NARCOLEPSY

CD4$^+$ T Cell Autoimmunity to Hypocretin/Orexin and Cross-Reactivity to a 2009 H1N1 Influenza A Epitope in Narcolepsy

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Narcolepsy, a disorder strongly associated with human leukocyte antigen (HLA)–DQA1*01:02/DQB1*06:02 (DQ0602), is characterized by excessive daytime sleepiness, cataplexy, and rapid eye movement sleep abnormalities. It is caused by the loss of ~70,000 posterior hypothalamic neurons that produce the wake-promoting neuropeptide hypocretin (HCRT) (orexin). We identified two DQ0602-binding HCRT epitopes, HCRT$_{56–68}$ and HCRT$_{87–99}$, that activated a subpopulation of CD4$^+$ T cells in narcolepsy patients but not in DQ0602-positive healthy control subjects. Because of the established association of narcolepsy with the 2009 H1N1 influenza A strain (pH1N1), we administered a seasonal influenza vaccine (containing pH1N1) to patients with narcolepsy and found an increased frequency of circulating HCRT$_{56–68}$– and HCRT$_{87–99}$–reactive T cells. We also identified a hemagglutinin (HA) pHA1 epitope specific to the 2009 H1N1 strain, pHA1$_{275–287}$, with homology to HCRT$_{56–68}$ and HCRT$_{87–99}$. In vitro stimulation of narcolepsy CD4$^+$ T cells with pH1N1 proteins or pHA1$_{275–287}$ increased the frequency of HCRT$_{56–68}$– and HCRT$_{87–99}$–reactive T cells. Our data indicate the presence of CD4$^+$ T cells that are reactive to HCRT in narcolepsy patients and possible molecular mimicry between HCRT and a similar epitope in influenza pH1N1, pHA1$_{275–287}$.

INTRODUCTION

Type 1 narcolepsy is a lifelong disorder characterized by sleepiness, cataplexy, and rapid transitions from wakefulness to rapid eye movement sleep. The disease affects ~1 in 3000 individuals, and onset is typically in childhood or early adolescence (*1*–*3*). The disorder is caused by a lack of wake-promoting hypocretin (HCRT) signaling, resulting from the loss of the 70,000 hypothalamic neurons that normally produce the peptide (*4*, *5*).

An autoimmune basis for narcolepsy has long been suspected on the basis of a strong association with human leukocyte antigen (HLA)–DQ haplotype, DQA1*01:02/DQB1*06:02, encoding the DQ0602 heterodimer (*6*, *7*). Indeed, 98% of patients with narcolepsy and documented HCRT deficiency have this haplotype, whereas only 18 to 25% of the general U.S. population has it (*6*–*9*). Other HLA- and immune-related polymorphisms, for example, in the T cell receptor (TCR) locus, also modulate disease predisposition (*6*, *7*, *10*, *11*), consistent with involvement of HLA-DQ presentation of an autoantigen to CD4$^+$ T cells and subsequent autoimmunity. Despite these associations with immune loci and the discovery of a likely autoimmune target, HCRT-producing neurons, autoimmune responses have not been demonstrated (*12*, *13*). Many investigations have searched for autoantibodies; although associations have been found (*14*–*16*), these have not been consistently reproduced (*12*–*14*, *17*).

As in other autoimmune disorders, the development of narcolepsy also requires environmental or stochastic effects, as demonstrated by the 25 to 33% monozygotic twin concordance (*1*). For example, in the late 2000s, cases of childhood narcolepsy that began after infections with *Streptococcus pyogenes* were reported, and increased levels of anti–streptolysin O antibodies were found in sera of patients with recent onset (*18*, *19*). Monitoring the incidence of narcolepsy in China, Han *et al.* (*20*) found strong seasonal variation, suggesting that winter infections could trigger the suspected autoimmune cascade.

In the spring of 2010, a cluster of sudden-onset narcolepsy cases was reported in Europe and Scandinavia after vaccination with the AS03-associated Pandemrix influenza vaccine (*17*, *21*). This vaccine was designed to prevent the pandemic H1N1 influenza A strain of swine origin that originally appeared in Mexico in 2009 and spread rapidly through the world (*22*–*24*). Like most vaccines against this outbreak of pH1N1, it used the A/California/7/2009 (H1N1)pdm09–like reassortant flu virus, in this case containing three genes derived from pH1N1—hemagglutinin (HA), neuraminidase (NA), and polymerase basic 1 (PB1)—embedded in a backbone from an innocuous older strain (PR8; A/Puerto Rico/8/1934H1N1) that is used for vaccine virus production. In Europe and some other countries, vaccines were made by using proteins derived from this reassortant (enriched for surface proteins, notably HA and NA), both without and with adjuvants. The adjuvants included MF-59, a squalene-based adjuvant, and AS03, a squalene-α-tocopherol mix adjuvant; in the United States, only unadjuvanted vaccines were used (*24*).

Since then, the use of Pandemrix in children has been confirmed to increase narcolepsy risk across Europe (odds ratio = 4 to 17), most strikingly in Scandinavia, where vaccine coverage was high (*21*, *25*–*29*). Only a small minority of children, ~ 1 of 15,000 Pandemrix vaccinees, developed narcolepsy, and the pH1N1 pandemic infection unfolded in parallel with the vaccination campaign in most countries, complicating interpretation (*21*, *25*). For instance, an epidemiological

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association was noted in China, where a large rise in childhood-onset cases was associated with the pH1N1 outbreak, but independent of vaccination.

To investigate further the autoimmune basis of narcolepsy and its relationship to pH1N1, we sought to identify DQ0602-presented epitopes in HCRT [a peptide that is present only in the sleep-controlling hypothalamic neurons affected in narcolepsy (*12*)] that could activate $CD4^+$ T cells in blood samples from patients with narcolepsy. We further tested whether pH1N1 influenza virus proteins contained any epitopes that might activate these same cells.

RESULTS

Identification of DQ0602-binding prepro-HCRT epitopes

Overlapping 15-mer peptides (11–amino acid overlap) covering the entire prepro-HCRT protein were screened for their ability to compete with $EBV_{490–503}$ for DQ0602 binding in vitro by using a peptide binding competition assay. With this approach, we found both weak and strong DQ0602 binders (Fig. 1A and table S1), including a previously reported DQ0602-binding peptide located in the N-terminal leader peptide of prepro-HCRT (*30*). We noted that binding to DQ0602 was strong for the C-terminal ends of the two functional, secreted peptides of prepro-HCRT: HCRT-1 and HCRT-2 (peptides 13–15 and 21–23 in Fig. 1A).

$CD4^+$ T cell reactivity to $HCRT_{53–67}$ and $HCRT_{85–99}$

Although several studies have reported an absence of autoantibodies against HCRT in narcoleptic patients (*12*, *31*, *32*), we tested for the presence of T cell reactivity toward HCRT epitopes. To test for $CD4^+$ T cell reactivity, we created a transfectant cell line that would allow antigen presentation specifically by DQ0602 in a major histocompatibility complex (MHC) class I– and class II–deficient lymphoblastic T-B fusion cell line (T2.DQ0602) (*33*, *34*). Using these T2.DQ0602 cells as antigen-presenting cells (APCs), we tested $CD4^+$ T cells isolated from our subjects with each of the HCRT sequences that we had identified to have high DQ0602 affinity by

