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T cell reactivity to Regulatory Factor X4 in Type 1 Narcolepsy¹

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Abstract

Type 1 narcolepsy is strongly (98%) associated with human leukocyte antigen (HLA) class II DQA1*01:02/DQB1*06:02 (DQ0602) and highly associated with T cell receptor (TCR) alpha locus polymorphism as well as other immune regulatory loci. Increased incidence of narcolepsy was detected following the 2009 H1N1 pandemic and linked to Pandemrix® vaccination, strongly supporting that narcolepsy is an autoimmune disorder. Although recent results suggest CD4+ T cell reactivity to neuropeptide hypocretin/orexin and cross-reactive flu peptide is involved, identification of other autoantigens has remained elusive. Here we study whether autoimmunity directed against Regulatory Factor X4 (RFX4), a protein co-localized with hypocretin, is involved in some cases. Studying human serum, we found that autoantibodies against RFX4 were rare. Using RFX4 peptides bound to DQ0602 tetramers, antigen RFX4-86, -95, and -60 specific human CD4+ T cells were detected in 4/10 patients and 2 unaffected siblings but not in others. Following culture with each cognate peptide, enriched autoreactive TCR $\alpha\beta$ clones were isolated by single-cell sorting and TCR sequenced. Homologous clones bearing TRBV4-2 and recognizing RFX4-86 in patients and one twin control of patient were identified. These results suggest the involvement of RFX4 CD4+ T cell autoreactivity in some cases of narcolepsy.

Keywords: narcolepsy, RFX4, DQ0602, tetramer, TCR

Introduction

Type 1 Narcolepsy (T1N) is a disabling neurological disorder that affects 1/2,000 individuals. It is characterized by excessive daytime sleepiness, disturbed nocturnal sleep, sleep paralysis, hypnagogic hallucinations and sudden episodes of bi-lateral losses of muscle tone, triggered by emotions (cataplexy) (1). The condition is caused by the loss of (>90%) orexin/hypocretin (HCRT)- producing neurons (2, 3) and is highly (98%) associated with human leukocyte antigen (HLA) class II haplotype DQA1*01:02/DQB1*06:02 (DQ0602) (4-11). Furthermore, this disorder is also strongly associated with T cell receptor (TCR) alpha polymorphism and other immune response genes, including cathepsin H (CTSH) and tumor necrosis factor (ligand) superfamily member 4 (TNFSF4) (4, 12, 13). An increased incidence of narcolepsy was detected, especially in children in Finland, Sweden, Europe and China, following the 2009-2010 influenza A H1N1 pandemic and associated vaccination campaign with Pandemrix® (14-24). It was also reported that cases of T1N were triggered by other environmental factors, including Streptococcus infection, which has previously been associated with a number of autoimmune diseases (15, 25-30). Taken together, this data strongly suggests that T1N is an autoimmune disorder.

So far, no consistent autoantibodies have been found in T1N in spite of large efforts. One of the most promising autoantigens first identified was TRIB2 (31), a protein initially thought to be enriched in HCRT cells. It was shown by three different studies, including one by our group, that TRIB2 autoantibody levels are elevated in recent onset narcolepsy cases (32). However, later efforts failed to consistently confirm this finding (33, 34).

Similarly, numerous efforts were exerted to screen for autoantibodies against HCRT, HCRT receptor 1 (HCRTR1) and HCRTR2 in T1N patients and healthy controls (35-37). Although Ahmed et al. (2015) report anti-HCRTR2 autoantibodies in the majority of post-Pandemrix® narcolepsy patients tested (17 of 20 sera) (38), others utilizing a similar protocol have reported conflicting results in both sporadic narcolepsy patients (5 of 181, 3 of 61 sera) and post-Pandemrix® narcolepsy patients (0 of 40 sera) (37). Results suggesting association of anti-streptolysin O (ASO) and anti-DNAse B (ADB) antibodies with recent narcolepsy onset could not be replicated either in China (25, 27, 39). Notably, Deloumeau and colleagues (2010) identified higher level of IgG complexed with HCRT1 in T1N patients compared to controls, whereby overall serum levels of IgG did not differ between the two groups (40, 41). Recent techniques used have included radioligand-binding (35), cell-based enzyme-linked immunosorbent (ELISA) (38) and flow cytometry-based assays (37, 42, 43), all with negative results. While the general consensus is now that a T cell mediated response is likely the primary mechanism underlying T1N, it yet remains an enigma whether this cascade may also generate a humoral response with the presence of any antibodies specifically and reliably associated with T1N remaining contentious.

