions [17], and now functionally defined HLA class I supertypes (Tables 1–3 and 5), all associate with various aspects of disease outcome after DENV exposure. This suggests that there is most likely a major concentration of genes in the human MHC that influence dengue disease expression. Our study complements previous bioinformatic predictions on the relative immunogenicity of the DENV proteome by revealing in a major urban transmission zone that the B44 supertype, which is predicted to target immune epitopes highly conserved across all DENV serotypes [23], is protective against the most severe consequences of secondary DENV infection (Table 5). Taken together, these data suggest that vaccines designed to replicate and enhance B44 supertype–driven immune responses to DENV may induce protection against severe disease.

Notes

Acknowledgments. We thank the staff, patients, and their families at Queen Sirikit National Institute of Child Health and Kamphaeng Phet Provincial Hospital for their participation in this study.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as reflecting the official views of the National Institutes of Health and the US Army or the US Department of Defense.

Financial support. This work was supported by a Program Project grant from the National Institutes of Health USA (NIH-P01AI34533) and the US Army Medical Research and Materiel Command. This study was granted human use approval by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the US Army Surgeon General's Office, and the University of Massachusetts Medical School.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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