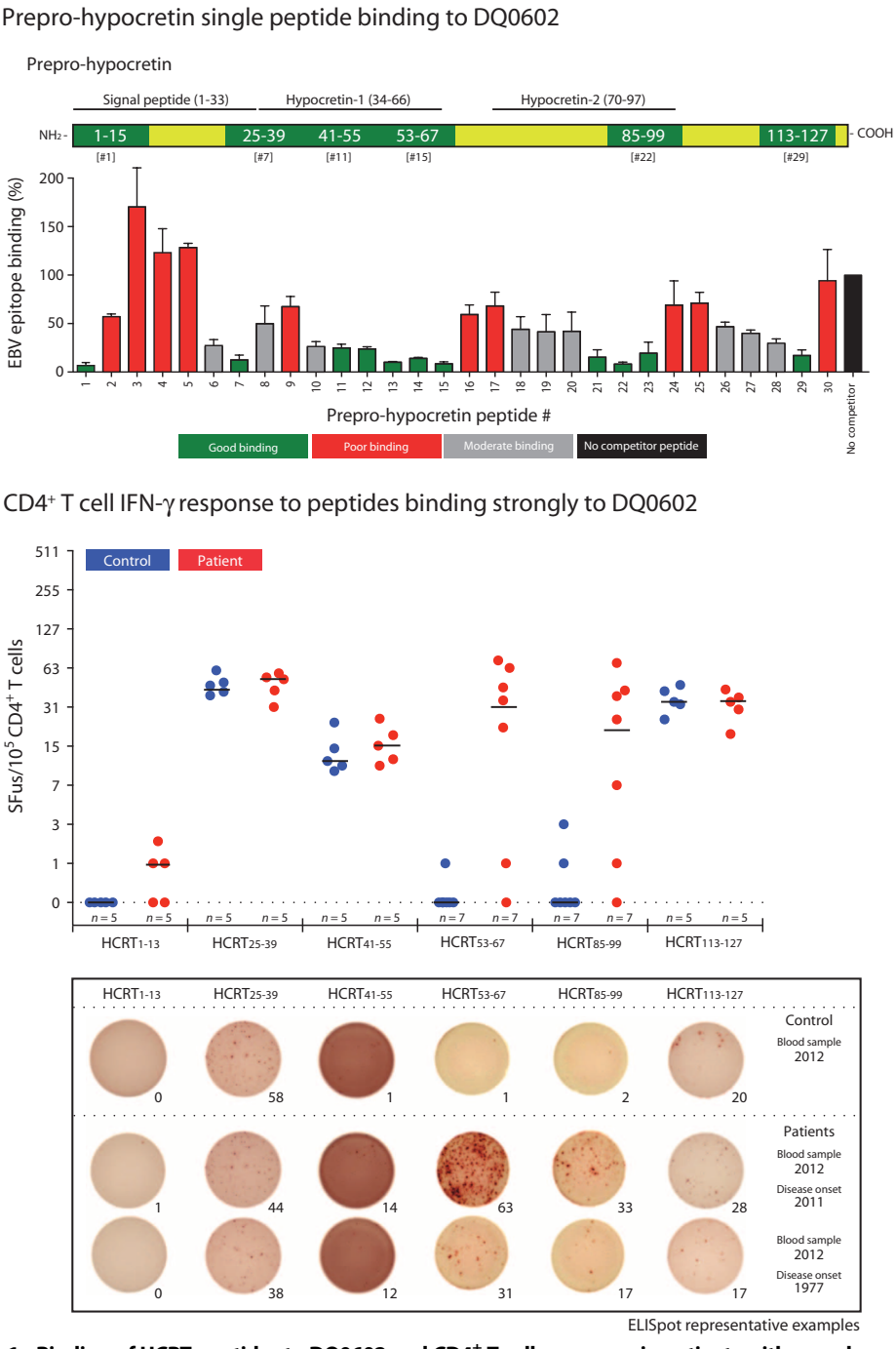


Fig. 1. Binding of HCRT peptides to DQ0602 and $CD4^+$ T cell responses in patients with narcolepsy. (**A**) Prepro-HCRT peptide binding to DQ0602. Overlapping 15-mer peptides (11–amino acid overlap) from the prepro-HCRT protein tested for competition with $EBV_{490–503}$ for DQ0602 binding in vitro. Binding was expressed as the percentage of the reference peptide ($EBV_{490–503}$) displaced; >75% decrease in signal is good binding, 50 to 75% decrease in signal is moderate binding, and <50% decrease in signal is poor binding. For details, see Materials and Methods. (**B**) $CD4^+$ T cell IFN-γ responses to peptides binding strongly to DQ0602 in cells from narcolepsy patients and control subjects. Top: ELISpot results (and median) from 24-hour in vitro stimulation of purified $CD4^+$ T cells (10^5 per well) from narcoleptic patients and DQB1*06:02-positive controls. Base 2 logarithmic scale. HCRT leader sequence ($HCRT_{1–13}$) and peptides $HCRT_{25–39}$, $HCRT_{41–55}$, and $HCRT_{113–127}$ were tested in five patients and five control subjects. Peptides $HCRT_{53–67}$ and $HCRT_{85–99}$ were tested in seven patients and seven controls. Bottom: Representative ELISpot images with spot-forming unit (SFU) counts for each peptide.

interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays.

We used $CD4^+$ T cells from patients with narcolepsy and from healthy controls and saw no reactivity in $CD4^+$ T cells collected from any of the subjects to the $HCRT_{1–13}$ leader sequence. In contrast, $CD4^+$ T cells from both patients and normal subjects contained cells that reacted to the other HCRT peptides, with two exceptions (Fig. 1B). $CD4^+$ T cells reactive to $HCRT_{53–67}$ and $HCRT_{85–99}$, a region corresponding to the C-terminal end of the two mature, secreted functional peptides, were present in patients with narcolepsy but not in healthy controls. Using shorter peptides (13-mer), we further defined the reactive epitopes in these two peptides as $HCRT_{56–68}$ and $HCRT_{87–99}$. $CD4^+$ T cell expression of tumor necrosis factor–α (TNF-α) by ELISpot was also stimulated by these two epitopes, suggesting a T helper 1 (T_H1) response (fig. S1).

Using $HCRT_{56–68}$ and $HCRT_{87–99}$, we next compared antigen presentation of these epitopes by autologous peripheral blood mononuclear cells (PBMCs), dendritic cells (DCs) differentiated in vitro from autologous monocytes, and T2.DQ0602 cells (with and without transfected HLA-DM). In all cases, there was greater reactivity in samples from narcoleptic patients than in those from healthy controls (Table 1). ELISpot responses in these assays correlated with each other, but additional background was observed with PBMCs when compared to other conditions (see intercepts in Table 2 and fig. S2). When we used the T2.DQ0602 cells, however, the amount of reactivity from control patients was close to zero, at the detection limit of the assay, resulting in a clear, narcolepsy-specific signal (Table 1 and fig. S2). We thus decided to use T2.DQ0602 cells (without DM) as APCs for all subsequent experiments.

Relation of $HCRT_{56–68}$ and $HCRT_{87–99}$ to disease onset and diagnostic value

We then used $HCRT_{56–68}$ and $HCRT_{87–99}$ presented by T2.DQ0602 cells to assess by ELISpot assays the reactivity of $CD4^+$ T cells from 23 patients with narcolepsy and 24 age- and gender-matched DQ0602-positive controls (Fig. 2A). Narcolepsy patients were selected to represent a broad spectrum, including recent and distant disease onset ($n = 9$, onset <2 years; $n = 8$, onset 3 to 9 years ago; $n = 6$, onset >13 years ago), and onset before ($n = 14$) and after ($n = 9$) the 2009 H1N1 pandemic. In almost all of these patients, we found strong $CD4^+$ T cell reactivity to $HCRT_{56–68}$ and $HCRT_{87–99}$, independent of collection date (ranging from 1991 to 2012); in contrast, samples from DQ0602-positive controls did not react (Fig. 2A).

To explore whether the amount of reactivity can be used as an indicator of disease, we used receiver operating characteristic (ROC) curve analysis, a statistical method to optimize sensitivity and specificity. We found that a cutoff of $HCRT_{56–68} \geq 3$ and $HCRT_{87–99} \geq 2$ SFUs/10^5 cells combined had a sensitivity of 0.83 (0.63 to 0.96) (all but four patients positive) and a specificity of 1.0 (1.0 to 1.0) (no control met criteria) (fig. S3). Three of the four patients without reactivity had long-lasting disease (9, 19, and 28 years), indicating that the autoimmune process may abate in some subjects over time. The fourth negative sample was drawn from a patient 1 year after onset in 1995. In two subjects with long-lasting disease (>18 years), anti-HCRT immunity was strong, suggesting that autoimmunity can also remain active or be reactivated by cross-reacting antigens.

$HCRT_{56–68}$ and $HCRT_{87–99}$ reactivity in discordant twin and post–Pandemrix vaccine subjects

Although our DQ0602-positive control samples were matched carefully (by age, gender, and time of collection), in four other patients, even more stringent controls were available in the form of monozygotic twins without narcolepsy. Within each pair of twins, $HCRT_{56–68}$ and $HCRT_{87–99}$ reactivity was observed only in the affected twin (Fig. 2B). We also studied 10 Irish children who developed narcolepsy after Pandemrix influenza vaccine administration and compared them

Table 1. Reactivity to HCRT peptides in samples from narcolepsy patients and control subjects. Data are shown as ELISpot count (IFN-γ SFUs/10^5 cells).

APCs	Patients	Controls	*P* (*U* test)
$HCRT_{56–68}$			
PBMCs	84 ± 17	40 ± 5	0.034
DCs	24 ± 3	5 ± 1	0.002
T2.DQ0602	32 ± 9	0.4 ± 0.4	0.010
T2.DQ0602 + DM	21 ± 3	0.5 ± 0.3	0.005
$HCRT_{87–99}$			
PBMCs	68 ± 21	31 ± 6	0.200
DCs	21 ± 4	7 ± 1	0.005
T2.DQ0602	26 ± 10	1 ± 1	0.007
T2.DQ0602 + DM	14 ± 1	0.6 ± 0.3	0.005

Table 2. Reactivity to HCRT peptides obtained with distinct APCs. For details, see fig. S2. r^2, correlation coefficient; *y*, best-fit linear equation.

APCs	$HCRT_{56–68}$	T2.0602 + DM	DCs	PBMCs
T2.DQ0602	r^2	0.96	0.86	0.92
	y	0.46*x* + 1.99	0.41*x* + 7.96	1.53*x* + 36.78
	P	<0.0001	<0.0001	<0.0001
T2.0602+DM	r^2		0.98	0.90
	y	1	1.01*x* + 3.45	3.24*x* + 29.37
	P		<0.0001	<0.0001
DCs	r^2			0.84
	y		1	2.91*x* + 19.01
	P			<0.0001
APCs	**$HCRT_{87–99}$**	**T2.0602 + DM**	**DCs**	**PBMCs**
T2.DQ0602	r^2	0.80	0.93	0.59
	y	0.26*x* + 3.45	0.45*x* + 7.97	1.14*x* + 33.71
	P	0.001	<0.0001	0.04
T2.0602+DM	r^2		0.81	0.68
	y	1	1.25*x* + 5.38	4.20*x* + 19.89
	P		0.001	0.02
DCs	r^2			0.50
	y		1	2.01*x* + 20.85
	P			0.08

to 7 of their unaffected siblings. The siblings that we selected as controls were DQ0602-positive and had also received the Pandemrix vaccine. Reactivity to the $HCRT_{56–68}$ and $HCRT_{87–99}$ epitopes was elevated only in those individuals with narcolepsy (Fig. 2C). These data demonstrate that T cell reactivity toward two HCRT epitopes presented by DQ0602 is very specific for type 1 narcolepsy.

$HCRT_{56–68}$ and $HCRT_{87–99}$ binding to narcolepsy-associated MHC class II and TCR recognition

We next analyzed $HCRT_{56–68}$ and $HCRT_{87–99}$ binding to DQ0602, and TCR responses to the DQ0602-peptide complex. Phenylalanine and lysine substitution scans (fig. S4) indicated that binding to the DQ0602 peptide-binding groove followed the register shown in Fig. 3A. Testing additional substitutions defined a broader motif tolerated by DQ0602: a preference for long side chains in P1, short side chains in P4, and medium hydrophobic side chains in P6, in line with reported binding motifs for DQ0602 (*35*). In contrast, P9 was uniquely promiscuous, accommodating any residue or a shorter peptide truncated at P10 ($HCRT_{56–66}$ and $HCRT_{87–97}$).

We also mapped putative TCR recognition/binding residues in the $HCRT_{56–68}$ epitope. Phenylalanine substitutions reduced T cell responses, strongly (P5 and P7), moderately (P8), or mildly (P2). These data imply that P5 and P7 are the most critical residues, similar to some other TCRs (*36*). Our data reflect assays of cells from five patients: T cells with $HCRT_{56–68}$ reactivity from other subjects may differ. The C-terminal P9-amidated variants of the shorter peptides $HCRT_{56–66–NH2}$ and $HCRT_{87–97–NH2}$, corresponding to the naturally processed/secreted HCRT-1 and HCRT-2, also bound DQ0602 (Fig. 3B).

