Next-Generation HLA Sequence Analysis Uncovers Shared Risk Alleles Between Clinically Distinct Forms of Childhood Uveitis

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Purpose. Classical alleles of the human leukocyte antigen (HLA) complex have been linked to specific entities of pediatric noninfectious uveitis, yet genetic predisposition encoded by the HLA super-locus across the patient population remains understudied.

Methods. We performed next-generation full-length sequencing of *HLA-A, HLA-B, HLA-C*, *HLA-DPB1, HLA-DQB1,* and *HLA-DRB1* in 280 cases. Dense genotype data from 499 Dutch controls from Genome of the Netherlands were imputed using an HLA-specific reference panel ($n = 5225$ samples from European ancestry). Cases and controls were compared using logistic regression models adjusting for sex.

Results. In total, 179 common and rare alleles were detected. Considering all cases and controls, *HLA-DQB1*04:02* and *HLA-DRB1*08:01* were identified as the principal HLA association, which was mainly driven by 92 cases with juvenile idiopathic arthritis-associated uveitis (JIA-U). The *HLA-DQB1*04:02-HLA-DRB1*08:01* haplotype was also the primary association for the phenotypically similar idiopathic chronic anterior uveitis without arthritis (CAU). Also, *HLA-DQB1*05:03* was an independent risk allele for CAU, but not in JIA-U. Analysis of 185 cases with other forms of uveitis revealed HLA-wide associations ($P < 2.79 \times 10^{-4}$) for *HLA-DRB1*01:02, HLA-DRB1*04:03,* and *HLA-DQB1*05:03*, which could be primarily attributed to cases with panuveitis. Finally, amino acid substitution modeling revealed that aspartic acid at position 57 that distinguishes the risk allele *HLA-DQB1*05:03* (for CAU and panuveitis) from nonrisk alleles, significantly increased the binding capacity of naturally presented ligands to HLA-DQ.

Conclusions. These results uncovered novel shared HLA associations among clinically distinct phenotypes of pediatric uveitis and highlight genetic predisposition affecting the antigen presentation pathway.

Keywords: noninfectious uveitis, pediatric uveitis, HLA, next-generation sequencing

Noninfectious pediatric uveitis poses a significant burden on the health and quality of life of affected children, because of its high risk of visual impairment and its lifelong dependency on specialized care.[1,2] Despite the variety of clinical phenotypes, little is understood of the underlying disease mechanisms that render some children prone to develop uveitis. Consequently, due to lack of understanding their causes, at least half of cases are considered idiopathic and therefore left to be classified according to the affected areas (i.e., anterior uveitis describes cases with a predominant involvement of the anterior segment of the eye).[3] Regardless, immune-mediated etiology is strongly suspected because pediatric uveitis occurs often in cases suffering from autoimmune diseases (e.g., rheumatic disease). In fact, the largest category (12%–30%) of pediatric uveitis is cases suffering from anterior uveitis associated with preexisting juvenile idiopathic arthritis (JIA).[4] In addition, immunosuppressive therapy regimes are effective in dampening eye inflammation and further support that derailed immunity is an important contributor of disease.[5–8]

Genetic studies that strongly linked several alleles of the family of human leukocyte antigen (HLA) genes on chromosome 6 to noninfectious uveitis add to the body of evidence.[9–15] HLA molecules are critically involved in adaptive immunity by presenting protein fragments sampled from the cellular (class I HLA molecules) and extracellular (class II HLA molecules) environment to surveilling T and natural killer cells to orchestrate antigen-specific immunity.[16] Genetic studies of pediatric uveitis have mostly been conducted in JIA-U cases of Western European

ancestry, which mapped the primary genetic risk for uveitis in JIA to amino acid positions 10 to 12 encoded by the HLA–DRB1 gene.[14] This uveitis motif exhibited a disease risk in females which was independent from the known genetic association of JIA with other amino acid positions in *HLA-DRB1*.[14] This example demonstrated that different polymorphisms in the same gene may confer independent disease risks and supported that uveitis is a distinct trait of the pediatric population.[14,15] Other HLA typing studies have been conducted in disease-specific contexts, such as sequence specific typing association studies in small cohorts of cases with tubulointerstitial nephritis and uveitis (TINU) syndrome.[17–22] At present, HLA typing studies across a representative sample of pediatric uveitis population are sparse. Here, we performed allelic resolution next-generation sequencing across all classical class I and class II HLA alleles to refine HLA associations and amino acids polymorphisms at the population level and stratify HLA haplotypes according to anatomical categories and specific entities.

Methods

Patient and Material Collection

The study was approved by the Medical Ethical Research Committee of the University Medical Center Utrecht in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients $\geq$ 18 years, from both parents and patients in cases between 12 to 18 years of age, and from parents in cases $<$ 12 years old. Peripheral blood samples (EDTA tubes) were obtained from patients with juvenile idiopathic arthritis-associated uveitis (JIA-U, $n = 92$), idiopathic chronic anterior uveitis (i.e., no JIA) (CAU, $n = 50$), (*HLA-B27*-positive) acute anterior uveitis ($n = 18$), intermediate uveitis (IU, $n = 39$), and panuveitis (PAN, $n = 81$). The diagnosis of uveitis was established by a trained uveitis specialist according to the SUN criteria.[23] All cases were recruited at the University Medical Center Utrecht, the Netherlands, a tertiary referral center. The study only considered patients with a diagnosis of noninfectious uveitis before the age of 18 years. Juvenile idiopathic arthritis was diagnosed according to the criteria of the International League of Associations for Rheumatology or by former criteria (e.g., European League Against Rheumatism).[24,25] Patients with JIA were screened by an ophthalmologist according to the guidelines of the Academy of Pediatrics.[26] Patients with 1+ cells or more in the anterior chamber and treated with at least topical steroids during ophthalmologic examinations, were diagnosed with JIA-U. The diagnosis for (probable or possible) TINU syndrome was made by an ophthalmologist specialized in pediatric uveitis and a pediatric nephrologist based on clinical criteria.[17]

HLA Typing by High-Resolution Next-Generation Sequencing

DNA was isolated from 300 µL of peripheral blood (EDTA tubes) using the MagNa Pure Compact (Roche Diagnostics, the Netherlands). From genomic DNA, coding sequences of the HLA region were enriched by PCR using multiplexed HLA amplification by NGSgo-MX6-1 PCR (GenDx, Utrecht, the Netherlands). Next-generation sequencing was performed on all samples using the Illumina sequencing system. The DNA fragments were analyzed with the NGSEngine software package version 2.14.0 (GenDx, Utrecht, The Netherlands) using the IPD-IMGT/HLA database version 3.36.0. After inspection of the full-length sequence, the genotype data for 179 distinct HLA alleles were reduced to second field nomenclature to reflect the unambiguous phenotype. The corresponding amino acid sequences for each of the 179 alleles was obtained from the the Immuno Polymorphism Database (IPD) and the international ImMunoGeneTics (IMGT)/HLA release 3.29[27] (Supplementary Table S1A-F). A total of 293 amino acid positions were considered polymorphic and used for fine-mapping of amino acid associations (Supplementary Table 6).