In contrast to the findings concerning antibody-mediated immunity, adaptive cell-mediated immunity has been increasingly and robustly implicated in the pathophysiology of narcolepsy (44, 45), a result that fits well with genetic data indicating association with HLA and TCR loci. Thanks to HLA class I and II multimers and fluorescence-activated cell sorting (FACS)-based single cell sorting and sequencing technologies, investigation of cellular immune responses to infectious agents and autoantigens in autoimmune diseases is now possible in more details (46, 47). Specifically, tetramer HLA-targeted T cells facilitates identification of antigen specific TCR-groups within highly diverse TCR repertoires (48, 49), which are stochastically generated by

V(D)J recombination, a process rearranging germline TCR loci in each T cell (50, 51). Using this technology, there is increasing evidence of CD4 autoreactivity directed against HCRT itself may indeed be a major autoreactive process in human narcolepsy (46, 52).

In this study, we hypothesize that regulatory factor X4 (RFX4) might be another important CD4+ T cell autoantigen in T1N, due to its high enrichment in HCRT cells (34, 53, 54). RFX members share a highly-conserved deoxyribonucleic acid (DNA)-binding domain (55) and regulate expression of their target genes, including major histocompatibility complex class II (MHCII) and interleukin-5 receptor α chain (IL-5R α) (56-63). Six isoforms of RFX4 have been identified (64). While RFX4 variant 1 (RFX4_v1), RFX4_v2 and RFX4_v4 are specifically expressed in testis (61, 65), expressions of both RFX4_v5 and RFX4_v6 is observed exclusively in gliomas (65). RFX4_v3 is specifically expressed in the brain and is crucial for brain morphogenesis, being involved in many aspects of brain development and disease (64-69). Of notable interest is the fact RFX4_v3 is expressed in the suprachiasmatic nucleus (SCN), the mammalian pacemaker responsible for generating a 24-h-circadian rhythm (70-72). It can also be induced by light exposure in a subjective night-specific manner, a typical characteristic of several other core SCN circadian genes (73), suggesting a role in circadian regulation. This hypothesis is further supported by findings of Glaser and colleagues (2005), who identified RFX4_v3 as a potential risk factor for Bipolar disorder (BPD) (74), a condition frequently featuring severe circadian instability (75-77). Intriguingly, RFX4 is highly enriched in-, and significantly specific to HCRT neurons, according to the results of translating ribosome affinity purification (TRAP) (34, 53, 54). Nevertheless, little is known about the regulatory network of RFX4 and the role it exerts on HCRT neurons. Taken together, the implication of RFX4 in circadian regulation, its hypocretin neuron specific expression

and the effect of related members on MHC class II genes, renders this protein an interesting candidate for investigation as an autoantigen in T1N. Of additional interest is the fact CD8+ directed T cell activity against this antigen has also been shown in a recent study of narcolepsy patients (45).

To further explore the hypothesis that immune reactivity directed toward RFX4 is relevant to narcolepsy pathophysiology and hypocretin cell loss, we searched for autoantibodies directed against RFX4_v1/v3/v4 in 86 T1N patients and 88 controls using high throughput flow cytometry-based (FACS) staining assays and explored T cell reactivity to RFX4 peptides bound to DQ0602. FACS positive samples for autoantibodies were confirmed using cell-based immunostaining and western blotting. While no autoantibodies were found, T cell binding DQ0602-RFX4 tetramers were found in four patients and two sibling controls of cases. The corresponding RFX4 antigen, DQ0602 restricted specific CD4+ T cells were next single-cell sorted for TCR sequencing and unique clone enrichment and preferential v-gene segments in TCR repertoires observed. Finally, activation of enriched TCR clones by RFX4 peptides was studied in vitro, showing functional effects. Altogether, our results provide evidence for the involvement of RFX4 in the pathophysiology of at least a few T1N cases.

Materials and Methods

Ethics Statement

This study has been reviewed and approved by the Stanford University Institutional Review Board (Protocol # 14325, Registration # 5136). Informed consent was obtained from each participant. We confirm that all methods were carried out in accordance with relevant guidelines and regulations.

