HLA-DQB1*05 subtypes and not DRB1*10:01 mediates risk in anti-IgLON5 disease

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Abstract

Anti-IgLON5 disease is a rare and likely underdiagnosed subtype of autoimmune encephalitis. The disease displays a heterogeneous phenotype that includes sleep, movement, and bulbar-associated dysfunction. Presence of IgLON5-antibodies in CSF/serum, together with a strong association with *HLA-DRB1*10:01~DQB1*05:01*, support an autoimmune basis. In this study, a multicentric cohort of 87 anti-IgLON5 patients was studied for HLA association. Genome-wide association study (GWAS) with imputation-derived and 4- and 8-digit resolution validation HLA typing revealed a stronger association with HLA-DQ than HLA-DR. Specifically, we identified a predisposing rank-wise association with *HLA-DQA1*01:05~DQB1*05:01, HLA-DQA1*01:01~DQB1*05:01* and *HLA-DQA1*01:04~DQB1*05:03* in 85% of patients. This association was further reflected in an increasingly later age of onset across each genotype group, with a delay of up to 11 years, while HLA-DQ-dosage dependent effects were also suggested by reduced risk in the presence

of non-predisposing DQ1 alleles. The functional relevance of the observed HLA-DQ molecules was studied with competition binding assays. These proof-of-concept experiments revealed binding of IgLON5 in a post-translationally modified, but not native, state to all three risk-associated HLA-DQ receptors. Further, a deamidated peptide from the Ig2-domain of IgLON5 activated T cells in two patients, compared to one control carrying *HLA-DQA1*01:05~DQB1*05:01*. Taken together, these *in silico* and *in vitro* data support a HLA-DQ mediated T cell response to post-translationally modified IgLON5 as a potentially key step in the initiation of autoimmunity in this disease.

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Abbreviations: HLA = Human Leukocyte Antigen; GWAS = Genome-wide association study; PBMC = Peripheral Blood Mononuclear Cell; SNP = single nucleotide polymorphism

Introduction

Anti-IgLON5 disease is a recently described autoimmune syndrome with distinctive and polymorphic clinical features[1]. It affects both sexes equally and has a late age of onset, with a mean age at diagnosis above 60[2] years of age. Symptoms may include a complex form of sleep disorder, with REM/NREM parasomnia, sleep apnea and stridor, gait and other movement abnormalities, as well as bulbar and cognitive impairment. All patients are positive for serum, and in most cases, CSF autoantibodies targeting the extracellular domain of IgLON5[1,2]. Early on in the disease, CSF pleocytosis is observed consistent with CNS inflammation[2]. Also, a neurodegenerative biology is supported by neuropathological[4] and PET[5] studies, showing the accumulation of hyperphosphorylated tau in the brains of patients. HOwever, neuropathological studies indicate rare cases may present without a tauopathy[13,14].

A clinical diagnosis can be difficult, as late onset and symptomatology overlap with that of neurodegenerative disorders and the only very recent description of this condition suggests it remains very likely under-recognised. Importantly, however, early and aggressive immunotherapy can stabilize or even ameliorate symptoms, although treatment remains poorly effective in >50% of patients[3]. However, a better understanding of the underlying pathophysiology of this devastating disorder may pave the way for alterative and improved therapeutics.

IgLON5 belongs to a family of five highly homologous cell surface immunoglobulin-like cell adhesion molecules that are widely expressed in the central nervous system[6,7] and myoblasts[8], whereby murine IgLON5 was found to be expressed at higher levels in the brainstem, thalamus, ventral striatum and olfactory bulb[9], thus mirroring the tauopathy-affected distribution[4]. Studies of cultured rat hippocampal neurons[10] and human neural stem cells[11] have shown that IgGs from patient sera can lead to disrupted cytoskeletal organization and neurodegenerative changes, while *in vivo* evidence from mouse models further supports disruptions to synaptic homeostasis and irreversible neuronal damage[12].

Altogether, these studies suggest that autoimmunity operates upstream of neurodegeneration, triggering neuronal structural damage and tau accumulation later in the course of the disease. Identification of key, early autoimmune pathways involved in triggering the generation of cytotoxic tau and neurodegeneration therefore may provide both early therapeutic targets for

the successful treatment of this disorder and beyond that, provide insights to crucial autoimmune-neurodegenerative mechanisms that are relevant beyond this condition as well.

An autoimmune origin for the disease is further supported by a unique Human Leukocyte Antigen (HLA) Class II association[1,15], similar to those found in other autoimmune diseases such as celiac disease [*DQ2*][16], rheumatoid arthritis [*DR4*][17] and, in CNS conditions, narcolepsy [*DQ0602*][18], and other autoimmune encephalitis syndromes associated with LGI1 and CASPR2 antibodies[19]. HLA class II molecules play a key role in initiating and driving autoimmunity by presenting (auto)antigens to $CD4^+$ T cells, leading to subsequent T and B cell activation[20,21]. However, T cell contributions, and the precise HLA molecules involved in T cell activation, have received limited attention. Although the mechanisms underlying autoreactivity are poorly understood, molecular mimicry with foreign antigens is strongly suggested in some diseases, such as Epstein-Barr virus in multiple sclerosis[22,23] or influenza in narcolepsy[24,25]. Interestingly, autoantigens are often post-translationally modified (PTM), such as citrullinated in rheumatoid arthritis[26,27] or cryptically fused in type 1 diabetes[28,29], in line with reduced negative selection for PTMs in the thymus[30].

The objective of this study was to conduct GWAS and HLA-association analysis in a large sample of anti-IgLON5 disease patients, to examine candidate autoimmune HLA-peptide binding and probe $CD4^+$ T cell reactivity to candidate binders, thereby ultimately allowing us to disentangle triggers and key events in the pathophysiology of this disease.