Reference Cohort

Genotype data from 499 unrelated and unaffected Dutch subjects from the Genome of the Netherlands (GoNL) were used as population controls.[28] The GoNL project is a human genetic project based on a representative sample of Dutch citizens from all provinces in the Netherlands, which captures the genetic variation in the Dutch population (for more information see http://www.nlgenome.nl). Next, we used BEAGLE package to prephase the GoNL single-nucleotide polymorphism (SNP) data for imputation. In other words, we estimated the haplotypes for each individual GoNL sample for efficient imputation of ungenotyped variants based on linkage disequilibrium.[29] We imputed classical HLA alleles using SNP data and analysis with the SNP2HLA pipeline, which given the strong linkage disequilibrium in the MHC region, can be used to tag the majority of HLA alleles. The SNP2HLA pipeline uses a reference panel assembled through HLA typing of 5225 European-ancestry individuals collected by the Type 1 Diabetes Genetics Consortium to ascertain accurate HLA imputation.[30]

HLA Association Analysis

Sequence data were converted to dosage data by coding the presence or absence of each classical allele or encoded amino acid residue at indicated positions to 0,1, or 2. This generated three possible genotypes: homozygous positive (2 alleles), heterozygous positive (1 allele), and homozygous negative (0 alleles) for any of the classical HLA alleles or amino acid residues at a given position. To visualize the ethnicity of the cases and controls, we used Uniform Manifold Approximation and Projection (UMAP)[31] based on the top 60 principal components calculated using the dosage data. For this analysis we also included HLA genotypes from populations of the 1000 Genomes Project (Supplementary Table 2).[32,33] HLA association testing was performed using PLINK 2.0 using an additive logistic regression model with sex as a covariate.[34] To identify potential independent associations for HLA alleles and amino acid residues, we performed a series of association analyses adjusting for the primary association (i.e., conditional analysis), by adding each significantly associated allele as a covariate to the model. Our data enabled multiple comparisons: 1) pediatric noninfectious uveitis versus controls, 2) JIA-U versus controls, 3) non-JIA-U versus controls, 4) CAU versus controls, 5) IU versus controls, and 6) PAN versus controls. A P value threshold of 2.79×10^{-4} (Bonferroni correction; 0.05/179 detected HLA alleles) was used as a threshold for

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significance to prioritize reporting of HLA associations and termed this threshold "HLA-wide" significance.

Amino Acid Substitution Modeling

To explore whether the presence of naturally occurring amino acids at position 57 of the different *HLA-DQB1*05* alleles affected the binding of peptide cargo to the HLA molecule, we extracted 157 ligands identified by mass spectrometry (from the HLA ligand atlas) from elutions of immunoprecipitations of *HLA-DQB1*05:01, HLA-DQB1*05:02*, and *HLA-DQB1*05:03*.[35] Replacement of position 57 with the amino acids at that position in the *HLA-DQB1*05* alleles was done in the NetMHCIIpan 4.0 server using the amino acid sequence of *HLA-DQB1*05* (UniProt accession: Q9GIK3) and the binding of each of the 157 predicted ligands using default settings.[36] Differences in the median binding score were calculated using a Wilcoxon signed rank test. We computed the peptide motif of the 157 peptide ligands using the artificial neural network model of NNAlign-2.0, which used the amino acid sequences and binding scores for each peptide for *HLA-DQB*05:03* as input (trained with a motif length of 9 and default settings for "MHC CLASS II ligands") to return a peptide core sequence alignment.[37] We also did this for a subset of the 157 peptides with a relatively stronger binding score for *HLA-DQB*05:03* (D57) compared to *DQB1:05:02* (V57), by computing the ratio of the binding scores (the log-transformed IC50 affinity scores from NetMHCIIpan 4.0) for all 157 peptides for those two alleles and extracting the top 50 peptides with the largest ratio.

Results

In total 280 cases with uveitis were genotyped of which 160 (57%) patients were diagnosed with anterior uveitis, 39 (14%) patients with intermediate uveitis, and 81 (29%) patients with panuveitis (Table 1). Summary of the HLA genotypes at four-digit resolution revealed 110 unique common alleles (≥1% detected in controls) and 69 rare alleles (<1% in controls) of which included 32 *HLA-A* alleles, 51 *HLA-B* alleles, 22 *HLA-C* alleles, 23 *HLA-DQB1* alleles, 13 *HLA-DPB1* alleles, and 38 *HLA-DRB1* alleles (Supplementary Table 3). Uniform manifold approximation and projection was used to visualize the ethnicity of the cases and controls (Supplementary Table 2). As expected, cases and controls were predominantly mapped to the European population (Fig. 1).

Table 1. Diagnosis of the 280 Uveitis Cases

Diagnosis	Number of Cases
Pediatric noninfectious uveitis	280
Anterior uveitis, No. (%)	160 (57)
JIA associated uveitis, No. (%)	92 (33)
Idiopathic chronic anterior uveitis, No. (%)	50 (18)
Acute anterior uveitis, No. (%)*	18 (6)
Non anterior uveitis, No. (%)	120 (43)
Intermediate uveitis, No. (%)†	39 (14)
Panuveitis, No. (%)‡	81 (29)

* *HLA B27* positive associated uveitis ($n = 7$), sarcoidosis ($n = 1$), and TINU syndrome ($n = 2$).
† Tubulointerstitial nephritis and uveitis syndrome ($n = 1$).
‡ Tubulointerstitial nephritis and uveitis syndrome ($n = 15$).

HLA-DRB1 and *HLA-DQB1* Are Risk Alleles for Pediatric Uveitis

Next, we tested for HLA associations considering all uveitis cases ($n = 280$). We identified seven associated HLA alleles and mapped the primary association to *HLA-DQB1*04:02* (odds ratio [OR] = 5.27, 95% confidence interval [95% CI] 3.04–9.12; $P = 2.98 \times 10^{-9}$), closely followed by *HLA-DRB1*08:01* (OR = 5.21 95% CI; 2.94–9.23; $P = 1.53 \times 10^{-8}$; Fig. 2A). The results from the association testing of the classical HLA alleles in all cases and controls are presented in Supplementary Table 3. All of the associated alleles were common (allele frequency in controls > 1%). Since the haplotype *DRB1*08:01-DQB1*04:02* has been previously associated with polyarticular rheumatoid factor-negative JIA,[38,39] we extracted the data from the 95 JIA cases and conducted association testing. For JIA-U versus controls, we ascertained the strong association to known JIA risk alleles *HLA-DRB1*08:01* (OR = 11.12, 95% CI 5.76–21.43; $P = 6.51 \times 10^{-13}$) and *HLA-DQB1*04:02* (OR = 10.11, 95% CI 5.32–19.22; $P = 1.69 \times 10^{-12}$; Fig. 2B). Next, we investigated all independent HLA associations by using conditional analysis. To this end we adjusted for the strongest HLA association by adding the allele as a covariate to the regression model. Adjusting for *HLA-DRB1*08:01* mitigated the association for *HLA-DQB1*04:02* due to strong linkage disequilibrium (i.e., correlation [LD]) between these alleles ($D'=0.97$, $r^2=0.86$ in our data, Table 2). However, adjusting for *HLA-DRB1*08:01* revealed an independent association for *HLA-DRB1*11:04* (Table 2). Finally, after adjusting for all three *HLA* alleles (*DRB1*08:01-DQB1*04:02-HLA-DRB1*11:04*), no residual HLA-wide association signal was observed ($P > 2.79 \times 10^{-4}$).