Effect of trivalent seasonal flu vaccination on HCRT-reacting $CD4^+$ T cells in narcolepsy patients

On the basis of the proposed relation between the Pandemrix influenza vaccine and narcolepsy (*21*, *25*–*29*), we studied nine patients and four DQ0602-positive controls before and 6 to 9 days after they had received a 2012 seasonal unadjuvanted influenza vaccination. We tested whether the pH1N1 antigens contained in the seasonal vaccine could reactivate HCRT-reactive cells. None of the patients reported any exacerbation of their narcolepsy after the vaccination; this result was expected because these patients have lost most of their HCRT cells (*4*, *5*). After vaccination, however, we observed increases in $CD4^+$ T cell responses to the $HCRT_{56–68}$ and $HCRT_{87–99}$ epitopes (Fig. 4A). These findings raised the possibility that the flu vaccine contained an epitope similar to the HCRT epitopes that might be activating T cells by molecular mimicry. They are also consistent with data showing that

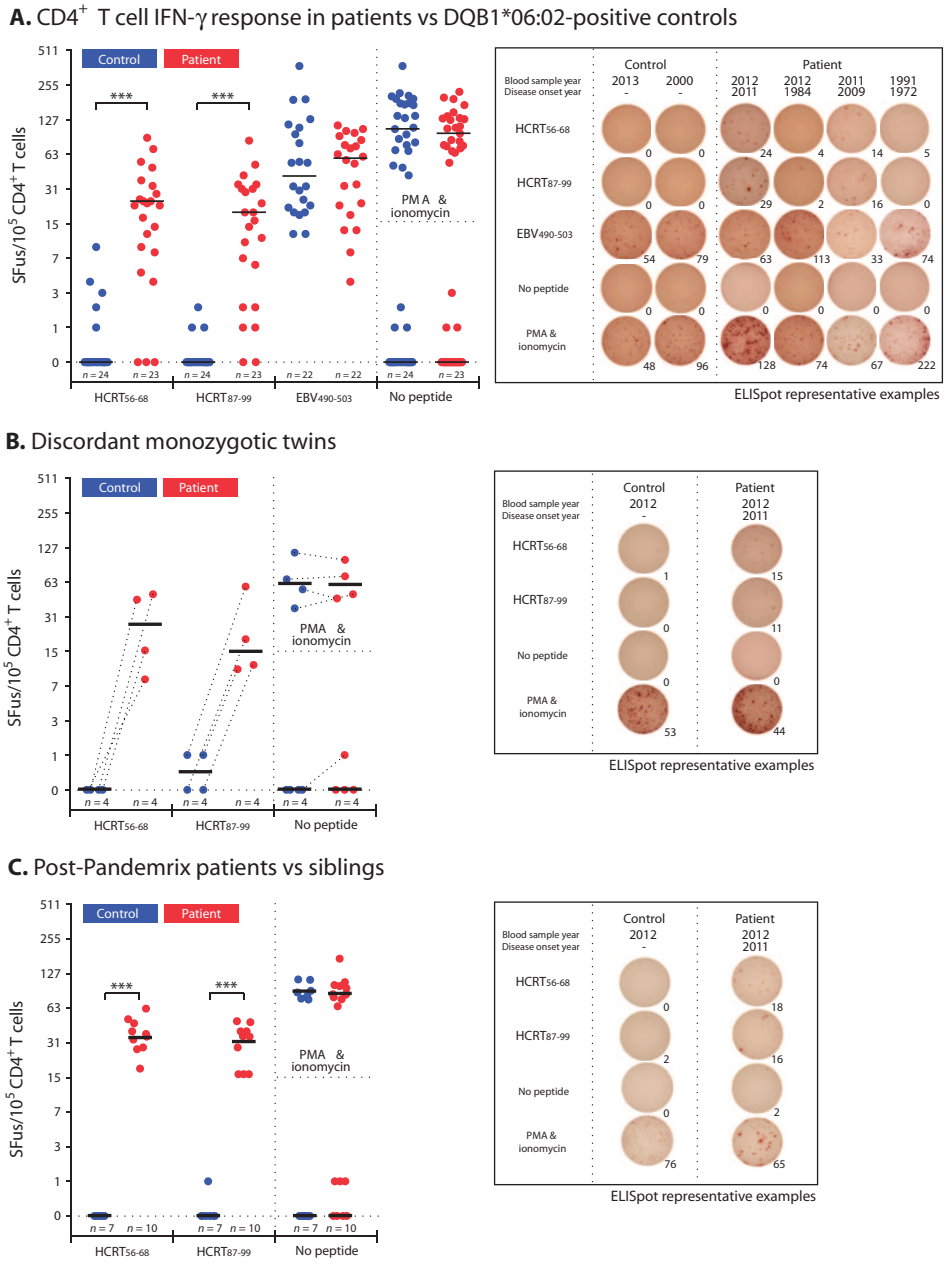


Fig. 2. HCRT peptides ($HCRT_{56–68}$ and $HCRT_{87–99}$) activate $CD4^+$ T cells in narcoleptic patients but not in healthy controls. HCRT peptides and $EBV_{490–503}$ were presented by T2.DQ0602 cells to purified $CD4^+$ T cells, and responding cells were detected by IFN-γ ELISpot. At right, representative ELISpot images with SFU counts are shown. (**A**) ELISpot results (and median) from 24-hour in vitro stimulation of purified $CD4^+$ T cells (10^5 per well) from narcoleptic patients (n = 23) and matched DQB1*06:02-positive controls (n = 24). (**B**) ELISpot results from four discordant monozygotic twin pairs (pairs marked with lines). (**C**) ELISpot results from Irish children who developed narcolepsy after Pandemrix vaccination (n = 10) and their vaccinated but healthy siblings (n = 7). PMA, phorbol 12-myristate 13-acetate. ***P < 0.001, Mann-Whitney-Wilcoxon U test.

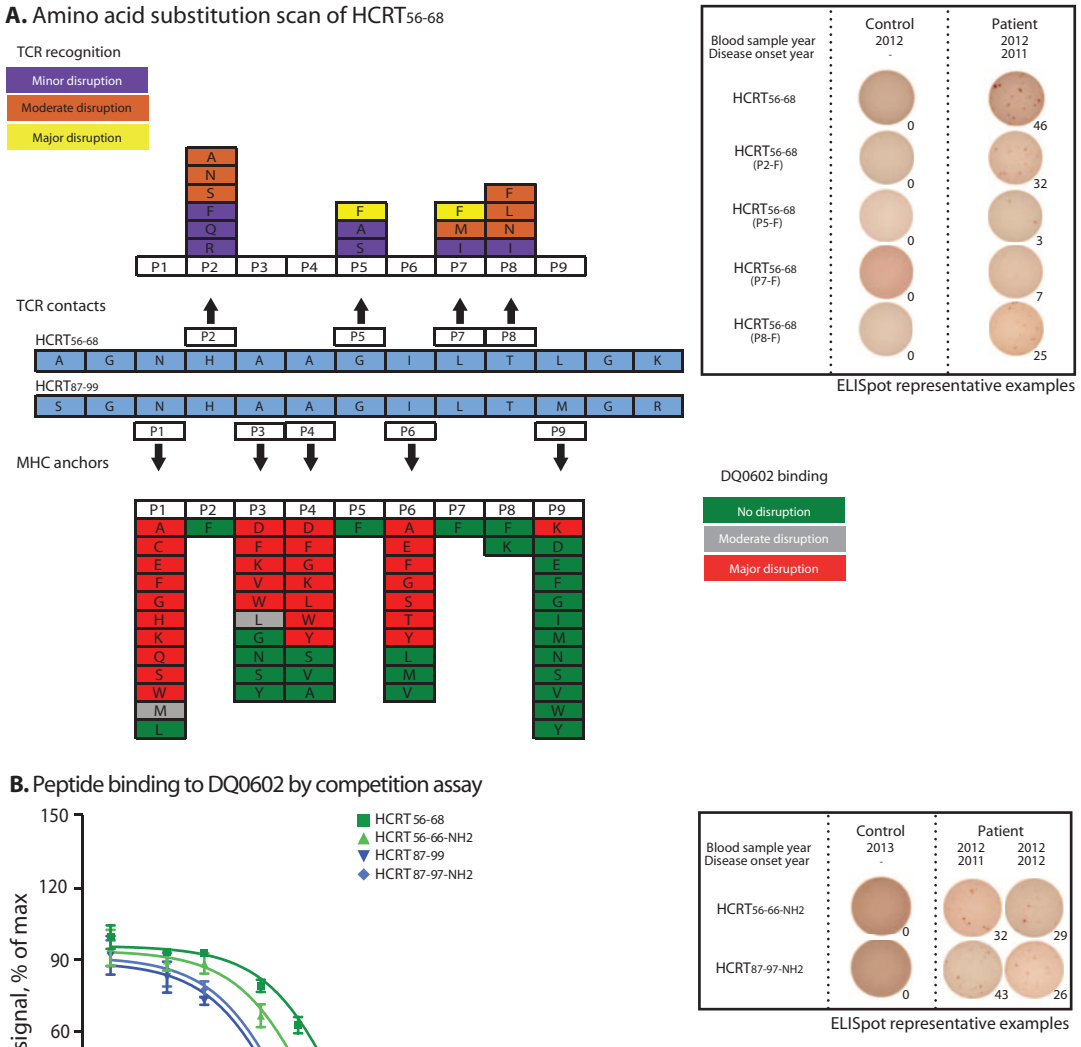


Fig. 3. Characterization of DQ0602-binding register and TCR contacts in narcolepsy-related HCRT epitopes. (**A**) Schematics of the effects of various single amino acid substitutions on TCR recognition (top, ELISpots assays of cells from five patients) and DQ0602 binding (bottom, displacement of biotinylated reference indicator peptide, $EBV_{490–503}$, on DQB0602; details in fig. S4). Shown are anchor residues for $HCRT_{56–68}$ and $HCRT_{87–99}$ binding to DQ0602 (P1, P3, P4, P6, and P9) and residues involved in subsequent TCR activation (P2, P5, P7, and P8) and the effects of various substitutions. (**B**) Inhibition of DQ0602 binding of biotinylated $EBV_{490–503}$ by $HCRT_{56–68}$, $HCRT_{87–99}$, and HCRT peptides with N-amidated C-terminal end (from secreted HCRT-1 and HCRT-2). Data are means ± SEM of three experiments, each performed in duplicate. EU signal, Eu^{3+}-labeled biotin measured with dissociation-enhanced time-resolved fluorometry.

vaccination with pH1N1 can activate cross-reactive T cells, for example, to epitopes of other infectious agents (*37*).