Materials and methods

Participants

A total of 87 patients with anti-IgLON5 disease enrolled across Spain (51), Italy (2), France (18) [French samples were banked in NeuroBioTec Hospices Civils de Lyon BRC (France, AC-2013-1867, NFS96-900)], Germany (4), Switzerland (5), Brazil (1), Japan (1), USA (4) and the UK (1) were used. All patients were positive for IgLON5 autoantibodies in serum. Data of 44 of these patients have previously been published[1,15,31-37] (Supplementary Table 1). A subset of 75 patients had DNA available for GWAS; these were used for HLA analysis vs controls matched by ethnicity using PCA (case control analysis). An additional 12 subjects

had HLA typing and clinical data available; these were used in addition to the 75 to maximize power for analyses comparing symptoms across HLA type categories within anti-IgLON5 disease patients (phenotypic analysis). Clinical data was collected from clinical records or assessed as previously described[1,32].

HLA and Genotyping

DNA was available from 75 patients and genotyped using the PMRA Affymetrix Array (AKESOgen). All quality control and genotype operations were conducted using PLINK[38]. Genotypes with imputation probability lower than 0.3 were removed. Haplotypes were phased and merged using QCTOOL and imputed to 1,000 Genome Phase III. Patients were matched to controls by principal component analysis (PCA, Eucleadian distance-based measure[39]) at a 1:8 ratio from a set of 2,503 controls, giving rise to 232 matched controls (1:3.1, Figure 1C). HLA Genotype Imputation with Attribute Bagging (HIBAG)[40] was used to perform HLA imputation. Calls with a probability below 0.75 (*n=4*) were additionally HLA typed using next generation sequencing (NGS)[41-43]. Prior 4-digit resolution HLA typing data was available from 50 patients. To further validate imputation, an additional 14 randomly selected participants were typed using 8-digit resolution NGS HLA genotyping (Supplementary Table 1).

Statistical Analyses

For genetic analyses, categorical variables are presented as percentages (*%*) and total number of subjects (*n*) for any group reported in brackets. For allele carrier frequencies, a generalized logistic model (GLM) was fit to each allele, controlling by the first three principal components. The analysis was repeated after conditioning for specific haplotypes, as previously described[44]. For haplotype analyses, a count of heterozygous and homozygous cases and controls was conducted, whereby controls were matched to cases by PCA (see above). $\chi 2$ statistics and odds ratio (OR) are reported. Age at disease onset between different HLA-carrier groups was compared using an independent t-test, with datapoints shown for all groups together with a box plot showing interquartile range (IQR), mean and whiskers extending to the smallest and largest data points within 1.5 times the IQR and violin plots visualizing data distribution. Total number *n* is reported for all clinical and demographic parameters. For peptide binding experiments, mean of data and standard error of the mean

(*sem*) is shown for all datapoints. All analyses were performed using R and Python. Significance levels were set at $*p < 0.05$ and $**p < 0.01$. All p values are FDR corrected.

Transfection and Purification of HLA Class II Monomers

Plasmids encoding HLA-DQA1*01:01, HLA-DQA1*01:04, HLA-DQA1*01:05, HLA-DQB1*05:01, HLA-DQB1*05:03, HLA-DRA1*01:01 and HLA-DRB1*10:01 were purified according to manufacturer's instructions (Qiagen, Cat#12662) and transfected into Sf9 cells using BestBac2.0 (Cat# 91-002, Expression Systems), with P0 stock virus harvested after five days. Successful transfection was confirmed using a Baculovirus Titering Kit (Cat# 97-101, Expression Systems) and Flow Cytometric Analysis. Following this, 100ml of Sf9 cells were seeded at a concentration of ~1 x 10^6 cells/ml together with 100µl of P0 in a 27°C shaking incubator. On day three, cells were re-suspended in fresh medium (Sf-900™ III SFM (Thermo Scientific, Cat#12658019), supplemented with 1% GlutaMAX™ (Thermo Fisher Scientific, Cat#35050061) and 10% FBS (Gibco, Cat# 26140079) and subsequently sat down for seven more days. P1 was harvested and tested using a Baculovirus Titering Kit (Cat# 97-101, Expression Systems), Flow Cytometric Analysis and a Lentivirus Titer Kit (Cell Biolabs, Cat#VPK-107).

Amplification of virus titers was repeated for four-six generations and tested using a minimum protein expression protocol, whereby 300ml of Hi5 cells at a density of 2 x 10^6 cells/ml were incubated with 1.5ml of amplified P virus at 27°C in a shaking incubator for three days. Following the three-day incubation, supernatant was harvested and proteins purified using a Protino Ni-NTA Agarose for His-tag protein purification kit (Machery-Nagel, Cat#745400.100). The eluted protein was mixed with 4X Laemmli Buffer (Bio-Rad, Cat#1610747) and boiled for 10 minutes at 95°C before loading onto a mini-PROTEAN® TGX® precast gel for SDS-PAGE (Bio-Rad, Cat#4561093) and stained with Comassie Blue (Bio-Rad, Cat#1610786). Presence of protein was confirmed using 10-180 kDa Prestained Protein Ladder (Fisher Scientific, Cat#26616).

Purification of HLA-DQA1*01:01/DQB1*05:01 (DQ0101), HLA-DQA1*01:04/DQB1*05:03 (DQ0104), HLA-DQA1*01:05/DQB1*05:01 (DQ0105), HLA-DRA1*01:01/DRB1*10:01 (DR10) (also referred collectively as DQ5, although DQ5 also include common subtypes with DQA1*01:012 or DQB1*05:02 not associated here) was repeated in 5L, as previously described[45]. Protein concentration was measured using

NanoDrop A280 and injection into a Superdex® 200 Increase 10/300 GL (GE Healthcare, Cat#GE17-5174-01) was used to further purify proteins by Akta Pure FPLC.

