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H1N1 hemagglutinin-specific HLA-DQ6-restricted CD4+ T cells can be readily detected in narcolepsy type 1 patients and healthy controls

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ABSTRACT

Following the 2009 H1N1 influenza pandemic, an increased risk of narcolepsy type 1 was observed. Homology between an H1N1 hemagglutinin and two hypocretin sequences has been reported.

T cell reactivity to these peptides was assessed in 81 narcolepsy type 1 patients and 19 HLA-DQ6-matched healthy controls.

HLA-DQ6-restricted H1N1 hemagglutinin-specific T cell responses were detected in 28.4% of patients and 15.8% of controls. Despite structural homology between HLA-DQ6-hypocretin and -H1N1 peptide complexes, T cell cross-reactivity was not detected.

These results indicate that it is unlikely that cross-reactivity between H1N1 hemagglutinin and hypocretin peptides presented by HLA-DQ6 is involved in the development of narcolepsy.

1. Introduction

Narcolepsy type 1 (NT1) is a rare disorder of the regulation of sleep and wakefulness with an incidence of 1 per 100,000 person years and a prevalence ranging between 20 and 50 per 100,000 individuals (Ohayon et al., 2002; Wijnans et al., 2013). The disorder is characterised by five core symptoms: excessive daytime sleepiness, cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nocturnal sleep. These symptoms arise as a result of the destruction of over 90% of hypocretin (Hcrt)-producing neurons in the lateral hypothalamus (Peyron et al., 2000; Thannickal et al., 2000). Unfortunately, causal treatment of the disorder is not yet available.

In pursuit of the disease mechanism, two findings have shifted the focus of narcolepsy research to the hypothesis that the destruction of Hcrt-producing neurons is caused by an auto-immune process. First, 95% of NT1 patients carry the *HLA-DQA1*01:02 / DQB1*06:02* haplotype encoding HLA-DQ6, an HLA-class II molecule expressed on antigen-presenting cells (Juji et al., 1984; Mignot et al., 1997; Tafti et al., 2014), which was later complemented by genome-wide association studies that showed variants within immune system-regulating genes in narcolepsy patients (Faraco et al., 2013; Han et al., 2013; Hor et al., 2010). Second, an increase in the incidence of NT1 has been observed in several European countries after the 2009 H1N1 influenza pandemic, and the subsequent vaccination campaign (Dauvilliers et al., 2013;

Abbrevations: H1N1-HA, H1N1-hemagglutinin; Hcrt, Hypocretin; HLA-DQ6, HLA-molecule encoded by the *HLA-DQA1*01:02 / DQB1*06:02* haplotype; ICSD-3, International Classification of Sleep Disorders, 3rd edition; mAb, Monoclonal antibody; NT1, Narcolepsy type 1; PBMC, Peripheral blood mononuclear cell; PCR, Polymerase chain reaction; SI, Stimulation index; TCC, T cell clone; TCR, T cell receptor

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Feltelius et al., 2015; Lind et al., 2014; Partinen et al., 2012). Even though there was no wide-spread vaccination campaign in Asian countries, an increased incidence has also been reported in China (Han et al., 2011). This suggests that NT1 might develop as the result of a cross-reactive anti-viral immune response that leads to the destruction of Hcrt-producing neurons. More insight in this reaction could pave the way for causal treatment development in NT1 and potentially also prevention of the disease by identifying individuals at-risk.

Research on the auto-immune reaction has focused on two candidate immune cell types that could drive this reaction leading to NT1: cross-reactive B cells and cross- or autoreactive CD4+ T cells. Four studies (Black et al., 2005; Overeem et al., 2006; Tanaka et al., 2006; Van Der Heide et al., 2015) failed to detect autoreactive B cells or autoantibodies to Hcrt or Hcrt-receptors. Another group claimed to have identified autoreactive CD4+ T cells specific for Hcrt (De La Herran-Arita et al., 2013; De La Herran-Arita et al., 2014), but the article was subsequently retracted. While HLA-DR-restricted Hcrt-specific T cell responses have recently been described (Latorre et al., 2018), this does not explain the strong link with HLA-DQ6. In short, the molecular mechanism underlying the HLA-DQ6 association remains to be determined.

Based on the increased incidence of NT1 after the H1N1 influenza pandemic mentioned above, we aimed to assess whether H1N1 influenza reactive CD4+ T cells are present in NT1 patients. A secondary aim was to assess whether these H1N1 influenza specific CD4+ T cells cross-react with Hcrt. We determined the crystal structure of one earlier identified H1N1 hemagglutinin peptide and two Hcrt peptides bound to the disease-predisposing HLA-DQ6 molecules to assess structural homology. Subsequently, we stimulated peripheral blood mononuclear cells with these peptides to generate specific T cell lines and clones. We then performed proliferation tests on these clones to assess both H1N1- and Hcrt-reactivity and assess HLA-DQ6 restriction, cross-reactivity and T cell receptor sequence of these clones.

2. Materials and methods

2.1. Subjects

Between March 2014 and June 2016, we included all consecutive narcolepsy type 1 patients after informed consent, recruited from the sleep clinic of the department of Neurology, Leiden University Medical Center and the Sleep-Wake Centre of Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede. All patients were diagnosed with narcolepsy type 1 according to the International Classification of Sleep Disorders (ICSD-3; (American Academy of Sleep Medicine, 2014)). Symptom onset in all patients was after the 2009 H1N1 pandemic. Healthy controls were included in the same time period as the narcolepsy type 1 patients and matched for HLA and gender.

2.2. Hcrt-1 measurements

CSF samples were drawn for Hcrt-1 measurement in 34 narcolepsy type 1 patients. Hypocretin-1 concentrations were measured in duplicate with an I^{125} hypocretin-1 radioimmunoassay (Phoenix Pharmaceuticals, Mountain View, CA, USA). This assay has an intra-assay variability of < 5% and a detection limit of 50 pg/mL. To adjust for inter-assay variability, Stanford reference CSF samples were included in the assay (Ripley et al., 2001; Mignot et al., 2002).

2.3. Peptides

All peptides used were produced at the Peptide Synthesis Facility of the Department of Immunohematology and Blood Transfusions of the Leiden University Medical Center. Two Hcrt peptides, $Hcrt_{56–68}$ and $Hcrt_{87–99}$, and one 2009 H1N1 influenza A hemagglutinin (H1N1-HA) peptide, H1N1-$HA_{275–287}$, were selected based on sequence similarity between these peptides and those described in the aforementioned retracted article (De La Herran-Arita et al., 2013; De La Herran-Arita et al., 2014). All peptide sequences can be found in Supplemental Table 1.

For follow-up experiments, we used processed Hcrt peptides: two truncated peptides lacking the first 4 residues (including histidines on position 59 and 90 of $Hcrt_{56–68}$ and $Hcrt_{87–99}$, respectively), thereby increasing homology between H1N1-$HA_{275–287}$ and the Hcrt peptides. Additionally, the histidine residue on position 59 of the first Hcrt peptide ($Hcrt_{56–68}$) was replaced by an oxo-histidine or alanine residue rendering the two peptides more homologous to H1N1-$HA_{275–287}$.

Soluble complexes of HLA-DQ6 containing the peptides H1N1-$HA_{275–287}$, $Hcrt_{56–68}$ and $Hcrt_{87–99}$ were produced essentially as described previously for HLA-DQ2 (Henderson et al., 2007; Petersen et al., 2014). Briefly, the αβ-heterodimer of the HLA-DQ6 extracellular domain was expressed in Hi5 insect cells, with each peptide linked to the N-terminus of the HLA-DQ6 β-chain. The C-termini of the constructs contained an enterokinase cleavable Fos/Jun zippers, and, at the C terminus of the β-chain, a BirA biotinylation sequence followed by a His10-Tag. The complexes were purified via diafiltration, metal affinity, size exclusion and ion exchange chromatography. For crystallisation experiments the Fos/Jun zippers were removed by enterokinase cleavage and ion exchange chromatography.