Association testing in the 185 uveitis cases without JIA revealed a primary association for *HLA-DRB1*01:02* (OR = 5.58, 95% CI 2.70–11.55; $P = 3.60 \times 10^{-6}$; Fig. 2C). Adjusting for *HLA-DRB1*01:02* revealed an independent association for *HLA-DRB1*04:03 (*OR = 11.67, 95% CI 3.82–35.66; $P = 1.62 \times 10^{-5}$). Adjusting for both *DRB1*01:02* and *DRB1*04:03* revealed an additional independent association for *HLA-DQB1*04:02* (OR = 3.82, 95% CI 2.01–7.25; $P = 4.21 \times 10^{-5}$). Next, adjusting for *DRB1*01:02, DRB1*04:03,* and *DQB1*04:02,* revealed an independent association for *HLA-DQB1*05:03* (OR = 3.31, 95% CI 1.87–5.83; $P = 3.65 \times 10^{-4}$). And finally, adjusting for *DRB1*01:02, DRB1*04:03, DQB1*04:02,* and *DQB1*05:03,* identified an independent association for *HLA-DRB1*14:01* (OR = 0.01, 95% CI 0.002–0.12; $P = 8.96 \times 10^{-5}$) (Table 2).After adjusting for *DRB1*01:02, DRB1*04:03, HLA-DQB1*04:02, DQB1*05:03,* and *DRB1*14:01,* no additional HLA-wide associations were detected ($P > 2.79 \times 10^{-4}$).

The JIA Risk *HLA-DRB1*08:01-HLA-DQB1*04:02* Haplotype Is Associated with Idiopathic Chronic Anterior Uveitis

Next, we stratified patients according to the anatomical location of uveitis. The frequency of the classical HLA alleles in all cases according to the anatomical location of their inflammation and controls are presented in Supplementary Table 4.

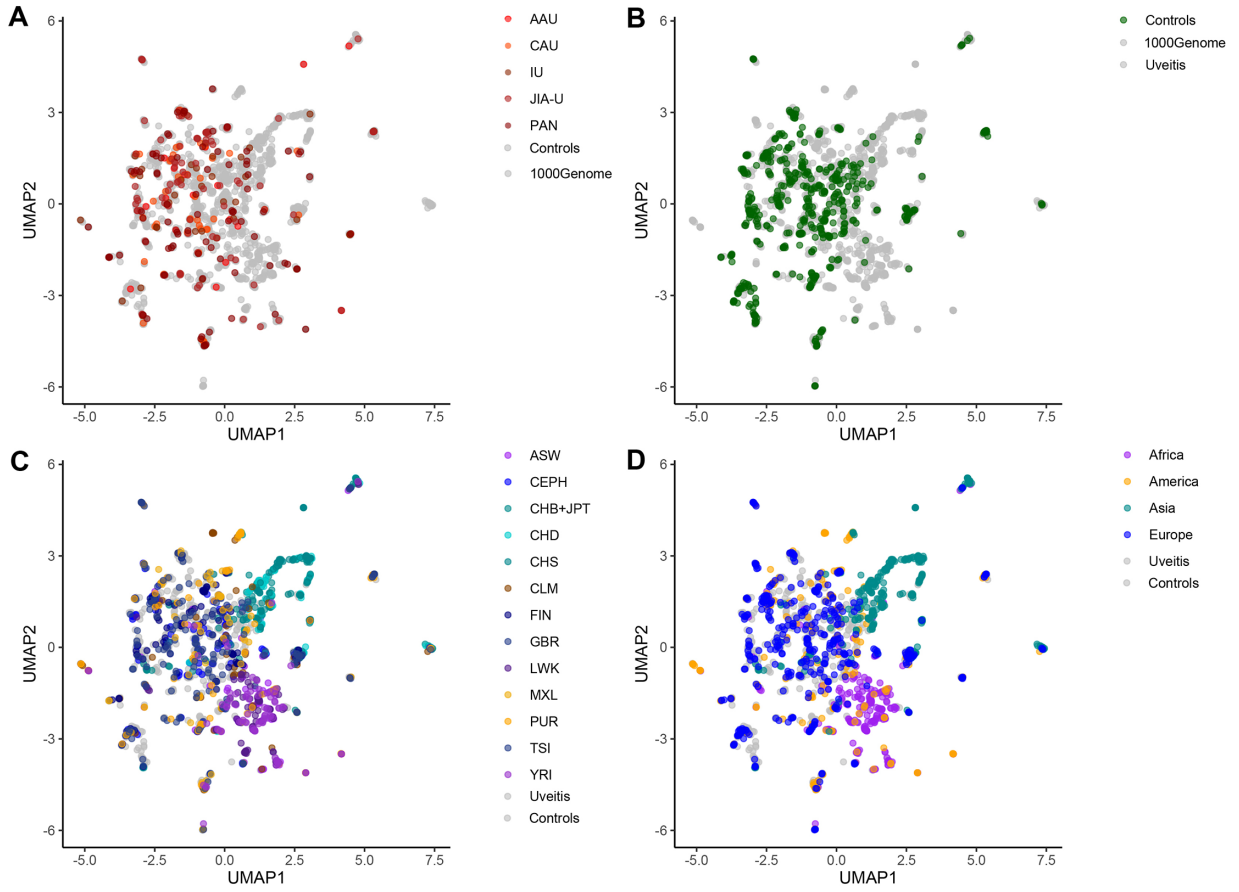

Figure 1. Uniform manifold approximation and projection of the HLA genotypes data. Ancestry populations from the 1000 genome dataset[32,33] (Supplementary Table 2) and Dutch subjects from the Genome of the Netherlands was used as reference data to analyze ethnicity. The UMAP projection mapped cases and controls predominantly to the European population. **A)** Visualization of data from uveitis samples (n = 280) used in this study. The different colors represent the five different forms of pediatric uveitis. **B)** Visualization of data from the control samples (n = 499) from the Genome of the Netherlands. **C)** Visualization of data from the 1000 genome per population code. **D)** Visualization of data from the 1000 genome per super population. AAU, acute anterior uveitis; CAU, idiopathic chronic associated uveitis; JIA-U, juvenile idiopathic arthritis associated uveitis; IU, intermediate uveitis; PAN, panuveitis; African (LWK, Luhya in Kenya; YRI, Yoruba in Nigeria; ASW Americans of African Ancestry in Southwest US); European (CEPH, Utah residents with Northern/Western European ancestry; FIN, Finnish in Finland; GBR, British in England and Scotland; TSI, Tuscans in Italy); East Asian (CHB, Han Chinese in Beijing; CHS, Southern Han Chinese; JPT, Japanese in Tokyo, and CHD, Chinese in Metropolitan Denver, CO, USA); and Mixed American (CLM, Colombian in Medellin, Colombia; MXL, Mexican in Los Angeles, California, USA, and PUR, Puerto Rican).