Identification of strong binder ligands specific of each HLA molecule

To identify possible reference ligands for HLA binding competition assays, we computationally predicted binding of different viral peptides to DQ0101, DQ0104, DQ0105 and DR10[46]. Seventeen 15-mer peptides were narrowed down from this analysis as possible strong binders and synthesized biotin-conjugated at GenScript NJ with >90% purity. Binding of these peptides to each HLA molecule was tested as described elsewhere[45]. In brief, CLIP peptides of purified HLA-monomers were removed using thrombin cleavage (Millipore, Cat#69671). In each well of a 96-well plate (Biorad, Cat#MLP9601), 25nM of HLA-monomer was incubated with 10µM of biotin-conjugated ligand and 1X protease inhibitor (Sigma Aldrich, Cat#P8849), whereby the final reaction volume was brought up to 30µl using reaction buffer (100 mM acetate, pH = 4.6, 150 mM NaCl, 1% BSA, 0.5% Nonidet P-40). All reactions were tested both with and without addition of 25nM HLA-DM and all reactions were tested at least in duplicate. Controls were run for each plate by substituting biotin-conjugated ligands for dimethyl sulfoxide (DMSO). Plates were sealed and incubated for three days at 37°C. The reaction was neutralized with 60 µl neutralization buffer (100 mM Tris-HCl, pH = 8.6, 150 mM NaCl, 1% BSA, 0.5% Nonidet P-40, 0.1% NaN3) and transferred to a plate coated with monoclonal anti-DQ (Biotium, Cat#BNUM0200-50) or anti-DR antibody (BioLegend, Cat#327002). Plates were incubated at room temperature for one hour, washed five times with 300µl 1XPBS supplemented with 0.05% Tween-20 (pH = 7.4) and subsequently incubated for one hour with 100 µl Europium-labelled streptavidin (Cat# 1244-360, PerkinElmer) at a 1:1,000 dilution in PBS with 1% BSA (pH = 7.4). Plates were washed as described above, 100 µl enhancement solution (Cat# 1244-105, PerkinElmer) added to each well and plates placed on a shaker for five minutes. DELFIA® time-resolved fluorescence (TRF) intensity was detected using a Tecan Infinite® M1000. Following repetition of candidate binders using the above protocol in titrations of 100uM, 10um and 1uM, KNIYIYLTAGKEVRR (bio-PLXC1, derived from Plexin C1) was confirmed as a strong-binding ligand to DQ0101, DQ0104 and DQ0105, and EDEIRGYKLHVEVAK (bio-HTSF1, derived from HIV Tat-specific factor 1) as a strong-binding ligand to DR10.

Competition binding assay

Fifteen-mer peptides with an 11-amino acid overlap covering the full length of IgLON5 and selected post-translational modifications predicted using MusiteDeep[47], NetPhos3.1[48], NetNGlyc1.0[49] and mass spectrometry data from IgLON family members[50] (Figure 3A, Supplementary Table 2) were synthesized with >90% purity at GenScript NJ and dissolved in DMSO at a stock concentration of 10mM. Competition binding assays were conducted using the same plate-binding assay used for identification of strong binders. Specifically, each reaction contained 25nM of thrombin-cleaved HLA-monomer, 400uM of IgLON5 peptide and 1uM of bio-PLXC1 or bio-HTSF1 for DQ0101/DQ0104/DQ0105 and DR10 containing reactions, respectively. All reactions were tested both with and without the addition of 25nM HLA-DM and in duplicate. Controls were run for each plate by substituting both IgLON5 and biotin-conjugated peptides for DMSO (= negative control) or just IgLON5 peptides for DMSO (= positive control). Peptides with Eu TRF intensity between 25-50% of that of bio-PLXC1/HTSF1 alone were considered weak binders, whereas any with intensity below 25% were considered strong binders. Candidate binders were further tested in titration at competitor-to-peptide ratios of 1:40, 1:100 and 1:200.

Biotinylation of HLA-DQA1*01:05-DQB1*05:01 (DQ105), peptide exchange and spheromer assembly

DQ0105 monomer was biotinylated (bio-DQ0105) using a BirA biotin-protein ligase standard reaction kit (Avidity, Cat#BirA500) and extra free biotin removed by injection into a Superdex® 200 Increase 10/300 GL (GE Healthcare, Cat#GE17-5174-01). Bio-DQ0105 was next transferred to a plate coated with monoclonal anti-DQ antibody (Biotium, Cat#BNUM0200-50), incubated for 1h at RT and processed as described for the identification of strong binders. Biotinylation efficiency of monomer was determined by detecting TRF intensity using a Tecan Infinite® M1000. Following this, bio-DQ0105 was cleaved and peptides exchanged as previously described[45] with ^{125}VYLIVHVPARIVDIS^{139} (bio-DQ0105/IgLON5). HLA-DM was removed and 5µl of bio-DQ0105/IgLON5 (10uM) incubated with 9.47µl PE-labelled streptavidin (2.78 uM, BioLegend, Cat#405203) and 64.6µl PBS (pH 7.4) for 30 min at RT. Uncleaved, unloaded bio-DQ0105 (bio-DQ0105/CLIP) (2.64µM) was also incubated with 0.75µl A488-labelled streptavidin (33.3µM, BioLegend, Cat#405235) and 59.3µl PBS (pH 7.4) for 30 min at RT. The

spheromers assembled with bio-DQ0105/CLIP monomers were used as a control for non-specific staining. 0.9µl of spheromer scaffold (471µM)[51] and 20µl PBS (pH 7.4) were added to each reaction and following brief vortexing, incubated at room temperature for 30 min in the dark on a shaking incubator.