Effect of in vitro stimulation with pH1N1 antigens on HCRT-reacting $CD4^+$ T cells

We further tested for possible molecular mimicry by stimulating, in vitro, PBMCs from 14 narcoleptic patients and 5 DQ0602-positive controls with vaccine antigen. PBMCs were incubated with the entire pH1N1 split vaccine proteins (detergent-solubilized vaccine protein extracts enriched in surface proteins) and expanded for 13 days with interleukin-2 (IL-2) and IL-7. We then isolated $CD4^+$ T cells from the samples and tested for HCRT epitope reactivity by ELISpot. The stimulation with pH1N1 antigens activated pHA1-reactive $CD4^+$ T cells, as expected, but it also activated $HCRT_{56–68}$– and $HCRT_{87–99}$–reactive cells. The pH1N1-induced reactivity was epitope-specific because it was not seen for a DQ0602-binding Epstein-Barr virus (EBV) epitope (Fig. 4B).

Identification of DQ0602-binding pH1N1 epitopes

On the basis of these results, we hypothesized that epitope(s) from variable regions of pH1N1 viral and vaccine proteins could stimulate and expand HCRT-reactive cells in vivo and in vitro. To search for such epitopes, we tested overlapping 15-mer peptides covering the pHA1, pNA1, and pPB1 proteins present in A/California/7/2009 (pH1N1) for DQ0602 binding and identified 31 strong and 70 weak binders (fig. S5 and tables S2 to S5). We performed a bioinformatics analysis of the DQ0602-binding pH1N1 influenza peptides from our screen to identify possible mimics of $HCRT_{56–68}$ and $HCRT_{87–99}$ (see Materials and Methods). Five candidate peptides (two from HA1, two from NA1, and one from PB1) were identified and tested in samples from narcolepsy and control subjects, after presentation by T2.DQ0602 cells.

Although these peptides gave rise to some T cell reactivity, it was generally variable among both patients and controls (fig. S6A), with the exception of $pHA1_{275–287}$, which produced consistent responses in most subjects independent of narcolepsy status (fig. S6B) and date of collection (26 ± 5 versus 26 ± 9 SFUs/10^5 cells for samples collected before versus after 2009, respectively). This epitope was particularly interesting because it is specific to pandemic 2009 H1N1 and has homology at P5, P7, and P8 to the $HCRT_{56–68}$ and $HCRT_{87–99}$ epitopes (Fig. 5A). A phenylalanine substitution scan of $pHA1_{275–287}$

was performed (fig. S4, C and D) and implied the alignment shown in Fig. 5A. The median inhibitory concentration (IC_{50}) for in vitro binding of $pHA1_{275–287}$ to DQ0602 (IC_{50} = 1.9 ± 1.1 μM) was similar to that of the HCRT epitopes (IC_{50} = 16.7 ± 1.1 μM and 3.4 ± 1.1 μM, see also Figs. 3B and 5B), and $CD4^+$ reactivity was stimulated at a similar range of concentrations (fig. S6C).

Effect of in vitro stimulation with $pHA1_{275–287}$ on HCRT-reacting $CD4^+$ T cells

To test whether $pHA1_{275–287}$ could be involved in activating $HCRT_{56–68}$– and $HCRT_{87–99}$–reactive $CD4^+$ T cells after pH1N1 exposure, we again performed in vitro antigen stimulations (Fig. 6, A and B, and fig. S7A). PBMCs from 14 patients and 5 DQ0602-positive controls (all 19 samples collected after 2009) were stimulated with $pHA1_{275–287}$ (1 μM) in culture for 13 days, as described above for the vaccine antigens. Stimulation of samples from narcolepsy patients with $pHA1_{275–287}$ in vitro significantly increased the proportion of $HCRT_{56–68}$ and $HCRT_{87–99}$ epitope–specific T cells (Fig. 6A). The proportion of $pHA1_{275–287}$–reactive cells also increased, as expected. In contrast, the population of $EBV_{490–503}$–responsive T cells decreased, implying that other cells expand and dilute the $EBV_{490–503}$–reactive cells. We also stimulated PBMCs from these same subjects with $HCRT_{56–68}$ plus $HCRT_{87–99}$ (1 μM) (Fig. 6B). Although HCRT-reactive cells were significantly enriched following this expansion, results were more variable, possibly because peptide capture by other HLA subtypes may occur.

Effect of enrichment of $CD4^+$ T cells reactive to $pHA1_{275–287}$ by CD38 capture on HCRT-reacting $CD4^+$ T cells

Because the cross-culture studies described above are long-term experiments in which many cell subpopulations could change, we also conducted experiments using only 24 hours of stimulation with the two HCRT epitopes, $pHA1_{275–287}$, or $EBV_{490–503}$ presented by T2.DQ0602, followed by purification of activated $CD4^+$ T cells with an antibody to CD38 (*38*) (Fig. 6, C and D, and fig. S7B). This acute exposure to $HCRT_{56–68}$ plus $HCRT_{87–99}$, or $pHA1_{275–287}$ increased the proportion of $CD4^+$ T cells reacting to the antigens, although $HCRT_{56–68}$–reactive T cells were not increased significantly by $pHA1_{275–287}$ exposure (Fig. 6, C and D). In cultures stimulated acutely with the EBV epitope, EBV-

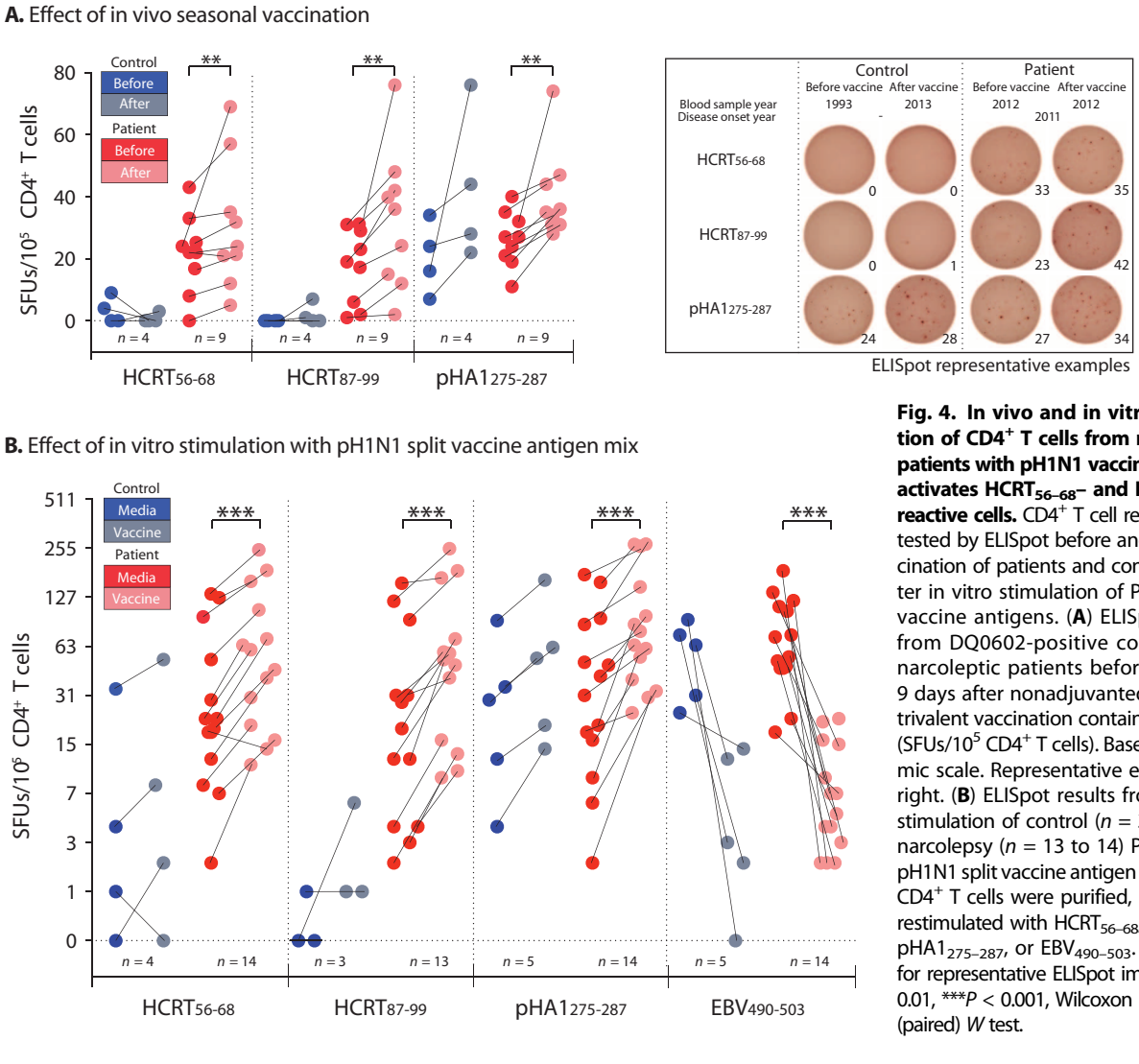


Fig. 4. In vivo and in vitro stimulation of $CD4^+$ T cells from narcolepsy patients with pH1N1 vaccine antigens activates $HCRT_{56–68}$– and $HCRT_{87–99}$–reactive cells. $CD4^+$ T cell reactivity was tested by ELISpot before and after vaccination of patients and controls, or after in vitro stimulation of PBMCs with vaccine antigens. (**A**) ELISpot results from DQ0602-positive controls and narcoleptic patients before and 5 to 9 days after nonadjuvanted influenza trivalent vaccination containing pH1N1 (SFUs/10^5 $CD4^+$ T cells). Base 2 logarithmic scale. Representative examples at right. (**B**) ELISpot results from in vitro stimulation of control (n = 3 to 5) and narcolepsy (n = 13 to 14) PBMCs with pH1N1 split vaccine antigen for 13 days. $CD4^+$ T cells were purified, rested, and restimulated with $HCRT_{56–68}$, $HCRT_{87–99}$, $pHA1_{275–287}$, or $EBV_{490–503}$. See fig. S7 for representative ELISpot images. $^{**}P < 0.01$, $^{***}P < 0.001$, Wilcoxon signed-rank (paired) W test.