2.4. Crystallisation, data collection, structure determination and refinement

Peptide-HLA-DQ6 complexes were crystallised at 20 °C via the hanging drop vapor diffusion method using equal volumes of mother liquor and protein solution at 10 mg/mL in a buffer containing 10 mM Tris (pH 8) and 150 mM NaCl. HLA-DQ6-$Hcrt_{56–68}$ and HLA-DQ6-$Hcrt_{87–99}$ were crystallised with mother liquor containing 16–20% PEG4000, 0.1 M NaOAc pH 4.5–5.0, and HLA-DQ6- $HA_{275–287}$ was crystallised with 23% PEG4000, 0.2 M NaI, 0.1 M HEPES pH 7. Prior to data collection the crystals were cryoprotected in mother liquor supplemented with 20% glycerol, or 20% ethylene glycol in the case of HLA-DQ6- $Hcrt_{87–99}$, and flash frozen in liquid N_2. X-ray diffraction data was collected at the mx2 beamline of the Australian Synchrotron using a ADSC Q315r detector and data processing was carried out with XDS (Kabsch, 2010) and Scala (Evans, 2006). The crystal structures were solved by molecular replacement in Phaser (Mccoy et al., 2007) using a published HLA-DQ6 structure (PDB code 1UVQ) as search model. The structural models were refined by iterative rounds of model building in Coot (Emsley et al., 2010) and restrained refinement in Phenix (Adams et al., 2010). For further details on data collection and refinement statistics, see Table 1.

2.5. Peripheral blood mononuclear cell (PBMC) isolation

Blood was drawn from all patients and healthy controls. PBMCs were extracted using Ficoll-Paque (GE Healthcare, Chicago, USA) gradient reagent. The first experiments were performed on fresh PBMCs, but in the remainder the isolated PBMCs were subsequently frozen in 10% dimethyl sulfoxide (DMSO; Sigma Aldrich, Saint Louis, USA) in fetal calf serum (FCS; Sigma Aldrich, Saint Louis, USA). These samples were stored until use in liquid nitrogen vessels.

2.6. Antigen-specific T cell lines

After isolating or thawing of PBMCs of narcolepsy type 1 patients and healthy controls, 1×10^6 cells were put into culture in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Basel, Switzerland) supplemented with L-glutamine (Thermo Fisher, Waltham, USA), 10% (pooled) human serum (NHS) and a mixture of the H1N1-$HA_{275–287}$, $Hcrt_{56–68}$ and $Hcrt_{87–99}$ peptides. After every 5 days r-interleukin 2 (20 U/mL final concentration; rIL-2; Novartis, Basel, Switzerland) and interleukin 15 (10 ng/mL final concentration, IL-15; R&D, Minneapolis,

Table 1
Data collection and refinement statistics.

Wavelength	HLA-DQ6-Hcrt$_{56–68}$	HLA-DQ6-Hcrt$_{87–99}$	HLA-DQ6-H1N1-HA$_{275–287}$
Resolution range	34.52–2.0 (2.071–2.0)	39.56–1.936 (2.005–1.936)	40.11–1.7 (1.761–1.7)
Space group	C 1 2 1	P 2 21 21	P 21 21 21
Unit cell	163.063 72.404 99.277	72.538 99.549148.372	55.764 89.58115.484
	90,113.763 90	90 90 90	90 90 90
Total reflections	137,291 (13803)	160,661 (15461)	126,644 (12583)
Unique reflections	70,012 (6989)	80,479 (7782)	64,298 (6352)
Multiplicity	2.0 (2.0)	2.0 (2.0)	2.0 (2.0)
Completeness (%)	97.65 (97.90)	99.54 (97.12)	99.84 (99.95)
Mean I/sigma(I)	7.34 (1.86)	9.85 (2.37)	10.47 (2.31)
Wilson B-factor	22.44	23.29	17.15
R-merge	0.06104 (0.3654)	0.03506 (0.2447)	0.04976 (0.3822)
CC1/2	0.995 (0.835)	0.999 (0.948)	0.994 (0.685)
CC*	0.999 (0.954)	1 (0.987)	0.999 (0.902)
R-work	0.1755 (0.2524)	0.1882 (0.2668)	0.1705 (0.2377)
R-free	0.1980 (0.2795)	0.2157 (0.2819)	0.1972 (0.2680)
Number of non-hydrogen atoms	6888	6696	3720
Macromolecules	6044	5990	3083
Ligands	76	90	95
Solvent	768	616	542
Protein residues	751	745	384
RMS (bonds)	0.008	0.009	0.009
RMS (angles)	1.17	1.25	1.46
Ramachandran favored (%)	98.09	97.65	97.61
Ramachandran allowed (%)	1.91	2.35	2.39
Ramachandran outliers (%)	0.00	0.00	0.00
Rotamer outliers (%)	0.60	1.21	0.59
Clashscore	5.44	5.58	4.36
Average B-factor	43.49	45.13	26.10
Macromolecules	43.27	44.94	22.61
Ligands	69.59	77.29	77.67
Solvent	42.63	42.29	36.94

HA = hemagglutinin; Hcrt = hypocretin.

USA) were added to each culture (Kooy-Winkelaar and Koning, 2015).

2.7. Peptide-specific T cell clone generation

Peptide-specific T cell clones (TCCs) were generated from T cell lines specific for H1N1-HA$_{275–287}$ from 3 narcolepsy patients by limiting dilution in culture medium containing 10^6 irradiated feeder cells/mL, 20 U/mL rIL-2, 10 ng/mL IL-15 and 1 μg/mL PHA ("feeder mix"; Remel, Lenexa, USA). The cells were stimulated with 20 U/mL rIL-2 and 10 ng/mL in 10% human serum/IMDM after 5 days. After 10 days, growing wells were transferred to 24-well plates and cultured in feeder mix until a confluent layer of cells was formed. For the T cell lines of 9 narcolepsy patients, streptavidin-PE-HLA-DQ6- H1N1-HA$_{275–287}$ and streptavidin-PE-HLA-DQ6-Hcrt$_{56–68}$ and -Hcrt$_{87–99}$ tetramers were used to directly stain HLA-H1N1-HA$_{275–287}$-, -Hcrt$_{56–68}$- and -Hcrt$_{87–99}$ -specific T cells. Tetramers were produced essentially as described (Ooi et al., 2017). These tetramer-positive CD4 + T cells were sorted by flow cytometry on a FACS-Aria III instrument (BD Biosciences) and expanded as described above. Clones were subsequently generated from the identified H1N1-HA$_{275–287}$-specific and/or Hcrt$_{56–68}$- or Hcrt$_{87–99}$-specific T cells, as described previously (Kooy-Winkelaar and Koning, 2015). All T cell lines used for TCC generation were derived from frozen narcolepsy patient samples.

2.8. Flow cytometry

Peptide-specific T cell lines generated from one patient were incubated for 30 min with 11 antibodies for surface staining and subsequently acquired on a LSRII instrument (BD Biosciences). Fluorochrome-labelled antibodies directed against CD3 (clone UCHT1), CD4 (clone SK3), CD5 (clone L17F12), CD7 (clone M-T701), CD14 (clone MφP9), CD27 (clone M-T271), CD28 (clone CD28.2), CD45RA (clone L48) and IgG1 (clone MOPC-21) were from BD Biosciences (San Jose, California, USA), anti-CD8 (clone 3B5) was from Invitrogen (Bleiswijk, the Netherlands) and anti-CD45 (clone HI30) from eBioscience (San Diego, California, USA). Results were analysed using FlowJo V.10 software (Schmitz et al., 2016).

2.9. T cell proliferation assay and assays for assessing HLA-DQ6 restriction

Proliferation assays were performed on T cell lines and TCCs in triplicate in 150 μl IMDM supplemented with 10% human serum in 96-well, flat-bottom plates (Corning Life Sciences, Tewksbury, USA) using 1×10^4 T cells stimulated with 1×10^5 irradiated *HLA-DQA1*01:02/DQB1*06:02* (HLA-DQ6)-matched allogeneic PBMCs (3000 RAD) in the presence or absence of either H1N1-HA$_{275–287}$ or Hcrt$_{56–68}$ and Hcrt$_{87–99}$ (10 μg/mL). In HLA-restriction experiments, the *HLA-DQA1*01:02/DQB1*06:02*-matched allogeneic PBMCs were replaced by PBMCs expressing either *HLA-DQA1*03:01/DQB1*03:02* (HLA-DQ8; no association with narcolepsy), *HLA-DQA1*01:02/DQB1*06:03* (less frequently found in narcolepsy compared to healthy controls (Tafti et al., 2014)) or a mix of *HLA-DQA1*01:02/DQB1*06:02* and *HLA-DQA1*01:02/DQB1*06:03*. Triplicate wells containing 10^4 T cells supplemented with 20 U/mL rIL-2 and 10 ng/mL IL-15 functioned as a positive control. After 48 h at 37 °C, cultures were pulsed with 10 μCi/mL of ^{3}H-thymidine and harvested 18 h later. Proliferation was measured using a MicroBeta Microplate Counter (PerkinElmer, Waltham, MA, USA). A positive response was defined as a stimulation index (SI) of 3 (defined as the mean count in the wells with peptides divided by the mean count in the wells without peptide) (Kooy-Winkelaar and Koning, 2015). Additionally, to confirm HLA restriction of the T cell lines, blocking experiments were performed in which either anti-HLA-class I (W6/32), anti-HLA-DP (B7/21), anti-HLA-DQ (SPV-L3) or anti-HLA-DR (B8.12.2) monoclonal antibodies (mAbs; locally produced) were added to the initial assay.