Idiopathic chronic anterior uveitis (CAU) is phenotypically similar to JIA-U, albeit without clinical evidence of arthritis. Remarkably, when testing for 50 CAU cases versus controls, we observed an HLA-wide significant association for the JIA risk loci *HLA-DQB1*04:02* (OR = 5.37, 95% CI 2.38–12.09; $P = 5.07 \times 10^{-5}$) and *HLA-DRB1*08:01* (OR = 5.30, 95% CI 2.26–12.44; $P = 1.30 \times 10^{-4}$; Table 3, Fig. 3A, and Supplementary Table 4). As expected, after adjusting for *HLA-DQB1*04:02,* the association signal for *HLA-DRB1*08:01* in CAU was lost, but in contrast to JIA-U revealed independent association for *HLA-DQB1*05:03* (OR = 5.86, 95% CI 2.52–13.62; $P = 3.92 \times 10^{-5}$; Fig. 3B). Further adjusting for *HLA-DQB1*05:03* removed the remainder of the signal (Fig. 3C). Note that although we considered the number of cases with acute anterior uveitis too low (n = 18) to be meaningful for disease-specific testing, the allele frequency of *HLA-DQB1*04:02* or *HLA-DRB1*08:01* was nearly identical to CAU, thus also higher compared to controls (Supplementary Table 4). In summary, these data show that the *HLA-DRB1*08:01-HLA-DQB1*04:02* haplotype is associated with pediatric anterior uveitis.

*HLA-DRB1*01:02, HLA-DRB1*04:03,* and *HLA-DQB1*05:03* Are Risk Alleles for Pediatric Panuveitis

We did not observe an HLA-wide significant association for intermediate uveitis—most likely due to the limited number of cases with this subtype in this study (n = 39)—but detected *HLA-DRB1*15:01* (OR = 2.18, 95% CI 1.26–3.77; P = 0.005) as the lead allele, which is in line with previous observations[11,12] (Table 3 and Supplementary Table 4). We detected *HLA-DRB1*01:02* (OR = 10.18, 95% CI 4.55–22.76; $P = 1.58 \times 10^{-8}$) as the primary association in patients with panuveitis (Table 3, Fig. 3D,

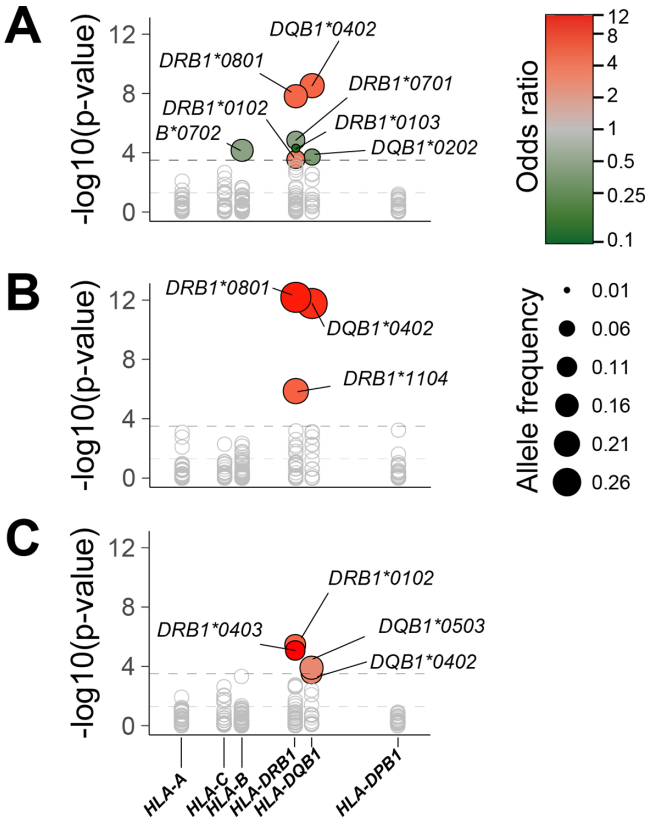

Figure 2. Association testing for human leukocyte alleles in all uveitis cases ($n = 280$), JIA-uveitis ($n = 92$), and uveitis without JIA-uveitis ($n = 185$) versus controls ($n = 499$). **A**) Initial association testing for all uveitis cases revealed a primary association for *HLA-DQB1*04:02* (AF = 0.09, OR = 5.27, 95% CI 3.04–9.12; $P = 2.98 \times 10^{-9}$), closely followed by *HLA-DRB1*08:01* (AF = 0.08, OR = 5.21 95% CI; 2.94–9.23; $P = 1.53 \times 10^{-8}$). **B**) Initial association testing for juvenile idiopathic associated uveitis cases ($n = 92$) showed a primary association for *HLA-DRB1*08:01* (AF = 0.16, OR = 11.12, 95% CI 5.76–21.43; $P = 6.51 \times 10^{-13}$) and *HLA-DQB1*04:02* (AF = 0.16, OR = 10.11, 95% CI 5.32–19.22; $P = 1.69 \times 10^{-12}$). **C**) Initial association testing for uveitis cases without juvenile idiopathic associated uveitis ($n = 185$) revealed a primary association for *HLA-DRB1*01:02* (AF = 0.06. OR = 5.58, 95% CI 2.70–11.55; $P = 3.60 \times 10^{-6}$). All indicated HLA alleles are associated at HLA-wide significance ($P < 2.79 \times 10^{-4}$, *dashed dark gray line*). The size and color of the dots represent the allele frequency and effect size (odds ratio), respectively. The *gray dots* represent the HLA alleles that did not reach significance. The *light gray dotted line* indicates nominal significance ($P = 0.05$). AF = allele frequency; OR = odds ratio; CI = confidence interval.

and Supplementary Table 4). We then adjusted for *HLA-DRB1*01:02,* which revealed independent association for the rare allele *HLA-DRB1*04:03* (OR = 29.61, 95% CI 7.96–110.22; $P = 4.35 \times 10^{-7}$; Fig. 3E). Adjusting for both *DRB1*01:02* and *DRB1*04:03*, we discovered an additional independent association for *HLA-DQB1*05:03* (OR = 4.51, 95% CI: 2.27–8.96; $P = 1.72 \times 10^{-5}$; Fig. 3F). Finally, conditioning on all three risk *HLA* alleles removed the remainder of the signal ($P > 2.79 \times 10^{-4}$; Fig. 3G). The haplotype *HLA-DRB1*01:02* and *HLA-DQB1*05* have been previously reported in patients suspected of TINU syndrome.[19–22] However, removing these patients ($n = 15$) from the analysis modestly affected the signal (*HLA-DRB1*01:02*, $P = 1.61 \times 10^{-6}$, and *HLA-DQB1*05:03*, $P = 8.59 \times 10^{-4}$) and indicates that the association signal at *DRB1*01:02* and *DQB1*05:03* represents a signal beyond the association with TINU.