A. Peptide sequence alignment

	P1	P2	P3	P4	P5	P6	P7	P8	P9
$HCRT_{56-68}$	A	G	N	H	A	A	G	I	L	T	L	G	K
$HCRT_{87-99}$	S	G	N	H	A	A	G	I	L	T	M	G	R
$pHA1_{275-287}$	E	R	N	A	G	S	G	I	I	I	S	D	T
A/New York/490/2003	S	R	G	F	G	S	G	I	I	I	S	N	A
A/Wisconsin/10/1998	K	R	N	S	G	S	G	I	I	I	S	D	T
A/Puerto Rico/8/1934	S	R	G	F	G	S	G	I	I	T	S	N	A
A/Brevig Mission/1/1918	A	L	N	R	G	S	G	S	G	I	I	T	S

B. Peptide binding to DQ0602 in vitro

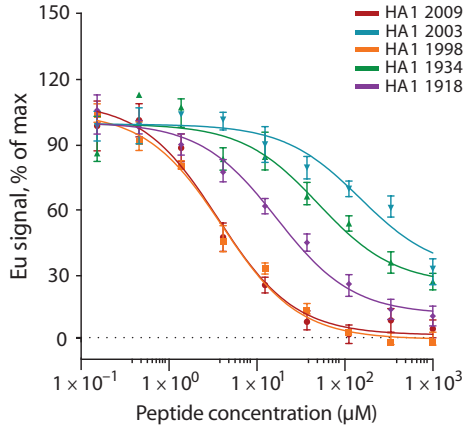


Fig. 5. Possible molecular mimicry between $pHA1_{275-287}$ (pH1N1) and $HCRT_{56-68}$/$HCRT_{87-99}$. (**A**) Alignment of $HCRT_{56-68}$, $HCRT_{87-99}$, and $pHA1_{275-287}$ with sequences from other seasonal and pandemic flu strains. Single amino acid substitution scans of $pHA1_{275-287}$ (fig. S4) established the binding register depicted here. (**B**) Inhibition of DQ0602 binding of $EBV_{490-503}$ by HA1 epitopes.

reacting cells increased, whereas pHA1 and the HCRT-reactive cells were diluted (Fig. 4E), indicating the specificity of the stimulation.

DISCUSSION

Here, we demonstrate that $CD4^+$ T cells reactive to the presentation of $HCRT_{56-68}$ and/or $HCRT_{87-99}$ by DQ0602 are present in abundance in most patients with narcolepsy. The epitopes in question are part of a functional, posttranslationally modified, secreted neurotransmitter, mature HCRT. The presence of these autoreactive T cells was most evident when presentation of the epitopes was restricted to DQ0602, the antigen-presenting molecule strongly associated with narcolepsy.

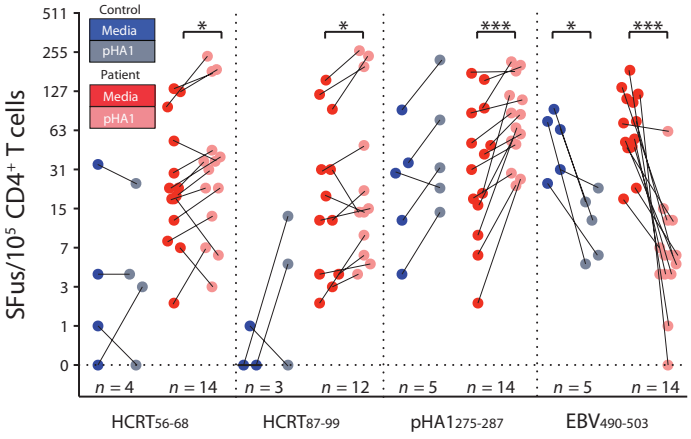
Using antigen presentation by T2.DQ0602 cells (an MHC class I– and class II–deficient lymphoblastic T-B fusion cell line) and IFN-γ production as a marker of $CD4^+$ T cell activation in an ELISpot assay, most patients with narcolepsy had circulating reactivity to $HCRT_{56-68}$ and $HCRT_{87-99}$, typically 10 to 80 SFUs/10^5 cells (that is, more than 1 of 10,000 cells), whereas samples from control subjects never had more than a few SFUs per 10^5 cells, and most often had none. Differences between controls and narcolepsy subjects were most striking in discordant monozygotic twins and in influenza-vaccinated (Pandemrix) DQ0602-positive controls and their siblings with narcolepsy. HLA DQ0602 and TCR affinity for the epitopes was high because T cell reactivity was detectable at peptide concentrations as low as 10 pM in narcoleptic patients. The autoantigens primarily elicited a T_H1 response, characteristic of many autoimmune diseases. These results establish that $CD4^+$ T cell reactivity to $HCRT_{56-68}$ and $HCRT_{87-99}$ is strongly associated with narcolepsy and could be the basis for a new approach to diagnosis of this disease.

When we used isolated human PBMCs as the APCs, a larger number of $HCRT_{56-68}$– and/or $HCRT_{87-99}$–activated cells were observed in both narcolepsy and controls. Because PBMCs express multiple HLA class I (A, B, and C) and class II (DR, DQ, and DP) alleles, and contain both $CD4^+$ and $CD8^+$ T cells, it is likely that $HCRT_{56-68}$ and/or $HCRT_{87-99}$, when added to the medium, bind HLA alleles other than DQ0602, resulting in the activation of other cell populations. When autologous DCs were used as APCs and $CD4^+$ T cells only were added, the specificity of the signal was largely restored (although more background was observed than when using T2.DQ0602 cells), suggesting that most of the nonspecific signal was a result of $CD8^+$ T cell activation, a signal that may not be physiological. Indeed, antigen processing and binding to HLA class I is typically intracellular; thus, class I presentation of these particular epitopes may never occur in vivo. The more homogeneous presentation of the peptides by T2.DQ0602 cells allowed for a clear distinction between narcolepsy patients and controls.

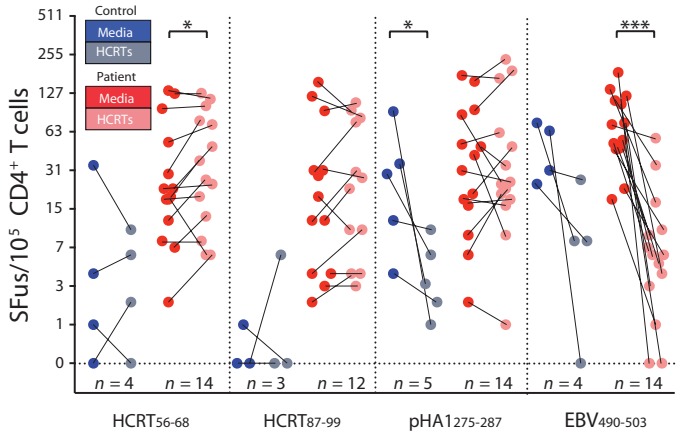
Because the loss of HCRT causes narcolepsy, and HCRT-containing neurons are apparently the only cells lost, $CD4^+$ T cell autoreactivity to DQ0602-HCRT is likely a critical contributor to disease pathophysiology, although it is possible that it is only a secondary reaction to another disease-initiating process. The sequence of events through which HCRT-reactive $CD4^+$ T cells would lead to neuronal death, however, remains obscure. Neurons do not express MHC class II, even in the presence of cytokines like IFN-γ that induce class II on other cell types (*39*). It is therefore unlikely that autoreactive $CD4^+$ T cells directly kill HCRT neurons via class II presentation of $HCRT_{56-68}$ and $HCRT_{87-99}$. Rather, other cells such as $CD8^+$ T cells could be involved, although, to date, there is no genetic, pathologic, or immunologic evidence for autoaggressive $CD8^+$ T cells in this disease (*12*, *39*). Unlike other HLA class II–associated diseases, there is no evidence for additional association of narcolepsy with alleles of HLA class I, the antigen-presenting molecule of $CD8^+$ T cells, although this has not been thoroughly explored. The lack of HLA class I association may suggest the contribution of an epitope with promiscuous binding to many HLA class I alleles, or another mechanism, for example, involving microglial cells. Antibody responses and epitope spreading may be limited in diseases like narcolepsy that selectively implicate neurons, perhaps explaining why autoantibodies directed toward HCRT cells have been difficult to detect.