2.10. T cell receptor (TCR) sequencing

TRAV, TRBV and CDR3 gene segment sequences of H1N1-HA$_{275–287}$-specific TCCs were amplified using PCR and a set of specifically designed primers. PCR products rendered in this way were cloned into a Promega pGEM-T Easy vector and subsequently sequenced. The *TRAV* and *TRBV* gene usage and CDR3 sequences for all generated clones were determined using IMGT/V-QUEST (Brochet et al., 2008).

2.11. Statistical analysis

Differences at baseline in participant characteristics were calculated with Student's *t*-tests and Pearson's chi-square test. Pearson's chi-square test was also used for comparing T cell proliferation in narcolepsy patients as compared to healthy controls. Differences between conditions in the HLA blocking experiments were calculated by one-way ANOVA with a Bonferroni post hoc analysis. Differences between *P*-values below 0.05 were deemed significant. Bonferroni corrections were executed when needed. All analyses were conducted using the IBM SPSS Statistics 23 software package.

3. Results

3.1. Patient characteristics

We included 81 narcolepsy type 1 patients and 19 healthy controls. Patient characteristics are shown in Table 2. Notably, all narcolepsy type 1 patients except one were *HLA-DQA1*01:02/DQB1*06:02* (HLA-DQ6) positive. Thirty-four patients had undergone a lumbar puncture for Hcrt-1 measurement; all had Hcrt-1 values in the cerebrospinal fluid that were below the cut-off value of 110 pg/mL based on the ICSD-3 criteria for narcolepsy type 1. Narcolepsy type 1 patients were younger than healthy controls, but the distribution of males and females was comparable between the two groups.

3.2. H1N1-HA$_{275–287}$, Hcrt$_{56–68}$ and Hcrt$_{87–99}$ presented by HLA-DQ6 show structural homology

Based on earlier described peptides of H1N1-HA and hypocretin that are able to bind HLA-DQ6 (De La Herran-Arita et al., 2013, 2014), we investigated the possibility of molecular mimicry between H1N1-HA and Hcrt peptides in the context of HLA-DQ6 presentation. We crystallised and determined the crystal structures of H1N1-HA$_{275–287}$, Hcrt$_{56–68}$ and Hcrt$_{87–99}$ presented by HLA-DQ6 at resolutions of 1.7 Å, 2.0 Å and 1.95 Å, respectively (Fig. 1). Alignment of the structures revealed that the two Hcrt peptide - HLA-DQ6 complexes, HLA-DQ6-Hcrt$_{56–68}$ and HLA-DQ6-Hcrt$_{87–99}$, were nearly indistinguishable in terms of peptide backbone positioning (Cα rmsd < 0.1 Å), peptide sidechain conformations and HLA substructure surrounding the peptide. Compared to the two Hcrt complexes, the H1N1-HA$_{275–287}$ peptide was bound to HLA-DQ6 in the expected homologous register and with overall similar backbone positioning (Cα rmsd < 0.35 Å) and sidechain conformations. Within the 9-mer core of the bound peptides, the most notable differences were observed in the exposed positions p2 and p8 of the peptides (p2-histidine and p8-threonine in the Hcrt complexes, and p2-alanine and p8-isoleucine in the H1N1-HA complex). In addition to the differences in peptide sidechains, we observed a difference in the β-chain helix, which was positioned closer to the peptide in the H1N1-HA complex when compared to the two Hcrt complexes. This homology prompted us to determine whether this could also lead to T cell cross-reactivity to the H1N1-HA and hypocretin peptides in functional experiments.

Table 2
Characteristics of study participants. Data indicate mean ± standard deviation. P-values result from Student's *t*-tests for the continuous variables and Pearson's chi-square tests for dichotomous variables.

	Narcolepsy type 1	Healthy controls	p-Value
N	81	19	
Age (years)	31.6 ± 19.2	55.0 ± 8.4	< 0.001
Males (%)	39 (48.1%)	9 (47.4%)	0.951
HLA-DQ6 +	80/81	19/19	0.626
Hypocretin-1 < 110 pg/mL	34/34	Not available	Not applicable
H1N1 vaccination (%)	15/47 (32%)	Not available	Not applicable

3.3. HLA-DQ6-H1N1-HA$_{275–287}$-specific CD4+ T cells are readily detectable in narcolepsy type 1 patients and healthy controls, but do not cross-react with HLA-DQ6-Hcrt$_{56–68}$ or -Hcrt$_{87–99}$

T cell lines were generated from PBMCs of narcolepsy type 1 patients and healthy controls by co-culture with a pool of the H1N1-HA$_{275–287}$, Hcrt$_{56–68}$ and Hcrt$_{87–99}$ peptides for 5 days followed by expansion in culture medium containing IL-2 and IL-15. Subsequently, the specificity of these T cell lines against the H1N1-HA$_{275–287}$ peptide was determined by co-culture of the T cell lines with irradiated HLA-DQ6 positive allogeneic PBMCs in the presence or absence of the H1N1-HA$_{275–287}$ peptide. Specific proliferation was measured by determining the incorporation of ^{3}H-thymidine after three days of culture. Proliferation was defined as a stimulation index equal or higher to 3. All T cell lines proliferated in the positive control condition which reinforced our earlier conclusion based on visual assessing T cell lines that they were in good condition. In 28.4% (23/81) of narcolepsy type 1 patients and 15.8% (3/19) of healthy controls, an H1N1-HA$_{275–287}$-specific T cell proliferative response was observed (Fig. 2). To assess the specificity of the T cell lines against the Hcrt peptides, they were also stimulated with the Hcrt$_{56–68}$ and Hcrt$_{87–99}$ peptides. No reactivity to Hcrt$_{56–68}$ or Hcrt$_{87–99}$ was observed in T cell lines of either narcolepsy type 1 patients or controls (Fig. 2), indicating the absence of cross-reactivity of H1N1-HA$_{275–287}$-specific T cells with Hcrt$_{56–68}$ and Hcrt$_{87–99}$ in the context of HLA-DQ6. FACS analysis was performed on 13 HLA-DQ6-H1N1-HA$_{275–287}$-specific T cell lines. Analyses revealed a dominance of CD4+ T cells in these T cell lines (Fig. 3).

3.4. T cell reactivity to H1N1-HA$_{275–287}$ is HLA-DQ6-restricted

T cell clones (TCCs) were generated from the H1N1-HA$_{275–287}$-reactive T cell lines of 12 narcolepsy type 1 patients by either limiting dilution (in T cell lines of 3 narcolepsy type 1 patients) or isolation using streptavidin-PE-HLA-DQ6- H1N1-HA$_{275–287}$ tetramers (in T cell lines of 9 narcolepsy type 1 patients) and tested for reactivity against H1N1-HA$_{275–287}$. In TCCs generated from all H1N1-HA$_{275–287}$-specific CD4+ T cell lines, H1N1-HA$_{275–287}$-specific clonal T cell responses were found (Table 3).

To assess HLA-DQ6 restriction, T cell proliferation experiments were performed with irradiated allogeneic PBMCs of different haplotypes: *HLA-DQA1*01:02/DQB1*06:03, HLA-DQA1*03:01/DQB1*03:02* (HLA-DQ8) or a mix of *HLA-DQA1*01:02/DQB1*06:02* and *HLA-DQA1*01:02/DQB1*06:03* next to those expressing *HLA-DQA1*01:02/DQB1*06:02*. T cell proliferation was only observed in the presence of HLA-DQ6-expressing PBMCs and the H1N1-HA$_{275–287}$ peptide. Furthermore, when T cell proliferation experiments were performed with irradiated allogeneic PBMCs expressing HLA-DQ6, an anti-HLA-DQ mAb was able to block T cell proliferation of the generated TCCs. An anti-HLA-class I mAb blocked T cell proliferation considerably less effectively, whereas anti-HLA-DR and –DP mAbs did not affect T cell proliferation (Fig. 4A-B). These experiments confirm that the detected H1N1-HA$_{275–287}$-specific CD4+ T cell proliferation is HLA-DQ6-restricted.