PBMC Culture, Staining, Isolation and sequencing of anti-IgLON5 antigen specific CD4$^+$ T cells

Frozen PBMCs were available from two patients with anti-IgLON5 disease carrying HLA-DQA1*01:05/DQB1*05:01 and from one HLA-matched control. Cells were thawed, counted and washed twice before settling in complete medium (RPMI 1640 (Sigma Aldrich, Cat#61770-036) supplemented with 10% heat-inactivated FBS (Gibco, Cat# 26140079) and 1X Penicillin-Streptomycin (Gibco, Cat#15140122) and filtered through a 0.2um PES Membrane (Nalgene, Cat#566-0020)) at a concentration of 700,000 cells/300µl/well in a 96-well flat bottom microplate (Thermo Fisher Scientific, Cat#167008). Each well was stimulated with 1.875µl of IgLON5-^{125}VYLIVHVPARIVDIS^{139} (1mM) and incubated in a tissue culture incubator at 37°C, 5% CO2 for ten days. Medium was changed every two-three days and cells stimulated with IL-2 on day eight. On day ten, cells were washed and re-suspended in 200µl FACS buffer (1XPBS pH 7.4 (Gibco, Cat#10010023) supplemented with 2mM EDTA (Sigma, Cat#E4884-500G) and 0.5% BSA (Sigma, Cat#A7888-50G), filtered through a 0.2µm PES Membrane (Nalgene, Cat#566-0020)). Cells were stained with 25µl of each bio-DQ0105/IgLON5 and bio-DQ0105/CLIP spheromer and incubated at 37°C, 5% CO2 for 90 min with gentle vortexing every 20 min. Cells were further stained with anti-CD3 (Biolegend, Cat#317343), anti-CD4 (Biolegend, Cat#300557), anti-CD8 (Biolegend, Cat#301014) and Aqua live/dead stain (Invitrogen, Cat#L34965) prior to analysis with FACS. CD4$^+$/bio-DQ0105/IgLON5$^+$ cells were sorted into 96-well plates filled with capture buffer (Takara, Cat#634439) and processed for single-cell sequencing, as previously described[45].

Standard Protocol Approvals, Registrations, and Patient Consents

This study was reviewed and approved by the Stanford University Institutional Review Board (Protocol #65073) as well as by local ethics committees of collaborating institutions. Written

informed consent was obtained from all patients for the storage and use of biological samples and clinical information for research purposes.

Results

A primary association with HLA-DQ rather than HLA-DR in anti-IgLON5 disease

DNA was available and genotyped from 75/87 patients with anti-IgLON5 disease (Supplementary Table 1) and matching to 232 controls by ethnicity using PCA, we found a strong signal within the HLA-Class II region on chromosome 6 (Fig 1A-C, Supplementary Figure 1, Supplementary Table 1). While chromosomes 2,11,13 and 16 contained suggestive peaks (Fig 1A), these were with low frequency SNPs, only sometimes supported by multiple markers. Hence, the HLA association was the only genome wide significance.

Following HLA imputation and typing, HLA association analysis revealed strong dominant associations with the highest frequency in *HLA-DQB1*05:01* (frequency = 0.747, 56/75 patients), followed by *HLA-DRB1*10:01* (frequency = 0.440, 33/75 patients) and *HLA-DQA1*01:05* (frequency = 0.413, 31/75 patients) in patients versus controls (Fig 1E(i), Table 1). As shown in previous studies[1,2,15], *HLA-DRB1*10:01* and *HLA-DQB1*05:01* showed the strongest association. Importantly, however, *HLA-DRB1*10:01*, *DQA1*01:05* and *DQB1*05:01* are in strong linkage disequilibrium (LD), forming a conserved haplotype[52] (Fig 1D, Supplementary Figure 2A). As such, association is with the entire haplotype, although risk is higher with *DRB1*10:01* and *DQA1*01:05*, as *DQB1*05:01* is also present in other haplotypes, most frequently in the context of *DRB1*01~DQA1*01:01~DQB1*05:01* haplotypes (Supplementary Figure 2A).

After excluding all carriers of *DRB1*10:01~DQA1*01:~DQB1*05:01* (33 cases and 6 controls), strong signals were observed with *HLA-DQB1*05:01* (frequency = 0.548, 23/42 patients), followed by *HLA-DQA1*01:01* (frequency = 0.548, 23/42 patients) and *HLA-DRB1*01:01* (frequency = 0.452, 19/42 patients) (Fig 1E(ii), Table 1). Once again, these alleles form a common conserved haplotype (Fig 1D, Supplementary Figure 2A), with only one subject carrying the *HLA-DRB1*01:02~DQA1*01:01~DQB1*05:01* haplotype. Given

the remaining 19 patients did not carry either *DRB1*10:01~DQA1*01:05-DQB1*05:01* or *DRB1*01~DQA1*01:01~HLA-DQB1*05:01*, we further conditioned the analysis by removing carriers of *DRB1*01:01/*01:02* alleles as well, which thus removed any carriers of *DQA1*01:01~DQA1*05:01*. This identified a remaining association with *HLA-DQB1*05:03* (frequency = 0.368, 7/19 patients), *HLA-DQA1*01:04* (frequency = 0.368, 7/19 patients), *HLA-DRB1*14:54* (frequency = 0.211, 4/19 patients) and *HLA-DRB1*14:01* (frequency = 0.105, 2/19 patients) (Fig 1E(iii), Table 1). Further conditioning with removal of *HLA-DQA1*01:04~DQB1*05:03* patients did not reveal additional significance (Fig 1E(iiii)).

Altogether, this analysis indicates a rank wise association with *HLA-DRB1*10:01~DQA1*01:05~DQB1*05:01*, *DRB1*01~DQA1*01:01~DQB1*05:01* and *DRB1*14~DQA1*01:04~DQB1*05:03* haplotypes, in order of descending predisposing risk. Importantly, conservation of *DQA1*01-DQB1*05* (*HLA-DQ5*) with 1-2 amino acid differences across all three of these haplotypes (Supplementary Figure 2B-C) indicate a primary role for HLA-DQ over HLA-DR.

HLA-DQ5 haplotypes associate with age at disease onset

Clinical data together with (at least) 4-digit resolution HLA data for all *DR~DQ* loci were available in 71 patients (Supplementary Table 1). Comparison of clinical characteristics identified a later in mean age at disease onset in line with carrier status for all three ranked *HLA-DQ5* haplotypes, which was significant for the two most dominant associations, *HLA-DQA1*01:05-DQB1*05:01* (Fig 2A(i)) and *HLA-DQA1*01:01-DQB1*05:01* (Fig 2A(ii)). Specifically, subjects with *HLA-DQA1*01:05-DQB1*05:01*, *DQA1*01:01-DQB1*05:01* and *DQA1*01:04-DQB1*05:03* had a 11.3, 8.6 and 0.9 years younger age of onset (Fig 2A(i-iii)), compared to non-risk HLA haplotype carriers (Fig 2A(iiii)), respectively.