When we screened pH1N1 proteins for DQ0602-binding epitopes, we identified $pHA1_{275-287}$ as a possible mimic of $HCRT_{56-68}$ and $HCRT_{87-99}$. Of avian influenza origin, $pHA1_{275-287}$ is located at the border of the HA1 globular domain (*40*). Cross-culture and cross-stimulation experiments with pH1N1 proteins or the pHA1 epitope $pHA1_{275-287}$ increased the proportion of HCRT-reactive cells in patients, suggesting cross-reactivity between these epitopes. The specificity of the effect was illustrated by the concomitant study of $EBV_{490-503}$–reactive T cells, which did not increase in frequency and were in fact diluted by the expansion of other populations. These experiments, however, were performed in vitro with high concentrations of $pHA1_{275-287}$, and, as the local concentration

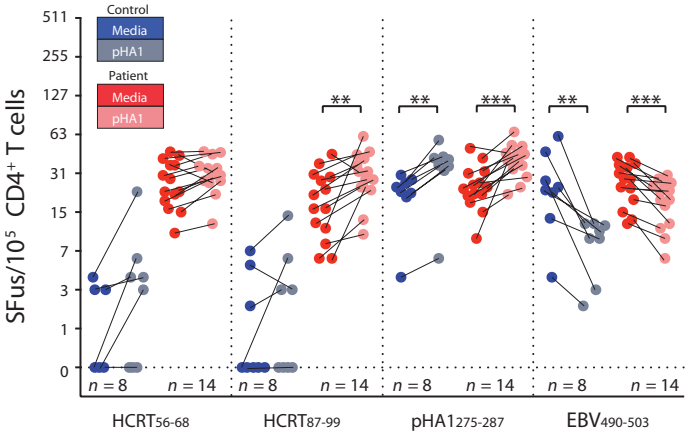
A. IFN-γ response after 13 days of PBMC in vitro stimulation with pHA1$_{275-287}$



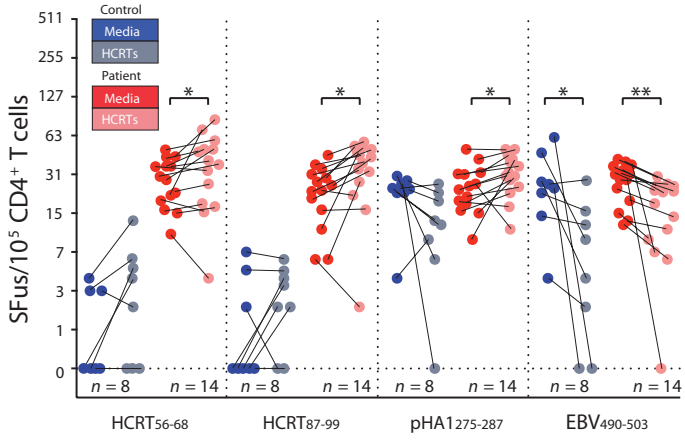
B. IFN-γ response after 13 days of PBMC in vitro stimulation with HCRT$_{56-68}$ and HCRT$_{87-99}$



C. CD38$^+$ T cell IFN-γ response after stimulation with pHA1$_{275-287}$



D. CD38$^+$ T cell IFN-γ response after stimulation with HCRT$_{56-68}$ and HCRT$_{87-99}$



E. CD38$^+$ T cell IFN-γ response after stimulation with EBV$_{490-503}$

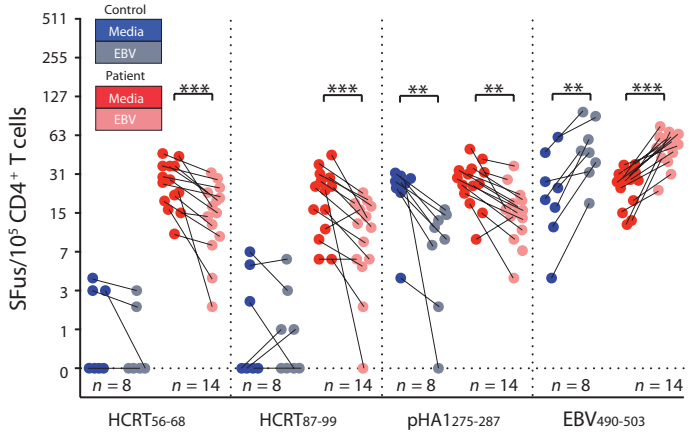


Fig. 6. Activation of HCRT-reactive CD4$^+$ T cells by pH1N1 pHA1$_{275-287}$. (**A** and **B**) ELISpot results from control (n = 3 to 5) and narcolepsy (n = 13 to 14) PBMCs cultured with pHA1$_{275-287}$ (A) or HCRT$_{56-68}$ plus HCRT$_{87-99}$ (B) for 13 days. CD4$^+$ T cells were purified, rested, and restimulated with HCRT$_{56-68}$, HCRT$_{87-99}$, pHA1$_{275-287}$, or EBV$_{490-503}$. (**C** to **E**) ELISpot results from T2. DQ0602 presentation of HCRT$_{56-68}$ + HCRT$_{87-99}$ (C), pHA1$_{275-287}$ (D), or EBV (E) epitopes (24 hours) to purified CD4$^+$ T cells from narcolepsy patients or control subjects. CD38$^+$ (activated) CD4$^+$ T cells were isolated and tested by ELISpot for reactivity to the same epitopes. Individual results from control (n = 8) and narcolepsy (n = 14) samples. See fig. S7 for representative ELISpot images. *P < 0.05, **P < 0.01, ***P < 0.001, Wilcoxon signed-rank (paired) W test.

of potential mimic epitopes in vivo is impossible to determine, the results may not extrapolate to the in vivo situation.

To address this issue, we hypothesized that in narcoleptic patients who developed narcolepsy after 2009, pH1N1 infections may have activated HCRT-responsive T cells. Consistent with this hypothesis, in vivo reexposure to pH1N1 with trivalent seasonal vaccination showed a small reactivation of preexisting HCRT cross-reactive cells in patients. The effect was small, perhaps because of the low potency of unadjuvanted seasonal vaccines in comparison to Pandemrix (*41*).

Although our data suggest that there is cross-reactivity between HCRT and $pHA1_{275–287}$, it appears that only a small subpopulation of T cells responds to both antigens in patients. Indeed, the number of $pHA1_{275–287}$–reactive cells appears similar in controls and patients (Fig. 6), indicating heterogeneity of $pHA1_{275–287}$–reactive TCRs. Further, many patients with narcolepsy have similar or higher numbers of HCRT-autoreactive cells than $pHA1_{275–287}$–reactive cells at baseline. Finally, both in vivo vaccination and in vitro cross-stimulation experiments with $pHA1_{275–287}$ produced only a small increase in HCRT-autoreactive cell frequency.

Our findings suggest that mimicry to $pHA1_{275–287}$ may have been involved in triggering some cases of narcolepsy after 2009. Nevertheless, other triggers must have existed before 2009. Before 2009, the incidence of narcolepsy in China was lower, but it was also seasonal (*42*), suggesting that winter-related infections contributed to the disease. We unexpectedly observed reactivity to the $pHA1_{275–285}$ epitope in samples collected before 2009, suggesting that before 2009, T cells cross-reactive to HCRT epitopes may have been selected by exposure to another influenza strain or other unrelated but homologous pathogen epitopes (*36*), particularly from 1957 to 1977 when influenza H1N1 was practically nonexistent in humans (*43*). To definitively confirm the existence of molecular mimicry, it will be necessary to identify and characterize cross-reactive TCRs.

In aligning H1N1 strains before 2009 with $pHA1_{275–287}$, we found few sequences with high homology and in vitro binding to DQ0602, confirming the specificity of this epitope for pH1N1. The influenza A 1998 variant with high homology is derived from a swine virus that has infected only a few humans (*44*). In the more common strains, binding to DQ0602 is either weaker or absent. The 1918 pandemic epitope bound moderately well and may align differently, as suggested in Fig. 5A. This may be significant as many seasonal H1N1 viruses have a glycosylation site at N287AS that limits epitope generation in this area, but the 2009 and 1918 pandemic strains do not (*40*).

Our results do not explain the particular association of narcolepsy with the Pandemrix vaccine. The strong stimulation of $CD4^+$ T cell responses by the AS03 adjuvant (*45*), together with other circumstantial factors (such as vaccine coverage and timing of pandemic), may have contributed in some countries. Alternatively, AS03-specific adjuvant effects or differences in H1N1 surface protein extraction protocols among vaccines could explain why most reports of narcolepsy have been associated with the Pandemrix vaccine. Similarly, co-infections such as *S. pyogenes* could have been involved in some cases, with streptococcal superantigens acting as "natural" adjuvants (*46*). Narcolepsy likely results from a sequence of events, including genetic predisposition (*6*, *7*, *10*), stochastic effects (generation of pathogenic TCRs in naïve T cells of only some individuals), lack of self-tolerance, activation by mimics and/or nonspecific immune effects (other infections and adjuvants), and finally central nervous system penetration of pathogenic T cells, possibly facilitated by fever or other events.

MATERIALS AND METHODS

Human subjects

All patients with narcolepsy ($n = 39$; 51% male; mean age ± SEM, 24.2 ± 2.5 years; range, 6 to 67 years) had cataplexy with an onset age of 17.5 ± 1.7 years (ranging from 5 to 51 years) and met the criteria for International Classification of Sleep Disorders 3 (ICSD3) for type 1 narcolepsy. The controls ($n = 35$; 51% male; age, 26.9 ± 2.9 years; range, 6 to 58 years) were either unrelated subjects, the healthy twin for twin pairs, or vaccinated siblings for Pandemrix probands. Patients and controls were all DQB1*06:02-positive, as typed by a PCR-SSP (polymerase chain reaction amplification with sequence-specific primers) method that used sequence-specific primers (forward, 5′-CCCGCAGAGGATTTCGTGTT-3′; reverse, 5′-AACTCCGCCCGGGTCCC-3′) and internal control primers (forward, 5′-TGCCAAGTGGAGCACCCAA-3′; reverse, 5′-GCATCTTGCTCTGTGCAGAT-3′). Genomic DNA was amplified at 94°C for 15 min, followed by 35 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1 min, and 72°C for 10 min. The amplification produced a 217–base pair (bp) band in the presence of DQB1*06:02, and a 700- to 730-bp internal control band.

A panel of 23 unrelated patients (57% male; age, 27.7 ± 3.0 years; range, 9 to 52 years) and 24 unrelated controls (52% male; age, 30.5 ± 3.6 years; range, 9 to 58 years) were first tested, followed by the 4 discordant twin pairs (50% male; age, 21.1 ± 7.0 years; range, 6 to 38 years) and then the Pandemrix-vaccinated subjects (10 cases: age, 16.3 ± 2.4 years; range, 8 to 30 years; 40% male; 7 vaccinated siblings: age, 14.3 ± 1.4 years; range, 10 to 20 years; 43% male). Subjects vaccinated with the trivalent seasonal 2012 flu vaccine (Fluzone, NDC 49281-705-55, 2012-2013 formula, Sanofi Pasteur) included nine patients (78% male; age, 35.7 ± 3.9 years; range, 12 to 52 years) and four controls (75% male; age, 38.4 ± 6.5 years; range, 22 to 53 years). Sample collection date ranged from 1991 to 2013, with similar numbers of narcolepsy patients and controls collected before and after September 2009, the onset of the H1N1 2009 pandemic in the United States (32 and 35% collected before 2009, respectively). Written consent was obtained in all cases in the context of Institutional Review Board–approved studies, following the guidelines for human subjects research under U.S. Department of Health and Human Services human subjects regulations (45 CFR Part 46).