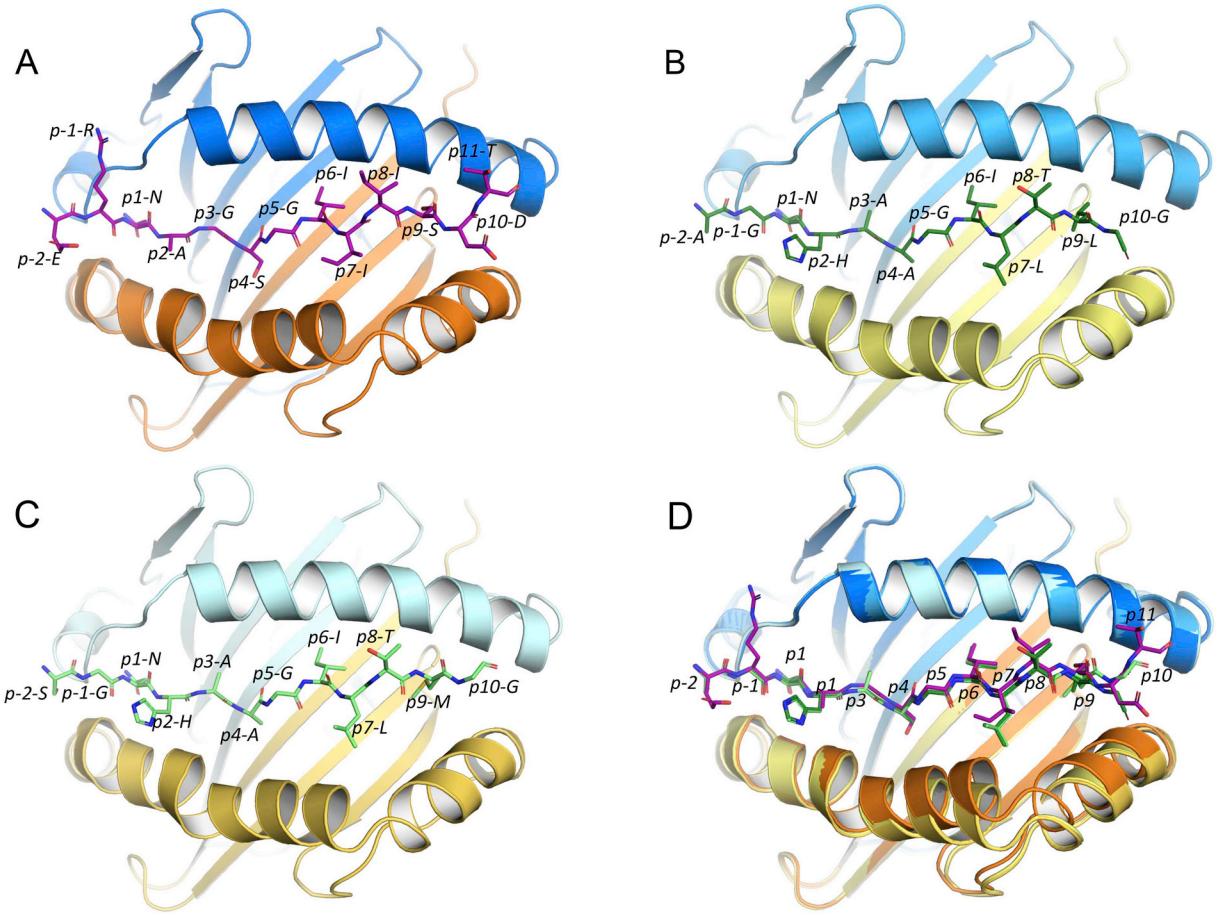


Fig. 1. HLA-DQ6 presenting H1N1-HA$_{275–287}$, Hcrt$_{56–68}$ and Hcrt$_{87–99}$ peptides show structural homology. A. H1N1-HA$_{275–287}$ peptide. B. Hcrt$_{56–68}$ peptide. C. Hcrt$_{87–99}$ peptide. D. Overlay A., B. and C.
HA = hemagglutinin; Hcrt = hypocretin.

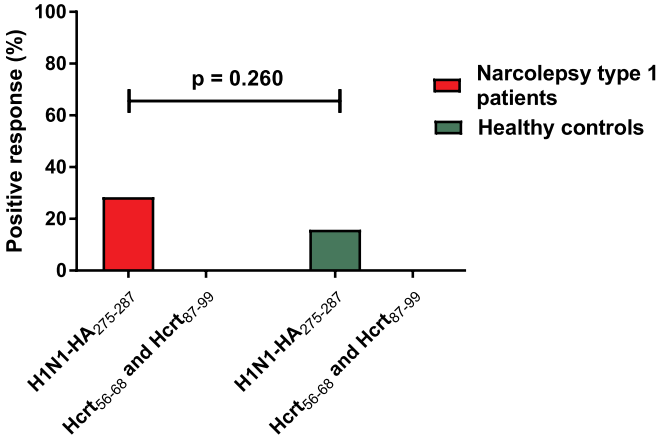


Fig. 2. Overview of T cell responses to H1N1-HA$_{275–287}$, Hcrt$_{56–68}$ and Hcrt$_{87–99}$ for narcolepsy type 1 patients (red) and healthy controls (green). The P-value results from a Mann-Whitney *U* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. The HLA-DQ6-H1N1-HA$_{275–287}$-specific T cell receptor repertoire in narcolepsy type 1 patients and healthy controls shows no biased expression

Recent reports describe a bias in TCR sequences for recognition of peptide-HLA complexes in CD4+ T cell mediated diseases (Qiao et al., 2014; Petersen et al., 2014). Since we were not able to show significant differences between percentages of narcolepsy type 1 patients and controls with HLA-DQ6-H1N1-HA$_{275–287}$-specific T cells, we searched for differences in the T cell repertoire used by narcolepsy type 1 patients and controls to mount immune responses to this antigen. TCRs expressed by 20 H1N1-HA$_{275–287}$-specific TCCs from 4 narcolepsy type 1 patients and 4 H1N1-HA$_{275–287}$-specific TCCs from 2 healthy controls were sequenced (Table 4). 18 TCR sequences were identified in TCCs of narcolepsy type 1 patients; 4 different TCR sequences in TCCs of healthy controls. There was expansion of some clones within a given patient, but these likely arose during the culturing process, with some T cell clones responding better to peptide hence expanding at a greater rate than others subsequently skewing the representative pool. Nevertheless, although only a small sample size was interrogated, no evidence for a biased *TRAV, TRBV* or CDR3 sequence motif was observed across unrelated individuals with narcolepsy type 1 or in healthy controls.

4. Discussion

HLA-DQ6-H1N1-HA peptide-specific CD4+ T cell responses were readily detected in both narcolepsy type 1 patients and healthy controls, with a higher proportion in the narcolepsy type 1 group. We did not detect HLA-DQ6-Hcrt peptide-specific T cell responses. Our experiments do not support the hypothesis that these Hcrt peptides are implicated in cross-reactivity leading to Hcrt-producing neuronal destruction and, thereby, to narcolepsy type 1.