As previously reported[15], we observed a higher proportion of male carriers with *HLA-DQA1*01:05-DQB1*05:01* (77% versus 49% are male, p = 0.37, Fig 2B). We did not confirm however presence of a sleep-dominant phenotype in *HLA-DRB1*10:01* carriers (43% versus 46% in non-*DRB1*10:01*, p=0.12) as initially suggested[15]. Rather, stratifying by HLA-DQ5 revealed a more diverse clinical picture in non-*HLA-DQ5* versus *HLA-DQ5* carriers, although numbers of non-DQ5 patients are too low to be conclusive for this subgroup (Fig 2C).

HLA-DQ5 Dosage Effects and Allele Competition are supported by haplotype frequencies and delayed age at disease onset

Given that *HLA-DQA1*01*-encoded alpha chains can heterodimerize with either *HLA-DQB1*05*- or *06*-encoded beta chains, giving rise to DQ molecules with distinct binding profiles[53] (Supplementary Figure 3), we investigated if alterations in disease risk could occur in double DQ1 heterozygotes, as reported for narcolepsy where allele competition of other DQ1 molecules occurs with the DQ0602 heterodimer[54]. As predicted, we observed reduced frequency of trans-located *DQB1*06* (DQ6, non-IgLON5-susceptibility associated *DQ1* haplotypes) in patients versus controls (Table 2). Further, age at disease onset was earliest in patients homozygous for *DQB1*05* (*DQ5/DQ5*), moderate for heterozygous carriers of *DQ5* with non-competing haplotypes (*DQ5*/other) and latest in *DQ5/DQ6* heterozygotes (Fig 2D), suggesting that competition with non-susceptibility DQ1 alleles modulates age of onset. While onset occurred significantly earlier in *DQ5/DQ5* (Fig 2C(i)) and *DQ5*/other (Fig 2C(ii)) subjects compared to non-*DQ5* carriers (Fig 2C(iiii)), this effect was abrogated when *DQ5* was inherited together with *DQ6* (Fig 2C(iii)). Altogether, these observations further support a functional role of *HLA-DQ5* in the development of anti-IgLON5 disease and suggest that rank wise and dosage-dependent effects stratify disease risk.

Deamidation of N-glycosylation sites in the Ig2-domain primes IgLON5 peptide immunogenicity

In order to identify potential immunogenic IgLON5 peptides acting as T cell auto-antigens, we derived 15-mer peptides (with 11-mer overlaps between peptides) encompassing the entire length of the IgLON5 protein (Fig 3A, Supplementary Table 2)) and screened these for binding (Fig 3B) to all risk-associated *HLA-DQ5* molecules: *HLA-DQA1*01:05-B1*05:01* (Fig 3C(i)), *DQA1*01:01-B1*05:01* (Fig 3C(ii)) and *DQA1*01:04-B1*05:03* (Fig 3C(iii)), as well as to *DRA1*01:01-DRB1*10:01* (Fig 3C(iiii)). While ^{141}PVTVNEGGNVNLLCL^{155} showed some weak binding to risk-associated *HLA-DQ5* molecules, we did not identify any strong binding native IgLON5-derived peptides, suggesting that native IgLON5 is unlikely to act as the primary T cell autoantigen.

In line with observations from other autoimmune diseases that showed preferential immunogenic binding of peptides in PTM form[30], we investigated possible modifications of IgLON5 (Fig 3A). Computational predictions suggested that several serine, threonine, and tyrosine residues could be phosphorylated[47,48], and several asparagine residues N-linked glycosylated[49] (Fig 3A). Notably, mass spectrometry confirmed several N-glycosylation sites in homologous IgLON peptides[50], consistent with previous work[55]. Similarly, asparagine and glutamine can also be chemically deamidated as a result of protein aging, which is a possibility considering that the half-life of surface-expressed IgLON5 is over 3 days *in vitro* and could be higher in neurons[56-58]. We computationally predicted binding of theses modified forms of IgLON5 to HLA molecules and subsequently narrowed down 124 15-mer peptides that we further tested using *in vitro* competition binding assay.

Using this additional collection of modified peptides, only a few peptides were found to bind the disease-associated *HLA-DQ5* monomers (Fig 3C), including ^{125}VYLIVHVPARIVNIS^{139}, ^{145}NEGGNVNLLCLAVGR^{159} and ^{149}NVNLLCLAVGRPEPT^{163}, located within the Immunoglobulin-like 2 (Ig2) domain (also targeted by IgLON5 autoantibodies[55]), but only when asparagine residues (N) N_{137}, N_{149} and N_{149}, respectively, were substituted for aspartic acid (D). N to D substitution mimics deamidation of key N-glycosylation sites[59], a PTM that is a known contributor to reducing self-tolerance and priming peptides for autoimmune T cell recognition [60,61]. Furthermore, a dosage-dependent increase in HLA-peptide binding further confirmed these peptides as relevant binders (Fig 3E), and comparison of the binding motifs of risk-associated HLA-DQ5 molecules shows that all three HLA-DQs bind D with high-affinity in pocket six of the nine-mer binding core, thus further substantiating that deamidation may be key to making these peptides immunogenic.

Taken together, these results suggest that deamidation of specific asparagine sites within the IgLON5-Ig2 domain might prime specific IgLON5 peptides for HLA class II presentation through risk-associated HLA-DQ5 molecules.