Peripheral blood mononuclear cells

To collect PBMCs, three procedures were used, depending on the donor and situation: (i) a simple blood draw (almost all cases); (ii) a blood donation (250 ml at the Stanford Blood Bank); or (iii) leukocyte apheresis (Stanford Blood Bank), 10 patients (50% male; age, 35.6 ± 4.3 years) and 7 controls (71% male; age, 36.6 ± 4.8 years). PBMCs were extracted using Ficoll gradient reagent, then frozen in 10% dimethyl sulfoxide in fetal bovine serum (FBS), and stored in liquid nitrogen until use. Leukocyte apheresis samples were used for most experiments requiring titration, changes of conditions, or large amounts of cells.

$CD4^+$ T cell isolation

Frozen PBMCs were thawed and washed twice in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin. Then, $CD4^+$ T cells were isolated from the PBMCs by negative selection with an "untouched" $CD4^+$ cell isolation kit according to the manufacturer's instructions (11346D, Invitrogen). Purified $CD4^+$ T cells were >98% pure as assessed by flow cytometry (fluorescence-activated cell sorting).

DC preparation

PBMCs (5×10^7) were incubated for 5 days in RPMI-10 (RPMI 1640 with L-glutamine and 10% human AB serum, 1% Hepes, and 0.2% gentamicin) together with IL-4 (200 IU/ml) (eBioscience, 14-8049-62) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 IU/ml) (Miltenyi Biotec, 130-093-862) in 10-cm culture plates. On day 6, floating immature DCs were collected, and 1.5×10^6 cells were incubated in 3 ml of RPMI-10 with IL-4 and GM-CSF as above using six-well culture plates. By day 7, 30 μl of MCM [IL-1β (5 ng/ml) and IL-6 (150 ng/ml) (R&D Systems, 201-LB-005 and 206-IL-050) and prostaglandin E2 (1 μg/ml) (Sigma, P6532)]. Matured DCs were harvested on day 8.

Generation of the T2.DQ0602 cell line

The α chain of DQ0602 (DQA1*01:02) was amplified by PCR using human complementary DNA (cDNA) made from PBMCs of an HLA-DQA1*01:02 homozygote donor.

The primers were 5′-CGGGATCCATGATCCTAAACAAAGCTCTGCTGCTGGG-3′ and 5′-ACGCGTCGACTCACAATGGCCCTTGGTGTCTGGA-3′ (including restriction sites for Bam HI and Sal I). The β chain of DQ0602 (DQB1*06:02) was amplified by PCR from the bicistronic pLNCXPOX vector containing the DQ0602 cDNA (a gift from W. Kwok from Benaroya Research Institute, Seattle, WA). The primers were 5′-CCGGAATTCATGTCTTGGAAGAAGGCTT-3′ and 5′-ACGCGTCGACTCAGTGCAGAAGCCCTTT-3′ (including restriction sites of Eco RI and Sal I). Both chains were digested with restriction enzymes indicated above and then ligated into the retroviral vector PBMN-ZIN-neo, which was cut with the same enzymes. Plasmids containing DQA1*01:02 and DQB1*06:02 cDNA, respectively, were sequentially introduced into T2 cells, a human T-B hybrid cell line (*32*), using the Phoenix Retroviral System, as described (*33*). Cells expressing DQ0602 dimers on the surface were enriched using phycoerythrin (PE)–conjugated anti-DQ antibody (Ia3, BD Biosciences) and anti-PE MACS microbeads (Miltenyi Biotec).

ELISpot assays

$CD4^+$ T cells (100,000 per well) and T2.DQ0602 cells (100,000 per well) were added to an ELISpot 96-well plate coated with a IFN-γ–specific antibody (551849, BD Biosciences) in the presence of 1 μM of the indicated peptide (200-μl volume per well). In PBMC studies, 100,000 cells per well were used. PMA/ionomycin stimulation served as both loading and positive control. After 24 hours of stimulation in the incubator (37°C, 5% CO_2, 92% relative humidity), the cell suspension was aspirated and collected for further experiments (surface marker staining). Further antibody incubations and development of the ELISpot plate were done according to the manufacturer's instructions (551849, BD Biosciences). Spots were counted with a fully automated computer-assisted CTL ELISpot reader using the ImmunoSpot 4.0.17 software. More detailed analyses of $CD4^+$ T cell responses were performed on selected peptides using concentrations ranging from 1 pM to 1 μM or at concentrations as indicated in the corresponding figure legends.

HLA-DQ0602 and HLA-DM protein production and isolation

For production of MHC-DQA1*01:02/DQB1*06:02 (DQ0602) with β chain N-terminally tethered thrombin-cleavable CLIP peptide, we subcloned the gene sequences into a pAcGP67A baculovirus transfer vector–based construct. As template, we used cDNA from human PBMCs from an HLA-DQA1*01:02/DQB1*06:02 homozygote donor. The primers for DQA1*01:02 were 5′-GAGGATCCGAAGGCATTGTGGCTGACCACG-3′ and 5′-GATCTAGACTCTGTGAGCTCTGACATAGGGG-3′. The primers for DQB1*06:02 were 5′-AGGTGGGTCCAGAGACTCTCCCGAGGATTT-3′ and 5′-CCTCTAGATTCAGACTGAGCCCGCCACTC-3′. The tethered thrombin-cleavable CLIP sequence (PVSKMRMATPLLMQA) was added at the N-terminal end of DQB by PCR stitching. For this, we used the primers 5′-GAGGATCCCGTGTCCAAGATGC-3′ and 5′-CCTCTAGATTCAGACTGAGCCCGCCACTC-3′ and the CLIP oligo 5′-GAGGATCCCGTGTCCAAGATGCGCATGGCCACCCCCCTGCTGATGCAGGCCGGGAATTCGGGCGGTGGCTCACTAGTGCCACGGGGCTCTGGAGGAGGTGGGTCCAGAGACTCTCCC-3′. Both DQA and DQB constructs contained the gp76 secretion signal, extracellular sequences of the respective DQ chain, an acid (DQA)–base (DQB) zipper region, and a 6×his tag on both chains, and the DQA construct included a C-terminal birA site. For production of HLA-DM, a similar approach was used with sequences of HLA-DMA and HLA-DMB derived from the same cDNA source as the DQ genes. The DM construct contained the same additions as described above, except the CLIP and thrombin sequences. The following primers were used: HLA-DMA, 5′-GAGGATCCTGAAGCTCCTACTCCAATGTGG-3′ and 5′-GATCTAGAATTCTCCAGCAGATCTGAGGGCAG-3′; HLA-DMB, 5′-GAGGATCCCACAGGAGCAGGTGGCTTCGTG-3′ and 5′-CCTCTAGAGGGGGACAGCCCAGGTGTCCA-3′.

The vectors were cotransfected with BaculoGold (560129, BD Biosciences) into SF9 cells using Cellfectin II (Invitrogen). Briefly, the cells were incubated with the transfection mixture for 3 hours at 27°C. The transfection mixture was then replaced with SF9 complete medium, and the cells were incubated at 27°C for 1 week. Then, the supernatants containing virus were harvested and amplified. The resulting P1 virus stock was stored at 4°C and used for protein production. For this, P1 virus was added to Hi5 cells, and the cells were incubated for 5 days at 27°C. Supernatants from the infected Hi5 cells were harvested, and buffer [50 mM tris-HCl (pH 8), 5 mM calcium chloride, and 1 mM Ni-sulfate] was added (while stirring). The resulting mixture was spun and filtered; 0.01% azide and 5 mM imidazole were added. A column was prepared using Ni-NTA agarose (Qiagen) and Ni-sulfate and washed with HBS [20 mM Hepes (pH 7.4), 150 mM NaCl] with 5 mM imidazole. The protein preparation was run through columns, washed with HBS + 5 mM imidazole, and finally eluted using 300 mM imidazole in HBS. The eluate was further purified by S200 size-exclusion fast protein liquid chromatography and finally stored in phosphate-buffered saline (PBS) with 50% glycerol at −20°C.

Peptide binding assays

Two slightly different protocols were used, but data were merged, because results were overlapping. In the first protocol, 250 nM DQ6-CLIP cleaved overnight at room temperature was used. It was incubated overnight at 37°C with 200 nM HLA-DM, 25 μM biotinylated reference indicator peptide, $EBV_{490–503}$ (biotin-GGGRALLARSHVERTTDEY) (*47*), and 40 mM citrate buffer (pH 5.2) and at different micromolar concentrations of competing peptide, as applicable (30 to 100 μM). In the second protocol, we used 30 nM DQ6-CLIP cleaved for 2 hours at room temperature in the presence of phenylmethylsulfonyl fluoride and incubated 4 to 21 hours at pH 4.6 with 100 nM HLA-DM and 1 μM biotinylated $EBV_{490–503}$. A 96-well plate (MaxiSorp, Nunc) was coated overnight at room temperature with anti-human class II antibody (555557, BD Biosciences) or, in the second protocol, with anti-DQ monoclonal antibody SPVL3 at room temperature for 2 hours. After being washed (0.05% Tween 20, 0.1% NaN_3 in PBS), the plates were blocked

with 10% FBS or 2% bovine serum albumin (BSA) in PBS 2 hours at room temperature and washed again. The peptide exchange reaction was stopped by adding one volume of 100 mM Hepes (pH 7.4) with 10% FBS or two volumes of neutralization buffer [100 mM tris-Cl (pH 8.3), 150 mM NaCl, 1% (w/v) BSA, 0.5% (v/v) NP-40, 0.1% (w/v) NaN_3] to the protein-peptide mixture, and the mixture was transferred to the washed antibody-coated Nunc plate and incubated for 2 hours at room temperature. For detection of biotinylated peptide bound by the DQ0602 protein, we used the DELFIA Eu-N1 Streptavidin System (PerkinElmer).