The HLA-DQ6-H1N1-HA peptide-specific TCCs did show some, but

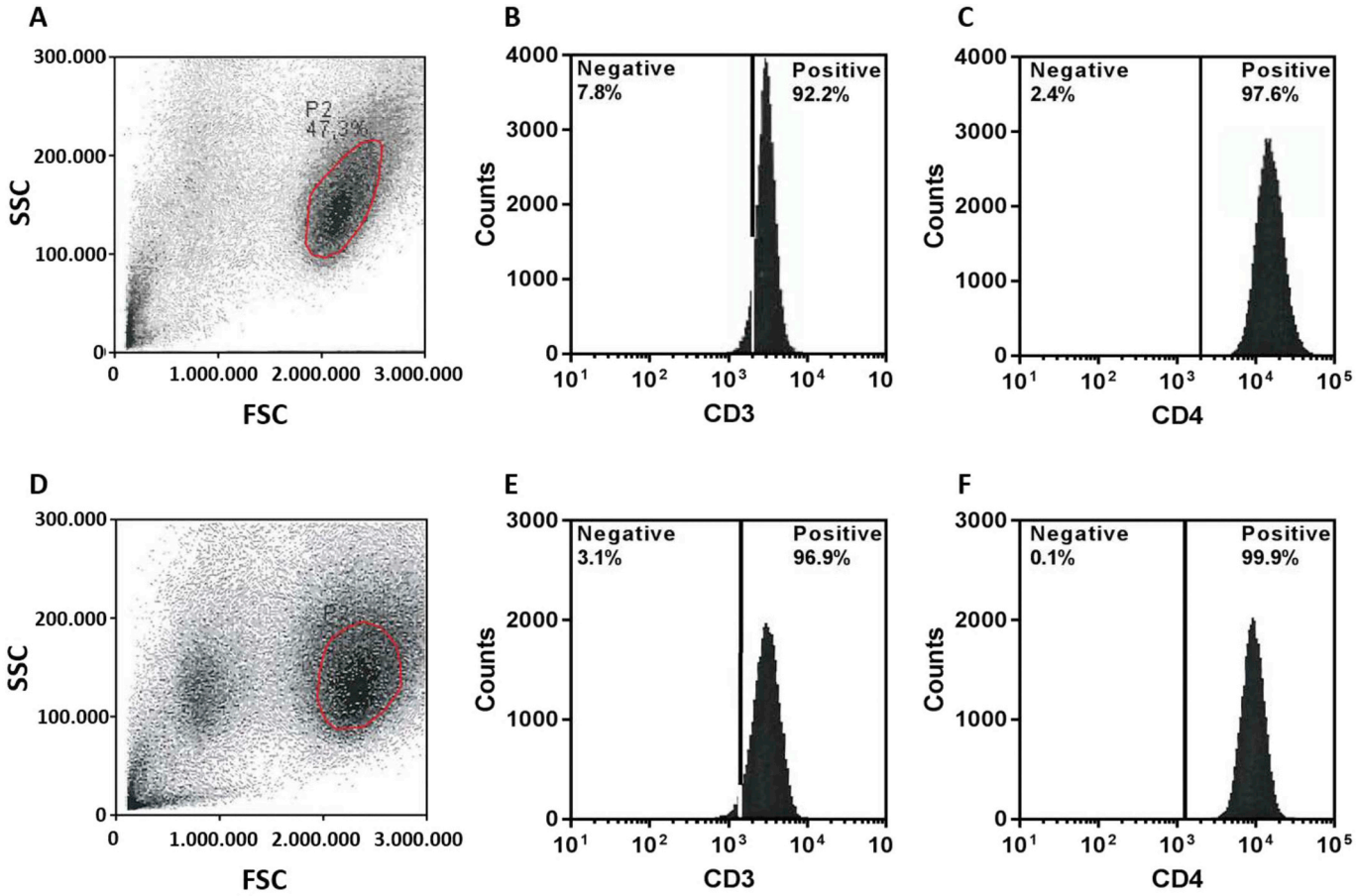


Fig. 3. H1N1-HA$_{275–287}$-specific T cell lines are predominantly CD4+. These fluorescence-activated cell sorting (FACS) plots show the gating strategy of two T cell lines in different patients (A-C and D-F). Living single cells are selected in the FSC-SSC plot (A and D). In the next plot CD3+ cells are selected (B and E), which show a high percentage of CD4+ cells in the last plot (C and F).

Table 3
H1N1-HA$_{275–287}$-specificity of the generated T cell clones (TCCs). All subjects are narcolepsy type 1 patients.

Subject number	H1N1 vaccination	TCCs tested	H1N1-HA$_{275–287}$-specific TCCs
19[a]	Yes	50	1
24	No	25	25
25	No	11	11
26	No	11	11
27[a]	Yes	109	19
29	Yes	5	5
32	Unknown	14	14
35	Unknown	12	12
38	Yes	11	10
39	Unknown	4	4
47	No	22	22
50[a]	Yes	130	23

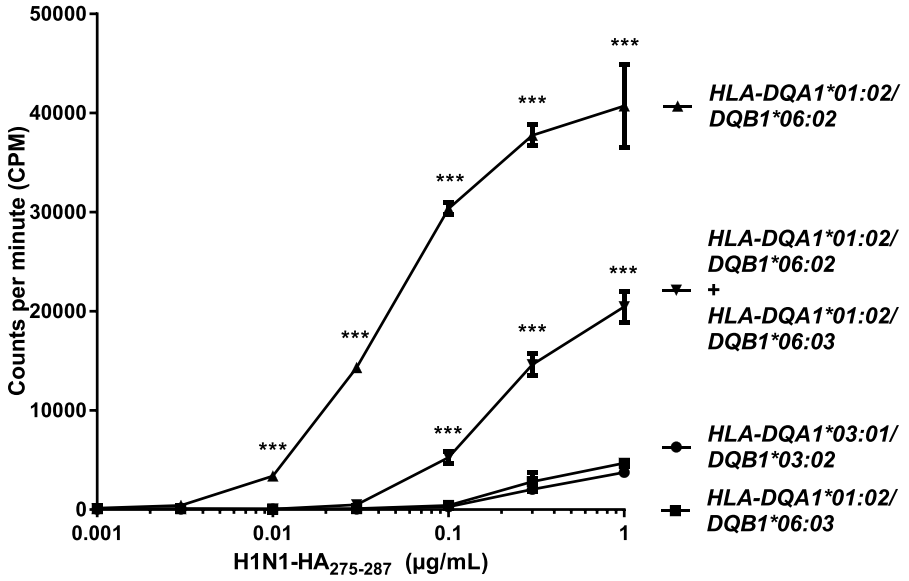
[a] TCC generation using limiting dilution instead of tetramer sorting.

no extensive clustering of T cell receptor sequences in narcolepsy type 1 patients. Larger studies would be required to determine if the H1N1-specific TCR repertoire in NT1 patients differs from controls.

Our findings add to those of others in the field who did show antibody-mediated reactivity to H1N1-HA in narcolepsy type 1 patients. Several groups found H1N1-HA specific antibodies in narcolepsy type 1 patients (Lind et al., 2017; Lind et al., 2014), but H1N1-HA specific T cells have not been described. The lack of reactivity to Hcrt peptides was reported in studies focusing on autoantibodies (Black et al., 2005) and CD4+ T cells (Ramberger et al., 2017; Kornum et al., 2017). HLA-DR-restricted Hcrt-specific T cell responses have recently been described (Latorre et al., 2018), but that study does not explain the strong link with HLA-DQ6. Interestingly, one other study (Ramberger et al., 2017) reported reactivity to Hcrt-peptide pools in a small minority of narcolepsy type 1 patients, although the peptides used differed from the ones in the current study.

Moreover, in our current study we focused on H1N1-HA and Hcrt peptides that display sequence homology, which does not rule out that other H1N1 peptides are involved. Another possibility is that the key differences between the peptides, a histidine residue at position 59 and 90 of the Hcrt$_{56–68}$ and Hcrt$_{87–99}$ peptides, respectively, as compared with an alanine residue in the corresponding position in the H1N1-HA$_{275–287}$ peptide, may prevent cross-reactive responses. We therefore also tested substitution variants of the Hcrt-peptides in which the histidine residues were replaced by an alanine, but we observed no cross-reactivity to these peptides as well, making it unlikely that post-translational modification of Hcrt-peptides underlies cross-reactivity (results not shown). In future studies we will therefore be testing T cell responses to peptide pools representing the H1N1 proteome and pre-prohypocretin.

One of the limitations in our study is that we have not been able to distinguish between narcolepsy type 1 patients and healthy controls that have actually encountered the H1N1 influenza virus and those who have not. We could therefore not be sure whether different exposure to the virus explains the lack of differences in H1N1-HA peptide-specific T cell responses between narcolepsy patients and healthy controls. However, T cell mediated cross-protective immunity generated by previous H1N1 infections was found to be common in the population (Miller et al., 2010).