Increased CD4+ T cell reactivity to a deamidated IgLON5-Ig2-derived peptide

To test whether deamidation may be a key modification to evoke a functional autoimmune response, we stimulated PBMCs of two *HLA-DQA1*01:05-DQB1*05:01*-carrying patients

and one HLA-matched control with an Ig2-derived 15-mer peptide, ^{125}VYLIVHVPARIVDIS^{139}, mimicking deamidation at N_{137} for 10 days, prior to isolating antigen-specific $CD4^+$ T cells using spheromer[51] (Fig 4A).] Both patients showed elevated $CD4^+$ T cell reactivity compared to the control, although this was more pronounced in patient 2 (Fig 4B). Antigen-specific $CD4^+$ T cells were sorted and sequenced in order to investigate cellular phenotypes. While there was some admixture, patient cells formed a distinct cluster relative to control cells (Fig 4C), suggesting similarities in cellular phenotype. Delineation into distinct T cell transcriptional states, namely naive (T_{naive}), effector (T_{em}), highly effector (T_{emra}) and regulatory (T_{reg}) T cell subsets (Fig 4D, E(i)) showed a higher expression of T_{emra} markers in the patient versus control population, while elevated activation (Fig 4E(ii)) in the former population further supports that antigen-specific T cells are more highly differentiated in patients. Taken together, these preliminary findings suggest that reactivity against deamidated Ig2-derived IgLON5 may be elevated in anti-IgLON5 disease and is associated with a more effector T cell phenotype.

Discussion

This study is the largest reported HLA-association analysis in anti-IgLON5 disease. Notably, it benefits from the inclusion of patients from multiple different ethnicities and countries, and rigorous matching of controls by PCA[39]. GWAS typically require a large sample size to detect statistically significant associations between genetic variants and complex traits[62]. While our current study is underpowered for identification of genome-wide associations, HLA genes are highly polymorphic and show strong LD within, and across genes, making them a powerful marker for association studies, even in small sample sizes. As a result, we were able to confirm and refine the association of anti-IgLON5 disease with *HLA-DQ* over *HLA-DR*[2,15]. Interestingly, *HLA-DQ5*-dosage effects modulate both disease predisposition (haplotype carrier frequency differences), and age at disease onset, with a protective effect of *DQ6*, possibly through allele competition with *DQ5*. Future studies investigating effects of these combinations on HLA-DQ5 expression and how trans-dimerization may affect HLA-peptide binding[63,64] will be needed to further substantiate these observations.

A ranked association with three *HLA-DQ5* haplotypes, *HLA-DQA1*01:05-05:01*, *HLA-DQA1*01:01-05:01* and *HLA-DQA1*01:04-05:03*, in order of descending risk was found in

85% (74/87) of all patients and is further reflected in a delayed age at disease onset. Altogether, this strongly supports that *HLA-DQ* is a more reliable marker for genetic disease risk than *HLA-DR*. Disease risk in the remaining patients could not be explained by HLA, however, the fact that non-*DQ5* carriers have a significantly later age at disease onset suggests that there might be a distinct trigger. While a previous report suggested more dominant presentation of sleep or bulbar symptoms in *HLA-DRB1*10:01*-carriers[15], we were not able to confirm this observation, nor decipher any other significant associations between HLA-haplotype and major clinical phenotypes. Interestingly, a retrospective analysis of clinical, polysomnographic and HLA data[65] showed a high prevalence of *HLA-DQB1*05:01* for different types of NREM parasomnias, thus suggesting that stratification of sleep-phenotypes may occur at the level of *HLA-DQB1*05* as supposed to distinct HLA-*DRB1~DQA1~DQB1* haplotypes. However, a recent report[66] showed that a PSP-like phenotype was underrepresented in *HLA-DRB1*10:0*1-carriers, while carrier status did not affect development of movement disorders more generally, suggesting that stratification of clinical phenotypes by HLA-carrier status may only occur at the level of specific clinical features. Future clinical reports should thus focus on a more fine-grained analysis of HLA-association with detailed clinical phenotypes.

We did not observe that native IgLON5 peptides strongly bound HLA-DQ5. Rather, in line with observations from other autoimmune diseases such as type-1 diabetes mellitus or rheumatoid arthritis[30], HLA-DQ5 was found to bind PTM-modified IgLON5 peptides containing aspartic acid residues in several positions. In line with this observation, it is worth noting that DQA1*01:05/DQB1*05:01, DQA1*01:01/DQB1*05:01 and DQA1*01:04/DB1*05:03 all bind aspartic acid (D) in pocket six with high affinity, although less preeminently so for DQA1*01:04/DQB1*05:03. Deamidation can occur as proteins are targeted for degradation and it has thus been suggested that this modification may serve as an age-dependent molecular clock[56,59], an intriguing concept considering the late age of onset of the disease and slow turnover of the protein. While deamidation of asparagine was previously shown to alter both HLA class II antigen presentation[61], as well as T cell recognition[67], the physiological relevance of this modification in anti-IgLON5 disease remains enigmatic. Our hypothesis is that deamidations occur *in vivo* as the result of simple chemical deamidation[59], enzymatic deamidation, or following de-glycosylation of N-linked glycopeptides at the corresponding residues[68], although this latter mechanism was primarily shown for HLA-class I presentation of intracellularly processed peptides[69]. Notably, deficiency of the enzyme

NGLY1, which catalyzes the removal of N-linked glycans and plays a key role in ER-associated degradation (ERAD), was recently discovered to be the cause of a severe, multi-symptomatic disorder that also involves a complex movement disorder[70,71], while abnormalities in the NGLY1–NRF1 pathway have been associated with immune dysregulation[72]. Whether abnormalities in enzymatic N-deglycosylation could play a role in this process may therefore also be considered. Overall, a considerable short coming in the field of anti-IgLON5 disease research is the concurrent lack of availability of mass spectrometric or comparable proteomic data needed to confirm PTM changes speculated to occur in this condition beyond computational predictions. The significance of the PTM modifications suggested here and their relevance and importance in triggering the disease therefore need to be confirmed *in vivo*[73].

Of note, it has previously been suggested that the IgLON5-derived peptides ^{10}LRLLAAAAL^{19} and ^{127}IVHVPARIV^{136} constitute potential causative immunogenic cores, due to their high computationally predicted binding affinity to HLA-DRA1*01:01-DRB1*10:01 and HLA-DRA1*01:01-DRB1*01:01[15]. Testing these peptides *in vitro*, however, we could not confirm binding of either peptide to HLA-DRA1*01:01-DRB1*10:01. Altogether, the fact that HLA-DRA1*01:01-DRB1*10:01 neither accounts for genetic risk in most patients, nor shows binding of the proposed peptides, does not favor a role of HLA-DR in the disease. Furthermore, these results also highlight the limitations of *in silico* predictions[74] and stress the importance of *in vitro* binding assays to substantiate autoimmune HLA-peptide interactions.