DQ0602 epitope identification

Screening for possible DQ0602-binding peptides was performed with peptide libraries consisting of 15 amino acids (15-mer) with 11 overlapping amino acids. The prepro-HCRT library was purchased from JPT Peptides Technologies GmbH. The influenza peptide libraries [hemagglutinin (pHA1), neuramidase (pNA1), and polymerase PB1 proteins (pPB1) from A/California/07/2009] were from BEI Resources [National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH)]. The pHA1 and pNA1 peptide libraries were screened in pools of eight consecutive peptides. Pools that gave >25% displacement of the reference peptide signal were divided into two pools of four consecutive peptides and screened again. Finally, all peptides from the four-peptide pools with displacement exceeding 25% of the reference peptide signal were screened separately. For prepro-HCRT and pPB1, we did not screen pools, but instead screened all individual peptides independently. All experiments were done in technical duplicate and replicated in three independent setups. The concentration of peptide was 400 μM in all screens. For further analysis, peptides that outcompeted >75% of the reference peptide signal were considered strong binders, and peptides outcompeting 50 to 75% of the signal were considered weak binders. More detailed analysis of peptide binding was performed on selected peptides using concentrations ranging from 0.01 μM to 1 mM.

Peptide binding register and motif analysis

To determine the register of binding of identified DQ0602 epitopes, the binding of 13-mer peptide variants with single amino acid substitutions was measured using the binding assay, as described above. Selected amino acids were tested at each position across the 13-mer of $HCRT_{56–68}$, $HCRT_{87–99}$, and $pHA1_{273–287}$. Assays were done with two to three technical replicates and two to three independent repeats. Lack of binding of the test peptide, as indicated by binding of reference peptide, was interpreted to indicate lack of tolerance to the substitution in the test peptide.

To test the effect of amino acid substitutions on $CD4^+$ T cell activation, $HCRT_{56–68}$ (1 nM) with phenylalanine and other substitutions seen in Fig. 2 at positions P2, P5, P7, and P8 were used in ELISpot assays with cells from five patients and three controls.

Database searches for influenza mimic epitopes

To identify possible mimics of $HCRT_{56–68}$ and $HCRT_{87–99}$, we used the entire library of 101 influenza peptides binding DQ0602 (fig. S5 and tables S2 to S5) and bioinformatics to identify the best possible 9-mer epitope of the 15-mer sequences using three Immune Epitope Database prediction algorithms (SMM_align, NN_align, and consensus) and similarity scores. The resulting registers (P1, P3, P4, P6, and P9) were next used to assess conservation of predicted TCR binding residues at P5 (G), P7 (L or I), and P8 (T, L, I, V, and M) for all these peptides (critical residues in $HCRT_{56–68}$ and $HCRT_{87–99}$ for the activation of narcolepsy-specific T cells) and ranked by conservation of the number and homology of residues with the $HCRT_{56–68}$ and $HCRT_{87–99}$ epitopes at these positions. Using the 101 influenza binders, the program identified $pHA1_{273–287}$ (match at P5, P7, and P8), followed by $pNA1_{253–267}$ and $pHA1_{549–563}$ (match at P5 and P7) within the strong binders, and $pNA1_{21–35}$ and $pPB1_{157–171}$ (match at P5 and P7) as weak or nonbinders. To identify epitopes similar to $pHA1_{275–287}$, we aligned the corresponding region of $pHA1_{275–287}$ in other strains as indicated in Fig. 5. We also used the Influenza Research Database (http://www.fludb.org) search tool for identifying short peptides in proteins, using ERNAGSGIIISD as input, fuzzy matching with a cutoff value of 50% identity, and the search was performed against all HA proteins in the database.

Epitope 13-day stimulation of PBMCs and reactivation

PBMCs were thawed in 15 ml of RPMI 1640 + 10% FBS with 1% penicillin (100 U/ml)–streptomycin (100 μg/ml), washed twice, counted, and suspended at a density of 2×10^6/ml in RPMI 1640 with 10% FBS medium, IL-2 (10 U/ml, BD Pharmingen), and IL-7 (20 ng/ml, PeproTech). Aliquots of 100 μl (200,000 cells) were resuspended in individual wells of 96-well round-bottomed plates. For stimulation, we added $HCRT_{56–68}$ plus $HCRT_{87–99}$ or $pHA1_{275–283}$ or the bulk Belgian vaccine antigen of the 2009 pH1N1 Pandemrix vaccine (AFLSIDA109, no adjuvant, mostly containing pHA1, minimal amounts of pNA1 and pPB1, and minimal amounts of matrix proteins from the PR8 strain, as characterized by mass spectrometric analysis) at final concentrations of 1 μg/ml. Medium only was added as a negative control. Cells were next incubated at 37°C, 5% CO_2 for 13 days. At days 7 and 11, all cultures were split in half, and fresh medium, IL-2, and IL-7 (concentration as above) were added. At day 13, cells were collected and counted, and $CD4^+$ T cells were isolated using Dynabeads Untouched Human $CD4^+$ T Cells (Invitrogen). The resulting $CD4^+$ T cells were allowed to rest overnight in medium without peptide before ELISpot.

Epitope 24-hour stimulation and $CD38^+$ isolation

$CD4^+$ T cells (100,000 per well) and T2.DQ0602 cells (100,000 per well) were stimulated by 1 μM of each peptide (200-μl volume per well). After 24 hours of stimulation in the incubator (37°C, 5% CO_2, 92% relative humidity), the cell suspension was aspirated and $CD38^+$ cells (activated cells) were sorted using a $CD38^+$ microbead kit (130-092-263, Miltenyi Biotec). The resulting $CD38^+$ $CD4^+$ T cells were allowed to rest for 24 hours in medium without peptide before ELISpot.

Sequences of epitopes used

1. Epstein-Barr virus

Chain A, Epstein-Barr virus nuclear antigen-1 residues 470 to 607EBV$_{490–503}$ RALLARSHVERTTDE (15-mer)

2. 2009 pandemic influenza

Hemagglutinin HA1, segment 4, influenza A/California/07/2009, H1N1
$pHA1_{275–287}$ ERNAGSGIIISDT (13-mer)
$pHA1_{273–287}$ AMERNAGSGIIISDT (15-mer)
$pHA1_{549–563}$ AISFWMCSNGSLQCR (15-mer)
Neuraminidase NA1, segment 6, influenza A/California/07/2009, H1N1
$pNA1_{21–35}$ NLILQIGNIISIWIS (15-mer)
$pNA1_{253–267}$ YKIFRIEKGKIVKSV (15-mer)
PB1 polymerase (basic) protein 1, segment 2, influenza A/California/07/2009, H1N1
$pPB1_{157–171}$ ANESGRLIDFLKDVM (15-mer)

3. Other influenza strains

1918. $HA1_{274–285}$ LNRGSGSGIITS (12-mer, hemagglutinin HA1, segment 4, influenza A/Brevig Mission/1/1918, pandemic H1N1 from 1918)
1934. $HA1_{273–284}$ LSRGFGSGIITS (12-mer, hemagglutinin HA1, segment 4, influenza A/Puerto Rico/8/1934, H1N1 also known as PR8 backbone of influenza vaccines)
1998. $HA1_{274–285}$ MKRNSGSGIIIS (12-mer, hemagglutinin HA1, segment 4, influenza A/Wisconsin/10/1998; HA1 swine virus transmitted to human)
2003. $HA1_{273–284}$ LSRGFGSGIIIS (12-mer, hemagglutinin HA1, segment 4, influenza A/New York/490/2003 H1N1)

4. Prepro-HCRT (prepro-orexin) epitopes

Leader peptide: $HCRT_{1–13}$ MNLPSTKVSWAAV (13-mer)
HCRT-1: $HCRT_{25–39}$ ALLSSGAAAQPLPDC (15-mer)
$HCRT_{41–55}$ RQKTCSCRLYELLHG (15-mer)
$HCRT_{53–67}$ LHGAGNHAAGILTLG (15-mer)
$HCRT_{56–68}$ AGNHAAGILTLGK (13-mer)
HCRT-2: $HCRT_{85–99}$ QASGNHAAGILTMGR (15-mer)
$HCRT_{87–99}$ SGNHAAGILTMGR (13-mer)
$HCRT_{113–127}$ RRCSAPAAASVAPGG (15-mer)

Statistics

Data are reported as means ± SEM or as percentages. To compare ELISpot numbers across conditions, we used two-tailed Mann-Whitney-Wilcoxon *U* tests or Wilcoxon signed-rank nonparametric paired tests. To assess the best ELISpot numbers for diagnostic cutoffs, we used ROC analyses and 100 bootstrap iterations to define 95% confidence intervals. To study the effects of onset time (before versus after September 2009), sample collection period (before versus after September 2009), or disease duration on ELISpot numbers, linear regression models were used in narcolepsy subjects with these variables added as categorical or continuous covariates. Significance was reported when $P < 0.05$.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/216/216ra176/DC1
Fig. S1. $CD4^+$ T cell TNF-α response to DQ0602 strong binding peptides.
Fig. S2. T cell response to HCRT epitopes with different APCs.
Fig. S3. ROC curves for ELISpot data.
Fig. S4. DQ0602 binding of HCRT and pHA1 peptides with amino acid substitutions.
Fig. S5. Binding of pH1N1 peptides to DQ0602.
Fig. S6. Possible mimics of HCRT epitopes in pH1N1 stimulate T cells from narcoleptic patients and controls.
Fig. S7. Representative ELISpot images corresponding to Figs. 4 and 6.
Table S1. Prepro-HCRT peptides showing binding to DQ0602.
Table S2. pHA1 peptides showing binding to DQ0602.
Table S3. pNA1 peptides showing binding to DQ0602.
Table S4. pPB1 peptides showing binding to DQ0602.
Table S5. Influenza A H1N1 strains used for vaccinations by year.
Reference (*48*)

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