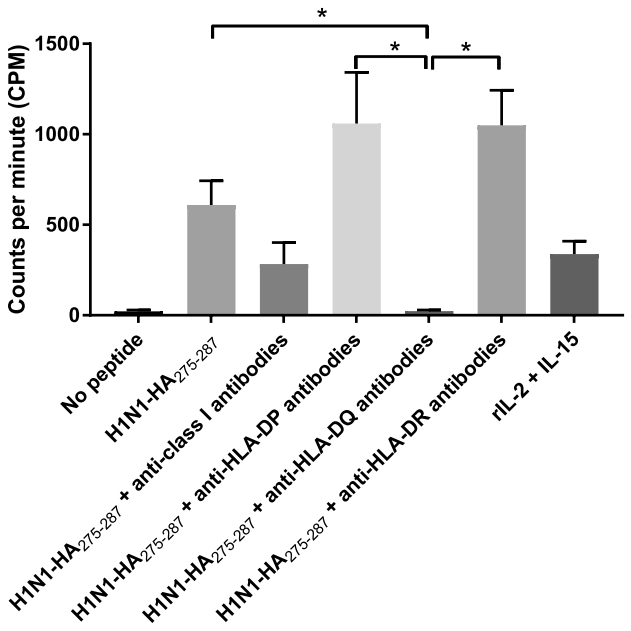


Fig. 4. A. A H1N1-HA$_{275–287}$ dose-dependent T cell response in a narcolepsy type 1 patient T cell clone (patient number 50, TCC 87) is found only in proliferation experiments performed with irradiated PBMCs that are *HLA-DQA1*01:02/ DQB1*06:02* (HLA-DQ6)-positive. Experiments were performed in triplicate wells. Significant differences relative to proliferation experiments with irradiated HLA-DQ8 (*HLA-DQA1*03:01/ DQB1*03:02*)-positive PBMCs. B. Anti-HLA-DQ mAb blocks T cell proliferation of the generated TCCs. An anti-HLA-class I mAb blocked T cell proliferation considerably less effectively, whereas anti-HLA-DR and –DP mAbs did not affect T cell proliferation.
* $p < .05$, ** $p < .01$, *** $p < .001$; mAb = monoclonal antibody; PBMCs = peripheral blood mononuclear cells; TCC = T cell clone.

Another limitation in our study is that we are performing our experiments in blood, while the actual destruction of Hcrt-producing neurons is taking place at the other side of the blood-brain barrier. It was recently described that T cell composition in cerebrospinal fluid differed between narcolepsy type 1 patients and healthy controls (Moresco et al., 2018). Repeating our experiments in cerebrospinal fluid would therefore be a better environment to test for cross-reactive T cells.

Additionally, detecting proliferation of a small immune subset that could have driven the destruction of the approximately 80,000 Hcrt-producing neurons (Thannickal et al., 2000) in a vast number of non-proliferating immune cells is technically difficult. The procedure used for peptide-specific TCC generation was also used in previous studies for the isolation of HLA-DQ-restricted gluten-specific T cell clones from patients with celiac disease in which we have determined the affinity of such T cells for the cognate HLA-DQ-gluten complexes. We observed that in cell-free assays the affinity values for some of these HLA-DQ-gluten specific TCRs were comparable with affinity values observed for microbial or non-self TCR–pMHC-II interactions, while others exhibited affinity values in line with those of low-affinity autoreactive TCR–pMHC complexes (Petersen et al., 2014). In cellular assays similar substantial differences were observed (Broughton et al., 2012). Therefore, this indicates that our method allows the identification of both high- and low-affinity T cell clones. However, we cannot exclude the possibility that with our technique we missed the detection of very low frequency T cells. The T cell library method that was performed in a recent publication on T cell reactivity to Hcrt (Latorre et al., 2018) would be an interesting technique to screen for these low frequency clones. Other novel approaches, such as mass cytometry, to address this rare immune subset problem are emerging with techniques that can isolate disease-specific immune subsets with unprecedented detail (Van Unen et al., 2017). Repeating our experiments with only those subsets that are specific for narcolepsy type 1, would significantly increase the odds of identifying rare cross-reactive immune cells in narcolepsy type 1 patients should they exist.

Table 4
T cell receptor sequences of 24 (20 narcolepsy type 1 patient, 4 healthy control) H1N1-$HA_{275–287}$-specific T cell clones (TCCs). Green and red values indicate receptor segments that are shared between TCCs of > 1 narcolepsy type 1 patient or healthy control.

Study number	Group	Clone	TRAV	TRAJ	CDR3	TRBV	TRBJ	TRBD	CDR3
19	Patient	4	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
19	Patient	8	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
19	Patient	11	25*01	38*01	CAGDAGNNRKLIW	4-2*01	1-4*01	1*01	CASSHQGINEKLFF
27	Patient	128	8-6*01	54*01	CAVTSPPIQGAQKLVF	4-2*01	2-1*01	1*01	CASSQGNGPYNEQFF
27	Patient	161	17*01	34*01	CATDAYNTDKLIF	4-2*01	1-2*01	1*01	CASSQASTGGSNYGYTF
27	Patient	161	21*01	43*01	CAVVHDMRF				
27	Patient	118	25*01	49*01	CAVNTGNQFYF	20-1*01	1-6*01	1*01	CSARVGQDSSPLHF
27	Patient	131	25*01	31*01	CAGGNNNARLMF	20-1*01	2-5*01	1*01	CSAAPGLRPQETQYF
27	Patient	145	13-1*01	37*01	CAPGSGNTGKLIF	12-3*01	2-5*01	2*01	CASSFQDYPQETQY
27	Patient	145	12-1*01	42*01	CVVNDIHYGGSQGNLIF				
27	Patient	125	26-2*01	23*01	CILRSHYNQGGKLIF				
38	Patient	108				4-3*01	2-1*01	1*01	CASSQGGGMGFDEQF
50	Patient	9	17*01	34*01	CATASYNTDKLIF	4-3*01	1-4*01	1*01	CASSRGTAATNEKLF
50	Patient	32	17*01	34*01	CATASYNTDKLIF				
50	Patient	37	19*01	30*01	CALSEDENRDDKIIF	3-1*01	1-6*01	1*01	CASSQSRVSSPLHF
50	Patient	38	17*01	37*01		4-1*01	1-1*01	2*02	CASSQSEGAEAFF
50	Patient	68	12-2*01	26*01	CAVNKGSNYGQNFVF				
50	Patient	80	17*01	34*01	CATSSYNTDKLIF	4-3*01	1-1*01	2*02	CASSSGRGSMNTEAFF
50	Patient	87	17*01	34*01	CATASYNTDKLIF	6-2*01	1-1*01	1*01	CASSAGTGAFF
50	Patient	99	12-2*01	26*01	CAVNKGSNYGQNFVF	20-1*01	1-5*01	1*01	CSAATGTGETFYNQPQHF
102	Control	9	12-2*01/02/03	49*01	CAVHHTNTGNQFYF	19*01/02/03	2-5*01	1*01	CASSRGTGGKETQYF
102	Control	11	26-2*01	3*01	CTQSSASKIIF	4-2*01/4-3*01/03/04	2-5*01	1*01	CASSQASGGLGETQYF
103	Control	1	12-3*01/02	6*01	CAMILSGGSYIPTF	20-1*01/02/03/04/05	1-1*01	1*01	CSAPKNTEAFF
103	Control	2	13-1*01	54*01	CAEGGIQGAQKLVF				

A = alanine; C = cysteine; CDR3 = complementarity-determining region 3; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; TRAJ = T-cell receptor alpha joining segment; TRAV = T-cell receptor alpha variable segment; TRBD = T-cell receptor beta diversity segment; TRBJ = T-cell receptor beta joining segment; TRBV = T-cell receptor beta variable segment; V = valine; W = tryptophan; Y = tyrosine.

5. Conclusions

We identified HLA-DQ6-restricted H1N1-HA peptide-specific T cell responses in a subset of narcolepsy type 1 patients and healthy controls. We did not find HLA-DQ6-H1N1-HA peptide-specific T cells cross-reactive to Hcrt-2 peptides. These results indicate that it is unlikely that cross-reactivity between H1N1-HA and Hcrt-2 peptides presented by HLA-DQ6 is involved in the development of narcolepsy.

Conflicts of interests

The authors confirm no conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.04.009.

References

Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., Mccoy, A.J., Moriarty, N.W., Oeffner, R., Read, R.J., Richardson, D.C., Richardson, J.S., Terwilliger, T.C., Zwart, P.H., 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221.

American Academy of Sleep Medicine, 2014. International Classification of Sleep Disorders. American Academy of Sleep Medicine, Darien, IL.