The three deamidated peptides we identified as HLA-DQ5-binders all lie within the Ig2 domain of IgLON5. Interestingly, the Ig2 domain was previously shown to be the key target site of patient autoantibody recognition[55]and treatment with bacterial-derived PNGase F (N-Glycosidase F, an endoglycosidic enzyme that deamidates N-glycosylated residues to aspartic acid through removal of oligosaccharides from glycoproteins) successfully abolished autoantibody binding to Iglon5 expressing cells[75]. The binding of deamidated peptides to risk-associated HLA-molecules we show here, therefore correspond with previously reported autoantibody target sites, both in terms of location along the IgLON5 protein, as well as the state of post-translational modification. This suggests that B cell and T cell epitopes involved in the diseases may overlap or be in close proximity, as often observed following epitope spreading[76]. T-B cell interactions are a critical component of the pathophysiological trajectory in most autoimmune diseases (Lanzavecchia 1985, Petersone, Edner et al. 2018) and our

study at hand lays an important foundation for the further investigation of this important interaction.

In the current study, we show elevated T cell reactivity against a single modified epitope, ^{125}VYLIVHVPARIVDIS^{139} in two patients compared to a single HLA-matched control. We used spheromers, a state-of-the-art multimer that allows for more efficient T cell staining and improved TCR-binding properties[51] that also detect low-affinity T cells that may be more disease relevant in autoimmunity[77,78]. While the T cell data we present suggests functional relevance of ^{125}VYLIVHVPARIVDIS^{139} in shaping an early autoimmune response in anti-IgLON5 disease driven by T cells in a late effectorness state[79], this data should be considered preliminary, subject to the small sample size. Future studies testing additional subjects and epitopes will be needed to confirm relevance of ^{125}VYLIVHVPARIVDIS^{139} and other epitopes in shaping anti-IgLON5 autoimmunity, as well as to define phenotypic signatures of disease-relevant, antigen-specific T cells. Whether reactivity against the same epitope is shared across carriers of distinct HLA-DQ5 molecules, as suggested by shared HLA-peptide binding properties shown in our current study, will also require further examination. We hypothesise that future studies focusing on the investigation of T cell reactivity against multiple IgLON5-derived epitopes in distinct HLA-DQ5 carriers may explain the ranked-HLA-DQ5 disease risk, which the current study promotes on the basis of genetic, demographic and molecular evidence.

Taken together, this study poses important clinical implications for diagnosis, stratification and treatment of anti-IgLON5 disease: a better understanding of HLA-association will allow us to recognise individuals at risk early, while distinction into different DQ5 subgroups may allow us to stratify different pathophysiologically differentiated syndromes, baring distinct treatment strategies. In summary, our study shows for the first time that anti-IgLON5 disease is primarily HLA-DQ associated, and that T cell autoimmunity directed toward deamidated IgLON5 sequences presented by these molecules may be involved. Future studies incorporating a larger number of patients, a difficult endeavor in a rare disease, will be needed to further elucidate genetic risk of this condition, also at a genome wide level. Concurrent to that, investigations of immune cell reactivity and phenotypes will elucidate key pathophysiological events, ultimately aiming to disentangle the intricate interplay of autoimmunity and neurodegeneration within, and beyond this condition. I think you should return to therapeutics here as otherwise it doesn’t fit with the introduction

Data Availability

Raw data are available on request to the corresponding author.

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Competing interests

S Irani has received honoraria/research support from UCB, Immunovant, MedImmun, Roche, Janssen, Cerebral therapeutics, ADC therapeutics, Brain, CSL Behring, and ONO Pharma and receives licensed royalties on patent application WO/2010/046716 entitled 'Neurological Autoimmune Disorders', and has filed two other patents entitled “Diagnostic method and therapy” (WO2019211633 and US-2021-0071249-A1; PCT application WO202189788A1) and “Biomarkers” (PCT/GB2022/050614 and WO202189788A1). S. Mariotto received speaker honoraria from Biogen, Sanofy, and Novartis. C. Hartmann has been serving as consultant for Univar and has received honoraria for lecturing and travel expenses / speaking honoraria from Abbott and Alexion, and research support from Abbott. A. McKeon patents issued for GFAP and MAP1B-IgGs and patents pending for PDE10A, Septins-5 and -7, and KLCHL11-IgGs, and has consulted for Janssen and Roche pharmaceuticals, without personal compensation. J. Dalmau holds a patent for the use of IgLON5 antibody testing and both he and F. Graus receive royalties from Euroimmun for the clinical use of this test.

Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1. GWAS and ranked HLA-association with three conserved DQB1*05:~ haplotypes. (A) Manhattan plot of GWAS. The blue dotted line depicts suggestive significance ($p < 1 \times 10^{-5}$) while the red dotted line shows genome-wide significance ($p < 5 \times 10^{-8}$). A clear signal can be seen within the HLA Class II region on chromosome 6. **(B)** Quantile-quantile plot of GWAS. **(C)** The first two principal components (PC1, PC2) are

shown to highlight ethnic diversity of study. Cases (purple) were matched at a 1:8 ratio to controls (pink) from a set of 2,503 controls (grey) by PCA. **(D)** Sankey plot shows conservation of risk-associated HLA-DRB1*~DQA1*~DQB1* haplotypes among cases. (**E) (i)** HLA association of anti-IgLON5 disease across HLA Class I and II is shown unconditioned **(ii)** under conditioned exclusion of HLA-DRB1*10:01 **(iii)** HLA-DRB1*10:01, HLA-DRB1*01:01 and HLA-DRB1*01:02 and **(iiii)** HLA-DRB1*10:01, HLA-DRB1*01:01, HLA-DRB1*14:01, HLA-DRB1*14:54 and HLA-DRB1*14:04.