Sex-Specific Differences in Associations with HLA Alleles

Because we previously detected sexual dimorphism in uveitis associated with JIA,[14] we were interested to test whether males and females showed differential association with HLA alleles (Supplementary Table 5). The distribution of male and females across each group is shown in Table 4. Several notable observations were made; Considering 27 males with JIA-U versus 250 male controls, we detected association for a novel risk allele *HLA-DRB1*11:01* (OR = 6.25, 95% CI 2.49–15.72; $P = 9.71 \times 10^{-5}$), which was not associated with females ($P = 0.32$). In analysis of 65 females with JIA-U, we observed a significant association for *HLA-DRB1*11:04* (OR = 10.16, 95% CI 3.88–26.58; $P = 2.32 \times 10^{-6}$), which was not associated with males ($P = 0.21$).

Testing female patients ($n = 33$) with CAU versus 249 female controls revealed *HLA-DQB1*05:03* (OR= 13, 95% CI 4.17–40.49; $P = 9.75 \times 10^{-6}$) as the primary association over *HLA-DQB1*04:02* ($P = 4.95 \times 10^{-4}$). However, our cohort of CAU cases may not have sufficient power to accurately assign one risk allele over another. Therefore, we ran the association test at *HLA-DQB1*05:03* for CAU and included sex as an interaction term in the model. With this test you can examine if sex influences the *HLA* association in general. This analysis showed a moderate, but significant effect for the interaction term ($P_{interaction} = 0.03$), indicating that the *HLA-DQB1*05:03* signal was indeed sex specific. We also ran interaction tests for the two JIA-U sex-specific alleles *HLA-DRB1*11:01* and *HLA-DRB1*11:04*, which revealed similar interactions with sex ($P_{interaction} = 0.0006$ and $P_{interaction} = 0.06$, respectively). These data show sex-specific differences in the association with risk HLA alleles.

HLA Amino Acid Associations With Uveitis

Next, we analyzed the association of HLA amino acid polymorphisms with uveitis to assess the contribution of specific amino acid positions (Supplementary Table 1A-F and Supplementary Table 6). Univariate analysis identified 12 amino acids at 12 positions for JIA-U and CAU, eight amino acids at eight positions for IU, and two amino acids at two positions for panuveitis (Supplementary Table 7A and 7B). However, none of the amino acids showed a stronger association compared to the classical alleles. (Supplementary Table 7A). In other words, no polymorphic amino acid position could explain the data better than the classical HLA alleles.

Aspartic Acid (D) at Position 57 Increases the Peptide Binding Affinity to HLA-DQ

We aimed to explore the functional implications of risk HLA alleles in pediatric uveitis. The amino acid position 86 distinguishes the JIA-U associated alleles *HLA-DRB1*11:01* (86-Glycine) in males, *HLA-DRB1*11:04* (86-Valine) in females (Supplementary Table 1E). This position has been shown to control DR alpha/beta dimer stability, but also peptide binding for the panuveitis-associated *HLA-DRB1*04:03*, and is therefore likely to influence antigen presentation.[40,41] There-

Table 2. HLA Associations Before and After Adjusting for the Primary Association in Juvenile Idiopathic Arthritis Associated Uveitis and Nonjuvenile Idiopathic Arthritis Associated Uveitis Versus Controls ($n = 499$)

	JIA Uveitis ($n = 92$)											
				Adjusting for HLA Alleles								
	Univariable Analysis			Step 1: *DRB1*08:01* Enters				Step 2: *DRB1*11:04* Enters				
***HLA* Allele**	**OR**	**(95% CI)**	***P***	**OR**	**(95% CI)**	***P***	**r^2 with DRB1*08:01**	**OR**	**(95% CI)**	***P***		
*HLA-DRB1*08:01*	11.12	(5.76–21.43)	6.51×10^{-13}	Conditioned				Conditioned				
*HLA-DQB1*04:02*	10.11	(5.32–19.22)	1.68×10^{-12}	1.51	(0.09–25.16)	0.78	0.86	1.46	(0.08–25.78)	0.79		
*HLA-DRB1*11:04*	5.63	(2.80–11.36)	1.37×10^{-6}	4.65	(2.23–9.72)	4.39×10^{-5}	0.01	Conditioned				

	Non-JIA Uveitis (n=185)																	
				Adjusting for HLA Alleles														
	Univariable Analysis			Step 1: *DRB1*01:02* Enters				Step 2: *DRB1*04:03* Enters			Step 3: *DQB1*04:02* Enters			Step 4: *DQB1*05:03* Enters				
***HLA* Allele**	**OR**	**(95% CI)**	***P***	**OR**	**(95% CI)**	***P***	**r^2 with *DRB1*01:02***	**OR**	**(95% CI)**	***P***	**OR**	**(95% CI)**	***P***	**OR**	**(95% CI)**	***P***		
*HLA-DRB1*01:02*	5.58	(2.70–11.55)	3.60×10^{-6}	Conditioned				Conditioned			Conditioned			Conditioned				
*HLA-DRB1*04:03*	11.98	(4.01–35.76)	8.48×10^{-6}	11.67	(3.82–35.66)	1.62×10^{-5}	0.009	Conditioned			Conditioned			Conditioned				
*HLA-DQB1*05:03*	2.94	(1.69–5.09)	1.23×10^{-4}	3.01	(1.72–5.25)	1.06×10^{-4}	0.00	3.19	(1.82–5.60)	5.09×10^{-5}	3.31	(1.87–5.83)	3.65×10^{-4}	Conditioned				
*HLA-DQB1*04:02*	3.20	(1.71–6.02)	2.96×10^{-4}	3.45	(1.83–6.52)	1.37×10^{-4}	0.001	3.82	(2.01–7.25)	4.21×10^{-5}	Conditioned			Conditioned				
*HLA-DRB1*14:01*	0.58	(0.21–1.57)	0.28	0.65	(0.24–1.77)	0.40	0.001	0.71	(0.26–1.94)	0.51	0.80	(0.29–2.19)	0.66	0.01	(0.002–0.12)	8.96×10^{-5}		

Table 3. Primary HLA Associations in Juvenile Idiopathic Arthritis Associated Uveitis, Idiopathic Chronic Anterior Uveitis (No JIA), Intermediate Uveitis, and Panuveitis Versus Controls ($n = 499$)