Black 3rd, J.L., Silber, M.H., Krahn, L.E., Avula, R.K., Walker, D.L., Pankratz, V.S., Fredrickson, P.A., Slocumb, N.L., 2005. Studies of humoral immunity to prepro-hypocretin in human leukocyte antigen DQB1*0602-positive narcoleptic subjects with cataplexy. Biol. Psychiatry 58, 504–509.

Brochet, X., Lefranc, M.P., Giudicelli, V., 2008. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 36, W503–W508.

Broughton, S.E., Petersen, J., Theodossis, A., Scally, S.W., Loh, K.L., Thompson, A., Van Bergen, J., Kooy-Winkelaar, Y., Henderson, K.N., Beddoe, T., Tye-Din, J.A., Mannering, S.I., Purcell, A.W., Mccluskey, J., Anderson, R.P., Koning, F., Reid, H.H., Rossjohn, J., 2012. Biased T cell receptor usage directed against human leukocyte antigen DQ8-restricted gliadin peptides is associated with celiac disease. Immunity 37, 611–621.

Dauvilliers, Y., Arnulf, I., Lecendreux, M., Monaca Charley, C., Franco, P., Drouot, X., D'ortho, M.P., Launois, S., Lignot, S., Bourgin, P., Nogues, B., Rey, M., Bayard, S., Scholz, S., Lavault, S., Tubert-Bitter, P., Saussier, C., Pariente, A., 2013. Increased risk of narcolepsy in children and adults after pandemic H1N1 vaccination in France. Brain 136, 2486–2496.

De La Herran-Arita, A.K., Kornum, B.R., Mahlios, J., Jiang, W., Lin, L., Hou, T., Macaubas, C., Einen, M., Plazzi, G., Crowe, C., Newell, E.W., Davis, M.M., Mellins, E.D., Mignot, E., 2013. CD4+ T cell autoimmunity to hypocretin/orexin and cross-reactivity to a 2009 H1N1 influenza A epitope in narcolepsy. Sci. Transl. Med. 5, 216ra176.

De La Herran-Arita, A.K., Kornum, B.R., Mahlios, J., Jiang, W., Lin, L., Hou, T., Macaubas, C., Einen, M., Plazzi, G., Crowe, C., Newell, E.W., Davis, M.M., Mellins, E.D., Mignot,