Figure 2. Delayed age of onset correlates with ranked HLA-risk and HLA-DQ5 dosage. **(A)** Age at disease onset **(B)** Sex demographics and **(C)** Major clinical features reported are shown for cases carrying the **(i)** HLA-DQA1*01:05-DQB1*05:01, **(ii)** HLA-DQA1*01:01-DQB1*05:01, **(iii)** HLA-DQA1*01:04-DQB1*05:03 or **(iiii)** None of the afore mentioned haplotypes. For **(A)(i)** Age range = 34-76 years ($\tilde{x}$ = 59.5 years), **(A)(ii)** Age range = 44-91 years ($\tilde{x}$ = 61.0 years), **(A)(iii)** Age range = 54-81 years ($\tilde{x}$ = 68.5 years), **(A)(iiii)** Age range = 59-81 years ($\tilde{x}$ = 71.0 years). In **(A)**, Individual markers indicate individual cases, whereby dark markers denote homozygous carriers of the given haplotypes. In **(C)**, numbers in the centre of pie charts indicate the total number of cases reporting the given clinical feature. **(D)** Age at disease onset is shown for carriers **(i)** Homozygous for HLA-DQB1*05:~, **(ii)** Heterozygous for HLA-DQB1*05:~ without HLA-DQB1*06:~ **(iii)** Heterozygous for HLA-DQB1*05:~ with HLA-DQB1*06:~ **(iiii)** Not carrying HLA-DQB1*05:~. For **(D)(i)** Age range = 49-80 years ($\tilde{x}$ = 60.5 years), **(D)(ii)** Age range = 34-91 years ($\tilde{x}$ = 62.0 years), **(D)(iii)** Age range = 54-70 years ($\tilde{x}$ = 65.0 years), **(A)(iiii)** Age range = 59-81 years ($\tilde{x}$ = 71.5 years). *n = total number of cases, μ_{age} = mean age, $\tilde{x}$ = median age, * $p < 0.05$, ** $p < 0.01$.*

Figure 3. Risk-associated HLA-DQ5 molecules preferentially bind IgLON5 in a deaminated form. (A) The entire length and sequence of the IgLON5 peptide is shown, highlighting sites of post-translational modification as predicted by MusiteDeep, NetPhos3.1, NetNGlyc1.0 and mass spectrometry data from Itoh et al. 2008. N_{glyc} = N-linked glycosylation, S_{phos} = Serine phosphorylation, T_{phos} = Threonine phosphorylation and Y_{phos} = Tyrosine phosphorylation. **(B)** Schematic shows how peptide-HLA binding is determined using competition binding assay. **(C)** Results from competition binding assay are shown for **i.** HLA-DQA1*01:05-DQB1*05:01, **(ii)** HLA-DQA1*01:01-DQB1*05:01, **(iii)** HLA-DQA1*01:04-DQB1*05:03 and **(iiii)** HLA-DRA1*01:01-DRB1*10:01. Light bars show

IgLON5 15mer peptides in physiological, and dark bars in post-translationally modified (ptm) form (see legend below graph. (**C**). The y-axis (log scale) shows mean fluorescent intensity (mfi) of IgLON5 peptide together with biotinylated-competitor in competition, divided by mfi of biotinylated-competitor alone. For **(i)**, **(ii)** and **(iii)**, bio-PLXC1 and for **(iiii)**, bio-HTSF1 was used as biotinylated-competitor. Along the x-axis, enumerated 15mer IgLON5 peptides, sequentially encompassing the entire length of the IgLON5 protein are shown (see supplementary table 2 for full list of enumerated peptides). The blue dotted line (0.5) shows the threshold for weak binders, and the red dotted line (0.25) the threshold for strong binders. **(D)** Binding of HLA-DQA1*01:05-DQB1*05:01 (green), HLA-DQA1*01:01-DQB1*05:01 (purple) and HLA-DQA1*01:04-DQB1*05:03 (yellow) to the three peptides **(i)** ^{125}VYLIVHVPARIV**N**IS^{139}, **(ii)** ^{145}NEGG**N**VNLLCLAVGR^{159} and **(iii)** ^{149}**N**VNLLCLAVGRPEPT^{163} is shown, with asparagine residues (**N**) in bold in an unmodified ('native', left), glycosylated ('GLcNac', middle) or deamidated ('deamidated',right) form. (Dotted lines as for **C.**; Y-axis is also as described for **C.**, but linear.) **(E)** Dose-dependant change in binding of peptides described in **(D) (i)**, **(ii)** and **(iii)** when deamidated at bold **N** residues (i.e., mimicking aspartic acid, **D**). Y-axis shows dose-dependant change in binding relative to baseline (40μM of IgLON5 peptide), given variation of IgLON5 peptide concentration shown along the x-axis. *Error bars in C and D, and shaded regions in E represent standard error of the mean (sem). All assays were repeated at least in duplicate.*

Figure 4. CD4$^+$ T cell reactivity and phenotypic profiling. (A) Schematic shows how peptide-HLA-spheromers are assembled and used to isolate antigen-specific CD4$^+$ T cells. **(B)** Proportion of CD4+ T cells reactive to ^{125}VYLIVHVPARIV**D**IS^{139}in one control and two patients. **(C)** Neighbourhood clustering of isolated single cells by **(i)** the Louvain method **(ii)** delineation into individuals (control, patient 1, patient 2). **(D)** T cell transcriptional states. **(i)** Tracksplot displaying the T cell marker expression profiles of individuals (control, patient 1 and patient 2, with the x-axis representing the individual cells and the y-axis denoting the different markers analyzed (right). The bar height indicates the level of expression of each marker. **(ii)** Dotplot displays the expression levels of T cell subset markers (left, same as D(i)) in the control versus patient (patient 1 and 2) group. **(E)** Marker expression by individual cells in the control versus patient (patient 1 and 2) group, displaying. **i.** Markers of T cell activation and **(ii)** T_{emra} markers. *For **B-E**, see legend for colour coding and descriptions. T_{naive}= naive; T_{em} = effector; T_{emra}= highly effector; T_{reg}= regulatory.*