JIA Uveitis ($n = 92$)			
	Univariable Analysis		
HLA Allele	**OR**	**(95% CI)**	***P***
*HLA-DRB1*08:01*	11.12	(5.76–21.43)	6.51×10^{-13}
*HLA-DQB1*04:02*	10.11	(5.32–19.22)	1.69×10^{-12}
*HLA-DRB1*11:04*	5.63	(2.80–11.36)	1.37×10^{-06}
Idiopathic chronic anterior uveitis (no JIA, $n = 50$)			
	Univariable Analysis		
HLA Allele	**OR**	**(95% CI)**	***P***
*HLA-DQB1*04:02*	5.37	(2.38–12.09)	5.07×10^{-05}
*HLA-DQB1*05:03*	5.10	(2.25–11.58)	9.73×10^{-05}
*HLA-DRB1*08:01*	5.30	(2.26–12.44)	1.30×10^{-04}
Intermediate uveitis ($n = 39$)			
	Univariable Analysis		
HLA Allele	**OR**	**(95% CI)**	***P***
*HLA-DRB1*15:01*	2.18	(1.26–3.77)	0.005
Panuveitis ($n = 81$)			
	Univariable Analysis		
HLA Allele	**OR**	**(95% CI)**	***P***
*HLA-DRB1*01:02*	10.18	(4.55–22.76)	1.58×10^{-08}
*HLA-DRB1*04:03*	29.30	(8.39–102.41)	1.22×10^{-07}
*HLA-DQB1*05:03*	3.71	(1.94–7.14)	7.97×10^{-05}

fore, we asked whether additional allele-defining positions affect the binding of peptides to HLA allotypes encoded by risk HLA alleles. The *HLA-DQB1*05:03* was found to be associated with two distinct forms of uveitis, CAU and PAN, and this allele is functionally understudied compared to other uveitis risk alleles detected in this study. This allele harbors an aspartic acid (D) at position 57 while other nonrisk alleles for *HLA-DQB1*05* have a serine (S) or valine (V) at position 57 (Fig. 4A). Curiously, the amino acid position 57 of the DQ betachain is located at the edge of the peptide binding groove (Fig. 4A) and confers risk to several autoimmune conditions suggesting it to be involved in peptide binding of HLA-DQ.[42–46] To assess whether the presence of aspartic acid at position 57 functionally affects binding of peptide cargo to HLA-DQ, we extracted ligands ($n = 157$ from the HLA ligand atlas) from immunopeptidomes of *HLA-DQB1*05:01* (57-S), *HLA-DQB1*05:02* (57-V), and *HLA-DQB1*05:03* (57-D) that only differ in the amino acid residue at position 57 (Supplementary Table 8).[35] Next, we modeled the binding affinity of the naturally occurring ligands to HLA-DQ, by using the full amino acid sequence of *HLA-DQB1*05* in NetMHCIIpan 4.0 and performed amino acid substitutions at position 57.[36] We observed that substitution of position 57 from S or V (nonrisk alleles) to the uveitis-associated amino acid aspartic acid (D) (equivalent to *DQB1*05:03*) resulted in a significant increased binding affinity score (S versus D; $P = 1.67 \times 10^{-27}$ and V versus D; $P = 1.64 \times 10^{-27}$; Fig. 4B). In more detail, major binding pockets for HLA-DQ are position P4, P6, and P9 in the peptide motif.[47] Therefore, we computed the peptide motif of the 157 *HLA-DQB1:05* ligands and the top 50 peptides with the largest difference in binding scores between HLA-*DQB1*05:02* (57-V) and *HLA-DQB1*05:03* (57-D) and compared the core sequence alignments to identify changes in specific amino acid position. This analysis revealed, as expected, a marked change in amino acid residue preference in particular at anchor position P9, which directly interacts with position 57 (Fig. 4C). Collectively, these data show that position 57 in the HLA-DQ beta chain modulates the peptide binding capacity and indicates risk HLA alleles associated with pediatric uveitis show altered antigen presentation capacity.

Table 4. The Distribution of Males and Females in the Investigated Groups

Diagnosis	Total Number of Cases	Number of Males	Number of Females
JIA associated uveitis, No. (%)	92 (33)	27 (29)	65 (71)
Idiopathic chronic anterior uveitis, No. (%)	50 (18)	17 (34)	33 (66)
Panuveitis, No. (%)	81 (29)	43 (53)	38 (47)

Discussion

The role of the human MHC locus in determination of the susceptibility to uveitis is evident from the routine screening for HLA alleles (more specifically *HLA-B27* and *HLA-A29*) to support the diagnosis of specific entities of noninfectious uveitis.[48] One of the outcomes of this study is that we detected a predominant contribution of common alleles to disease risk that allowed accurate comparison with available data from population controls,[28] but also detected the less common or *rare* allele *HLA-DRB1*04:03* (allele frequency of 0.7% in controls) as a novel risk allele for panuveitis. This supports that common and rare alleles define the susceptibility to uveitis in children.

Previous genome-wide association studies have consistently identified HLA class I and class II genes as the primary genetic risk factors for noninfectious uveitis.[10–15] However, these studies were based primarily on HLA imputation methods that despite their overall high accuracy to identify common alleles may be less adequate for the identification of rare or poorly characterized alleles. We therefore used sequencing data to comprehensively determine the full sequence at high resolution of class I and class II HLA alleles for a large cohort of patients with pediatric uveitis. Note imputed HLA alleles from population controls were nearly identical to the allele frequencies in Europe (Supplementary Table 9).[49]

Our study revealed that *HLA-DQB1*04:02* and *HLA-DRB1*08:01* (in strong LD) are risk alleles for both JIA-U and CAU. Idiopathic chronic anterior uveitis is clinically similar to JIA-U uveitis, but these patients do not present with any evidence for inflammatory arthritis.[50] A possible explanation for the shared genetic alleles could be that in the minority of JIA-U cases, uveitis may first manifest before the onset of arthritis (classified as CAU). A fraction of CAU cases may later develop arthritis and will be eventually classified as JIA-U.[50,51] However, in some of them, arthritis development might be suppressed by immunomodulating treatment for uveitis. In contrast to a previous GWAS in JIA-U, here we did