E., 2014. Retraction of the research article: "CD4(+) T cell autoimmunity to hypocretin/orexin and cross-reactivity to a 2009 H1N1 influenza A epitope in narcolepsy". Sci. Transl. Med. 6, 247rt1.
Emsley, P., Lohkamp, B., Scott, W.G., Cowtan, K., 2010. Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.
Evans, P., 2006. Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82.
Faraco, J., Lin, L., Kornum, B.R., Kenny, E.E., Trynka, G., Einen, M., Rico, T.J., Lichtner, P., Dauvilliers, Y., Arnulf, I., Lecendreux, M., Javidi, S., Geisler, P., Mayer, G., Pizza, F., Poli, F., Plazzi, G., Overeem, S., Lammers, G.J., Kemlink, D., Sonka, K., Nevsimalova, S., Rouleau, G., Desautels, A., Montplaisir, J., Frauscher, B., Ehrmann, L., Hogl, B., Jennum, P., Bourgin, P., Peraita-Adrados, R., Iranzo, A., Bassetti, C., Chen, W.M., Concannon, P., Thompson, S.D., Damotte, V., Fontaine, B., Breban, M., Gieger, C., Klopp, N., Deloukas, P., Wijmenga, C., Hallmayer, J., Onengut-Gumuscu, S., Rich, S.S., Winkelmann, J., Mignot, E., 2013. ImmunoChip study implicates antigen presentation to T cells in narcolepsy. PLoS Genet. 9, e1003270.
Feltelius, N., Persson, I., Ahlqvist-Rastad, J., Andersson, M., Arnheim-Dahlstrom, L., Bergman, P., Granath, F., Adori, C., Hokfelt, T., Kuhlmann-Berenzon, S., Liljestrom, P., Maeurer, M., Olsson, T., Ortqvist, A., Partinen, M., Salmonson, T., Zethelius, B., 2015. A coordinated cross-disciplinary research initiative to address an increased incidence of narcolepsy following the 2009–2010 Pandemrix vaccination programme in Sweden. J. Intern. Med. 278, 335–353.
Han, F., Lin, L., Warby, S.C., Faraco, J., Li, J., Dong, S.X., An, P., Zhao, L., Wang, L.H., Li, Q.Y., Yan, H., Gao, Z.C., Yuan, Y., Strohl, K.P., Mignot, E., 2011. Narcolepsy onset is seasonal and increased following the 2009 H1N1 pandemic in China. Ann. Neurol. 70, 410–417.
Han, F., Faraco, J., Dong, X.S., Ollila, H.M., Lin, L., Li, J., An, P., Wang, S., Jiang, K.W., Gao, Z.C., Zhao, L., Yan, H., Liu, Y.N., Li, Q.H., Zhang, X.Z., Hu, Y., Wang, J.Y., Lu, Y.H., Lu, C.J., Zhou, W., Hallmayer, J., Huang, Y.S., Strohl, K.P., Pollmacher, T., Mignot, E., 2013. Genome wide analysis of narcolepsy in China implicates novel immune loci and reveals changes in association prior to versus after the 2009 H1N1 influenza pandemic. PLoS Genet. 9, e1003880.
Henderson, K.N., Reid, H.H., Borg, N.A., Broughton, S.E., Huyton, T., Anderson, R.P., Mccluskey, J., Rossjohn, J., 2007. The production and crystallization of the human leukocyte antigen class II molecules HLA-DQ2 and HLA-DQ8 complexed with deamidated gliadin peptides implicated in coeliac disease. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 63, 1021–1025.
Hor, H., Kutalik, Z., Dauvilliers, Y., Valsesia, A., Lammers, G.J., Donjacour, C.E., Iranzo, A., Santamaria, J., Peraita Adrados, R., Vicario, J.L., Overeem, S., Arnulf, I., Theodorou, I., Jennum, P., Knudsen, S., Bassetti, C., Mathis, J., Lecendreux, M., Mayer, G., Geisler, P., Beneto, A., Petit, B., Pfister, C., Burki, J.V., Didelot, G., Billiard, M., Ercilla, G., Verduijn, W., Claas, F.H., Vollenweider, P., Waeber, G., Waterworth, D.M., Mooser, V., Heinzer, R., Beckmann, J.S., Bergmann, S., Tafti, M., 2010. Genome-wide association study identifies new HLA class II haplotypes strongly protective against narcolepsy. Nat. Genet. 42, 786–789.
Juji, T., Satake, M., Honda, Y., Doi, Y., 1984. HLA antigens in Japanese patients with narcolepsy. All the patients were DR2 positive. Tissue Antigens 24, 316–319.
Kabsch, W., 2010. XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132.
Kooy-Winkelaar, Y., Koning, F., 2015. Isolation and cloning of gluten-specific T cells in celiac disease. Methods Mol. Biol. 1326, 53–59.
Kornum, B.R., Burgdorf, K.S., Holm, A., Ullum, H., Jennum, P., Knudsen, S., 2017. Absence of autoreactive CD4(+) T-cells targeting HLA-DQA1*01:02/DQB1*06:02 restricted hypocretin/orexin epitopes in narcolepsy type 1 when detected by EliSpot. J. Neuroimmunol. 309, 7–11.
Latorre, D., Kallweit, U., Armentani, E., Foglierini, M., Mele, F., Cassotta, A., Jovic, S., Jarrossay, D., Mathis, J., Zellini, F., Becher, B., Lanzavecchia, A., Khatami, R., Manconi, M., Tafti, M., Bassetti, C.L., Sallusto, F., 2018. T cells in patients with narcolepsy target self-antigens of hypocretin neurons. Nature 562, 63–68.
Lind, A., Ramelius, A., Olsson, T., Arnheim-Dahlstrom, L., Lamb, F., Khademi, M., Ambati, A., Maeurer, M., Nilsson, A.L., Bomfim, I.L., Fink, K., Lernmark, A., 2014. A/H1N1 antibodies and TRIB2 autoantibodies in narcolepsy patients diagnosed in conjunction with the Pandemrix vaccination campaign in Sweden 2009–2010. J. Autoimmun. 50, 99–106.
Lind, A., Freyhult, E., Ramelius, A., Olsson, T., Arnheim-Dahlstrom, L., Lamb, F., Khademi, M., Ambati, A., Maeurer, M., Lima Bomfim, I., Fink, K., Fex, M., Torn, C., Elding Larsson, H., Lernmark, A., 2017. Antibody affinity against 2009 a/H1N1 influenza and Pandemrix vaccine nucleoproteins differs between childhood narcolepsy patients and controls. Viral Immunol. 30, 590–600.
Mccoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., Read, R.J., 2007. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674.
Mignot, E., Hayduk, R., Black, J., Grumet, F.C., Guilleminault, C., 1997. HLA DQB1*0602 is associated with cataplexy in 509 narcoleptic patients. Sleep 20, 1012–1020.
Mignot, E., Lammers, G.J., Ripley, B., Okun, M., Nevsimalova, S., Overeem, S., Vankova, J., Black, J., Harsh, J., Bassetti, C., Schrader, H., Nishino, S., 2002. The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. Arch. Neurol. 59, 1553–1562.
Miller, E., Hoschler, K., Hardelid, P., Stanford, E., Andrews, N., Zambon, M., 2010. Incidence of 2009 pandemic influenza a H1N1 infection in England: a cross-sectional serological study. Lancet 375, 1100–1108.
Moresco, M., Lecciso, M., Ocadlikova, D., Filardi, M., Melzi, S., Kornum, B.R., Antelmi, E., Pizza, F., Mignot, E., Curti, A., Plazzi, G., 2018. Flow cytometry analysis of T-cell subsets in cerebrospinal fluid of narcolepsy type 1 patients with long-lasting disease. Sleep Med. 44, 53–60.
Ohayon, M.M., Priest, R.G., Zulley, J., Smirne, S., Paiva, T., 2002. Prevalence of narcolepsy symptomatology and diagnosis in the European general population. Neurology 58, 1826–1833.
Ooi, J.D., Petersen, J., Tan, Y.H., Huynh, M., Willett, Z.J., Ramarathinam, S.H., Eggenhuizen, P.J., Loh, K.L., Watson, K.A., Gan, P.Y., Alikhan, M.A., Dudek, N.L., Handel, A., Hudson, B.G., Fugger, L., Power, D.A., Holt, S.G., Coates, P.T., Gregersen, J.W., Purcell, A.W., Holdsworth, S.R., La Gruta, N.L., Reid, H.H., Rossjohn, J., Kitching, A.R., 2017. Dominant protection from HLA-linked autoimmunity by antigen-specific regulatory T cells. Nature 545, 243–247.
Overeem, S., Verschuuren, J.J., Fronczek, R., Schreurs, L., Den Hertog, H., Hegeman-Kleinn, I.M., Van Duinen, S.G., Unmehopa, U.A., Swaab, D.F., Lammers, G.J., 2006. Immunohistochemical screening for autoantibodies against lateral hypothalamic neurons in human narcolepsy. J. Neuroimmunol. 174, 187–191.
Partinen, M., Saarenpaa-Heikkila, O., Ilveskoski, I., Hublin, C., Linna, M., Olsen, P., Nokelainen, P., Alen, R., Wallden, T., Espo, M., Rusanen, H., Olme, J., Satila, H., Arikka, H., Kaipainen, P., Julkunen, I., Kirjavainen, T., 2012. Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 pandemic vaccination campaign in Finland. PLoS One 7, e33723.
Petersen, J., Montserrat, V., Mujico, J.R., Loh, K.L., Beringer, D.X., Van Lummel, M., Thompson, A., Mearin, M.L., Schweizer, J., Kooy-Winkelaar, Y., Van Bergen, J., Drijfhout, J.W., Kan, W.T., La Gruta, N.L., Anderson, R.P., Reid, H.H., Koning, F., Rossjohn, J., 2014. T-cell receptor recognition of HLA-DQ2-gliadin complexes associated with celiac disease. Nat. Struct. Mol. Biol. 21, 480–488.
Peyron, C., Faraco, J., Rogers, W., Ripley, B., Overeem, S., Charnay, Y., Nevsimalova, S., Aldrich, M., Reynolds, D., Albin, R., Li, R., Hungs, M., Pedrazzoli, M., Padigaru, M., Kucherlapati, M., Fan, J., Maki, R., Lammers, G.J., Bouras, C., Kucherlapati, R., Nishino, S., Mignot, E., 2000. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat. Med. 6, 991–997.
Qiao, S.W., Christophersen, A., Lundin, K.E., Sollid, L.M., 2014. Biased usage and preferred pairing of alpha- and beta-chains of TCRs specific for an immunodominant gluten epitope in coeliac disease. Int. Immunol. 26, 13–19.
Ramberger, M., Hogl, B., Stefani, A., Mitterling, T., Reindl, M., Lutterotti, A., 2017. CD4+ T-cell reactivity to orexin/hypocretin in patients with narcolepsy type 1. Sleep 40.
Ripley, B., Overeem, S., Fujiki, N., Nevsimalova, S., Uchino, M., Yesavage, J., Di Monte, D., Dohi, K., Melberg, A., Lammers, G.J., Nishida, Y., Roelandse, F.W., Hungs, M., Mignot, E., Nishino, S., 2001. CSF hypocretin/orexin levels in narcolepsy and other neurological conditions. Neurology 57, 2253–2258.
Schmitz, F., Kooy-Winkelaar, Y., Wiekmeijer, A.S., Brugman, M.H., Mearin, M.L., Mulder, C., Chuva De Sousa Lopes, S., Mummery, C.L., Staal, F.J., Van Bergen, J., Koning, F., 2016. The composition and differentiation potential of the duodenal intraepithelial innate lymphocyte compartment is altered in coeliac disease. Gut 65, 1269–1278.
Tafti, M., Hor, H., Dauvilliers, Y., Lammers, G.J., Overeem, S., Mayer, G., Javidi, S., Iranzo, A., Santamaria, J., Peraita-Adrados, R., Vicario, J.L., Arnulf, I., Plazzi, G., Bayard, S., Poli, F., Pizza, F., Geisler, P., Wierzbicka, A., Bassetti, C.L., Mathis, J., Lecendreux, M., Donjacour, C.E., Van Der Heide, A., Heinzer, R., Haba-Rubio, J., Feketeova, E., Hogl, B., Frauscher, B., Beneto, A., Khatami, R., Canellas, F., Pfister, C., Scholz, S., Billiard, M., Baumann, C.R., Ercilla, G., Verduijn, W., Claas, F.H., Dubois, V., Nowak, J., Eberhard, H.P., Pradervand, S., Hor, C.N., Testi, M., Tiercy, J.M., Kutalik, Z., 2014. DQB1 locus alone explains most of the risk and protection in narcolepsy with cataplexy in Europe. Sleep 37, 19–25.
Tanaka, S., Honda, Y., Inoue, Y., Honda, M., 2006. Detection of autoantibodies against hypocretin, hcrtrl, and hcrtr2 in narcolepsy: anti-Hcrt system antibody in narcolepsy. Sleep 29, 633–638.
Thannickal, T.C., Moore, R.Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, J.M., 2000. Reduced number of hypocretin neurons in human narcolepsy. Neuron 27, 469–474.
Van Der Heide, A., Hegeman-Kleinn, I.M., Peeters, E., Lammers, G.J., Fronczek, R., 2015. Immunohistochemical screening for antibodies in recent onset type 1 narcolepsy and after H1N1 vaccination. J. Neuroimmunol. 283, 58–62.
Van Unen, V., Hollt, T., Pezzotti, N., Li, N., Reinders, M.J.T., Eisemann, E., Koning, F., Vilanova, A., Lelieveldt, B.P.F., 2017. Visual analysis of mass cytometry data by hierarchical stochastic neighbour embedding reveals rare cell types. Nat. Commun. 8, 1740.
Wijnans, L., Lecomte, C., De Vries, C., Weibel, D., Sammon, C., Hviid, A., Svanstrom, H., Molgaard-Nielsen, D., Heijbel, H., Dahlstrom, L.A., Hallgren, J., Sparen, P., Jennum, P., Mosseveld, M., Schuemie, M., Van Der Maas, N., Partinen, M., Romio, S., Trotta, F., Santuccio, C., Menna, A., Plazzi, G., Moghadam, K.K., Ferro, S., Lammers, G.J., Overeem, S., Johansen, K., Kramarz, P., Bonhoeffer, J., Sturkenboom, M.C., 2013. The incidence of narcolepsy in Europe: before, during, and after the influenza A(H1N1)pdm09 pandemic and vaccination campaigns. Vaccine 31, 1246–1254.