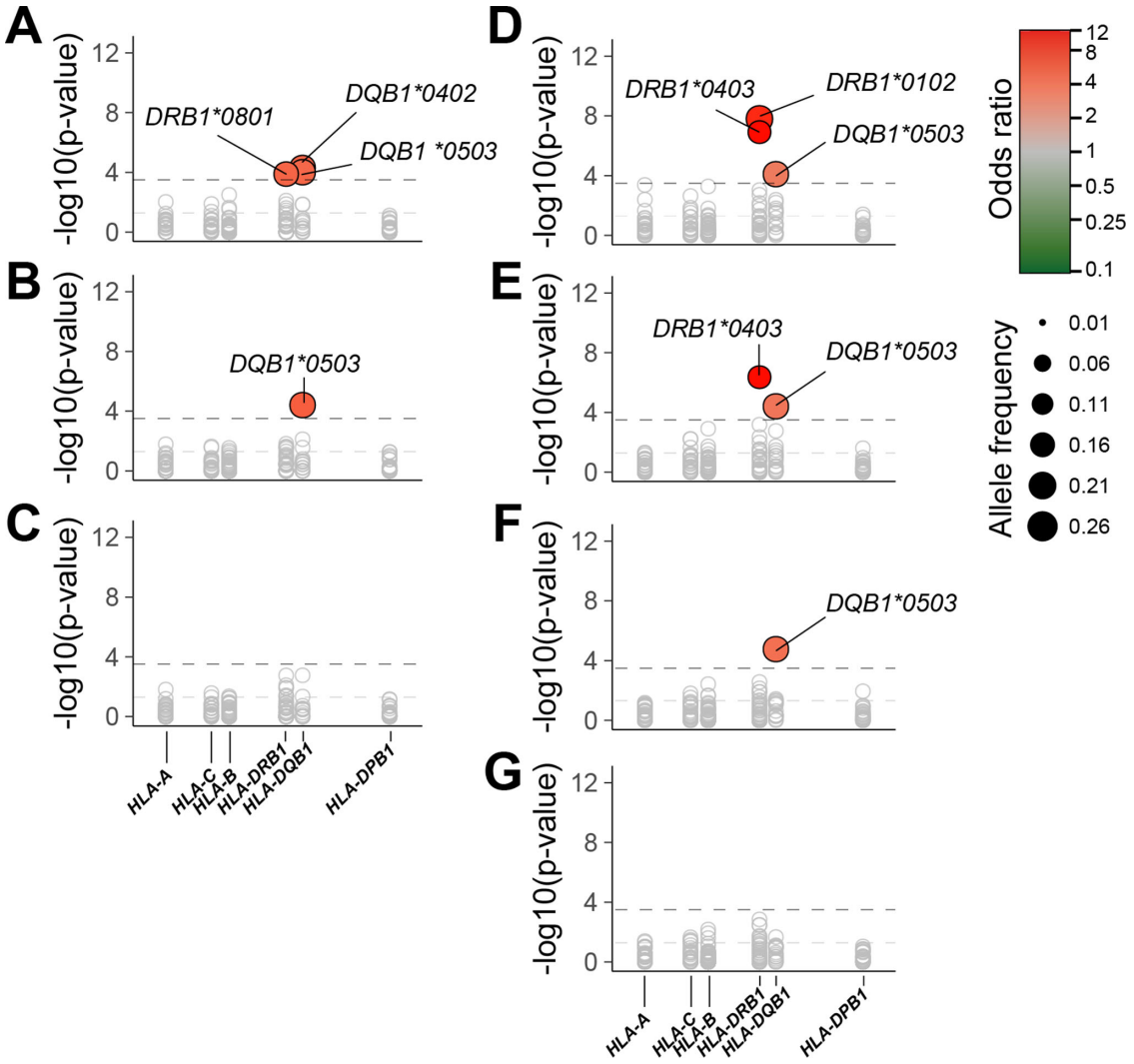

Figure 3. Independent HLA allele associations (i.e., conditional testing) in cases with idiopathic chronic anterior uveitis and panuveitis. **A**) Initial association testing in 50 chronic anterior uveitis cases detected the primary association for *HLA-DQB1*04:02* (AF = 0.10, OR = 5.37, 95% CI 2.38–12.09; $P = 5.07 \times 10^{-5}$). **B**) Adjusting for lead HLA allele (*HLA-DQB1*04:02)* revealed independent association for *HLA-DQB1*05:03* (AF = 0.10, OR 5.86, 95% CI 2.52–13.62; $P = 3.92 \times 10^{-5}$). **C**) After adjusting for *HLA-DQB1*04:02-HLA-DQB1*05:03* no additional independent HLA associations were detected. **D**) Initial association testing in 81 panuveitis cases mapped the primary association to *HLA-DRB1*01:02* (AF = 0.11, OR = 10.18, 95% CI 4.55–22.76; $P = 1.58 \times 10^{-8}$). **E**) After adjusting for *HLA-DRB1*01:02* an independent association for *HLA-DRB1*04:03* (AF = 0.07, OR = 29.61, 95% CI 7.96–110.22; $P = 4.35 \times 10^{-7}$) was detected. **F**) Adjusting for *HLA-DRB1*01:02* and *HLA-DRB1*04:03* discovered independent association for HLA-DQB1*05:03 (AF = 0.10, OR = 4.51, 95% CI 2.27–8.96; $P = 1.72 \times 10^{-5}$). **G**) Adjusting for *HLA-DRB1*01:02, HLA-DRB1*04:03, and HLA-DQB1*05:03* revealed no additional independent HLA associations. The size and color of the dots represent the allele frequency and effect size (odds ratio), respectively. The *gray dots* represent the *HLA* alleles that did not reach significance. HLA-wide significance threshold ($P = 2.79 \times 10^{-4}$) is indicated as a *dashed dark gray line* in the association plots). AF = allele frequency; OR = odds ratio; CI = confidence interval.

not include JIA patients without uveitis and therefore could not formally test whether the identified associations were related to uveitis or JIA. However, several lines of evidence link *DQB1*04:02-DRB1*08:1* to pediatric anterior uveitis. First, we showed that these alleles are genetically linked to CAU. Second, the allele frequency of these alleles in a previous GWAS was higher in patients with JIA-U compared to JIA patients without uveitis (0.19 vs. 0.11, respectively).[14] Finally, we showed that the allele frequency of *HLA-DQB1*04:02* and *HLA-DRB1*08:01* was also increased in patients with acute anterior uveitis, in line with previous observations.[52] These observations suggests that despite the association of these alleles with JIA in general these alleles may contribute to a common molecular mechanism that promotes anterior uveitis, perhaps also in JIA-uveitis.[38,39] Future studies that use additional genotype data to fine-map the MHC will provide sufficient resolution (extended haplotypes including noncoding polymorphisms) to perform bivariate analysis to determine the genetic correlation between these different types of anterior uveitis.

In addition, such studies should also aim to expand the cohort to represent non-European populations. A limitation of the current study is that cases and controls of the study were near exclusively from Western European ancestry as visualized by UMAP. Note that because UMAP dedicates relatively more visual space on populations with larger sample

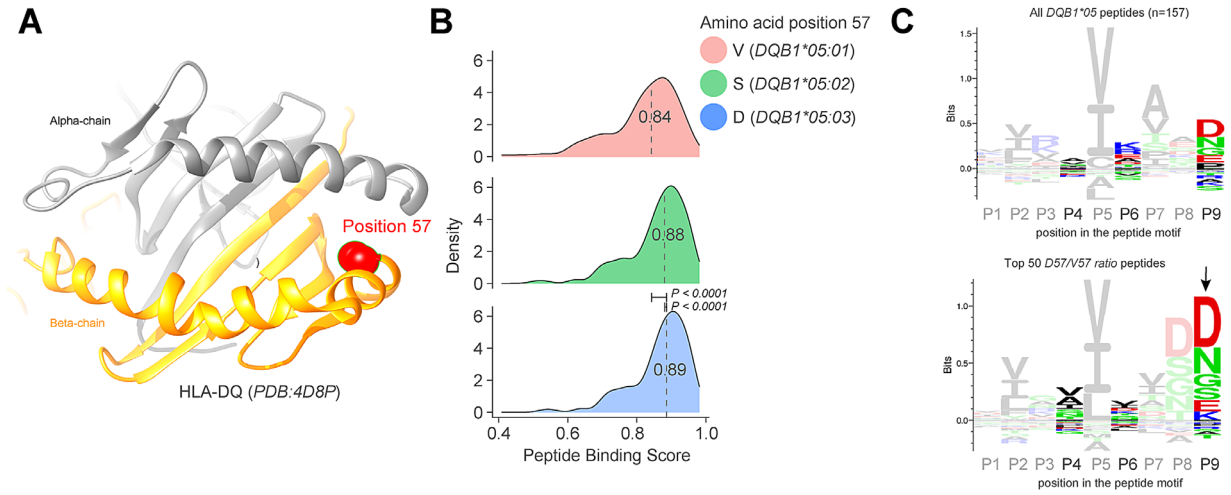

Figure 4. The effect of amino acid substitutions for position 57 on predicted binding affinity for natural ligands of *HLA-DQ*05* alleles. **A**) View into the peptide-binding groove of a three-dimensional ribbon model for *HLA-DQB1* (based on Protein Data Bank entry 4D8P).[46] Position 57 that distinguishes *DQB1*05* alleles is highlighted in *red*. **B**) The effect of amino acid substitution for position 57 on predicted binding affinity for naturally presented ligands eluted from *HLA-DQB1*05:01*, *HLA-DQB1*05:02*, and *HLA-DQB1*05:03* ($n = 157$, see Supplementary Table 8). Binding affinity scores were predicted using the full amino acid sequence of *HLA-DQB1*05* in NetMHCIIpan 4.0.[36] **C**) A sequence logo representation of the peptide motif of the 157 *HLA-DQB1:05* peptides from the HLA atlas as computed by the NNAlign 2.0 server.[37] The relative height of color-coded amino acid (letter code) at each position represent the information content in bits. Positive values show favored amino acids at that position and negative bits indicate amino acids that are not preferred at that position. The anchor position P4, P6, and P9 of HLA-DQ are highlighted.

size, this may exaggerate the *variation* within cases and controls in the projection. Regardless, it remains to be determined if the here identified risk loci for anterior uveitis are shared with other patient populations. For example, in East Asian populations the prevalence of uveitis in the JIA populations may be different compared to Europe.[53]

We identified three new independent HLA-wide associations *HLA-DRB1*01:02, HLA-DRB1*04:03,* and *HLA-DQB1*05:03* as being strongly associated with panuveitis. *HLA-DRB1*01:02* and *HLA-DQB1*05* associations have been previously reported in patients suspected for TINU syndrome by Levinson and co-workers.[20] A follow-up study from the same group, however, showed that the *HLA-DRB1*0102* and *HLA-DRB1*08:01* alleles were associated with patients with isolated acute anterior uveitis, but not in those with isolated biopsy-proven TIN without uveitis.[21] Perasaari et al. identified *HLA-DRB1*08:01* and *HLA-DRB1*14:01* as risk alleles rather than *HLA-DRB1*01:02* in patients with biopsy-proven TINU.[19] Our data also showed that in patients with acute anterior uveitis the allele frequency of *HLA-DRB1*08:01* was higher compared to controls (Supplementary Table 4). Of interest, work by Reddy et al. demonstrated that 14 out of 15 children with unexplained panuveitis also carried the *HLA-DRB1*01-HLA-DQB1*05* haplotype.[22] In our study, 15 of 81 patients with panuveitis were suspected for TINU syndrome. However, removing these patients ($n = 15$) from the analysis modestly affected the signal and indicates that the association signal at *DRB1*01:02* and *DQB1*05:03* might represent a signal beyond the association with TINU. In other words, we speculate that the alleles *HLA-DRB1*01* and *HLA-DQB1*05* might convey risk for panuveitis and *HLA-DRB1*08:01* may convey risk for anterior uveitis. Though, it should be noted that TINU is often asynchronous and therefore TINU might be underestimated. We further note that the diagnosis of some subtypes of uveitis may be prone to unintentional heterogeneity due to the intrinsic dependency to classify pediatric uveitis based on clinical expert opinion. Regardless, the diagnosis of uveitis according to the SUN criteria (as done in this study) may allow comparison between studies. These classification criteria appeared to perform well enough for use in clinical and translational research.[54] We detected *HLA-DRB1*15:01* as the lead allele in cases with IU, which is in line with previous observations.[11,12] Remarkably, this allele is also the primary association in multiple sclerosis, an inflammatory demyelinating disease of the central nervous system and is often accompanied by IU.[55] Furthermore, IU patients also show demyelinating lesions by MRI, supporting the close relation of IU and MS.[56] In our study, two cases that carried the *HLA-DRB1*15:01* allele showed demyelinating lesions by MRI.

We previously showed that uveitis in JIA is genetically linked to other amino acid positions by comparing to JIA without uveitis rather than an individual HLA allele.[14] In the current study, we demonstrated that the disease risk is detected best by the classical HLA alleles instead of specific amino acid positions, which are often shared by multiple HLA alleles. Alternatively, moderate-to-strong LD between HLA alleles and nearby non-HLA loci may suggest that risk HLA alleles serve as a *proxy* for variants in non-HLA genes implicated in pediatric uveitis. For example, *HLA-DQB1:04* is in LD with the TAP1 gene involved in antigen presentation.[57]

Further analyses revealed that the association signals in JIA-U (*HLA-DQB1*04:02*), CAU (*HLA-DQB1*05:03*), and panuveitis (*HLA-DRB1*01:02)* showed evidence for sexual dimorphism. We identified male-specific associations for *HLA-DRB1*11:01* in JIA-U, which showed a large difference in frequency with females. Conversely, *HLA-DRB1*11:04* was only associated with females. Our data supported sex-

specific effects based on interaction of sex with the risk for these alleles. Sex-specific HLA allele associations have been reported in other chronic inflammatory conditions.[14,58–60] This is in line with functional data on sex differences in the adaptive immune responses and the degree to which HLA molecules interact with T cells (i.e., selection and expansion).[61–63]

Other functional implications of our data became evident by functional modeling of allele-defining amino acid positions. Although such studies have been done more extensively for HLA-DR.[40,41] Since we previously showed that amino acid positions 10 to 12 encoded by the HLA-DRB1gene are the primary genetic risk factors for uveitis in JIA,[14] we focused in our study on *HLA DQB1*05:03*—especially since it revealed association with multiple clinically distinct types of uveitis. We demonstrated similar direct effects on peptide presentation for HLA-DQ. We showed that the risk allele *HLA-DQB1*05:03* was found to be associated with two forms of uveitis CAU and PAN. This allele harbors an aspartic acid (D) at position 57 while the other *HLA-DQB1*05* alleles have a serine (S) or valine (V) at that position. We observed that amino acid substitution of position 57 from S or V (in the nonuveitis associated allele *HLA-DQB1*05:01* and *HLA-DQB1*05:02*) to the uveitis-associated amino acid aspartic acid (D) in *DQB1*05:03* increased the binding of peptides, underscoring the critical involvement of this position in antigen presentation by HLA-DQ. Alternatively, although not evaluated in this study, this position may also influence the flexibility and possibility to accommodate longer peptides protruding out of the groove.

In conclusion, we conducted a comprehensive analysis of HLA alleles implicated in uveitis susceptibility in children. We ascertained previous and identified novel independent HLA associations. We further demonstrated that the phenotypically similar CAU and JIA-U share HLA associated alleles. This suggests a common molecular basis for these types of uveitis and might help to understand the molecular mechanism driving pediatric uveitis.

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