Participants

For anti-RFX4 autoantibody detection, two groups of matched T1N patients and controls were created. One group consists of 39 post-Pandemrix[®] T1N (PP-N) cases (mean age $\pm$ standard error of the mean (SEM): 14.9 ± 1.26 , 30% male) and 18 post-Pandemrix[®] controls (PP-C) (mean age $\pm$ SEM: 13.17 ± 0.97 , 39% male), the other group consists of 47 recent early onset T1N (EO-N) cases (mean age $\pm$ SEM: 12.95 ± 1.43 , 58% male) and 70 other controls (O-C) (mean age $\pm$ SEM: 18.85 ± 1.31 , 46% male) (Dataset 1). All patients meet international classification of sleep disorders 3 (ICSD3) criteria (<http://www.aasmnet.org/store/product.aspx?pid = 849>) for type 1 T1N. All are DQ0602 positive with one exception, a patient known to have low cerebrospinal fluid (CSF) hypocretin-1 levels. For T cell reactivity examination, 3 early onset T1N patients, 7 post-Pandemrix[®] T1N patients, 5 post-Pandemrix[®] controls, and 1 other control were tested and detailed information was summarized in Dataset 2. All 16 subjects are DQ0602 positive. Flow cytometry dot plots of 10 out of 16 subjects were also shown in S11 in Luo et al. (2018) (46).

Vaccine

Pandemrix® (A/California/7/2009 (H1N1) NYMC X-179A monovalent bulk (inactivated, sterile)) (Batch# AFLSFDA280) was used throughout the study. It was manufactured with HA content at 139 µg/ml (determined with single radial diffusion (SRD)) by GlaxoSmithKline (GSK) Dresden in January 2010. This batch has been used during the 2009-2010 vaccination campaign in Europe. Summary of Pandemrix® characteristics can be found in the European Medicines Agency library (http://www.ema.europa.eu/docs/en_GB/document_library/Other/2010/05/WC500091295.pdf).

RFX4 constructs

Original clones of RFX4_v1 (Cat# RG205481, OriGene), RFX4_v3 (Cat# RG223334, OriGene), RFX4_v4 (Cat# RG233113, OriGene) and green fluorescent protein (GFP) expression vector pCMV6-AC-GFP (Cat# PS100010, OriGene) were purchased from OriGene. These were transformed to TOP10 competent cells (Cat# C404003, Invitrogen) and plasmids purified from bacterial culture using EndoFree® plasmid maxi kit (Cat# 12362, QIAGEN).

Anti-RFX4 autoantibody detection with flow cytometry

Detailed protocol and quantitation analysis methods have been described previously (42, 43). Briefly, human embryonic kidney (HEK) 293T cells (ATCC® CRL-3216®) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cat# ATCC® 30-2002, ATCC), supplemented with 10% fetal bovine serum (FBS) (Cat# 26140079, Gibco) and 1% penicillin-streptomycin (Pen/Strep) (Cat# 15140-122, Gibco) in a 75cm² tissue culture flask (REF# 658175, CELLSTAR) at 37°C, 5% CO₂, and transfected with RFX4_v1, RFX4_v3, RFX4_v4 or pCMV6-AC-GFP using

the Lipofectamine[®] 3000 reagent (Cat# L3000015, Invitrogen) in Opti-MEM[®] I reduced serum medium (REF# 31985070, Gibco). After 24 hours, cells were harvested and stained with Zombie NIR[®] (Cat# 423106, BioLegend), followed by fixation and permeabilization with fix/perm buffer set (Cat# 421403, BioLegend) according to the manufacturer's instructions. Validation and sensitivity were detected using two positive antibodies: mouse anti-RFX4 antibody (Cat# H00005992-B01P, Abnova) (Supplemental Fig. 1) and rabbit anti-RFX4 antibody (Cat# NBP2-48967, Novus) (Supplemental Fig. 2), followed by staining with R-phycoerythrin (PE) anti-mouse immunoglobulin (Ig)G (REF# 12-4010-82, eBioscience) and PE anti-rabbit IgG (REF# 12-4739-81, eBioscience) (1:100), respectively. Cells were incubated with human serum (1:20) for 1 hour at 4°C, followed by staining with PE anti-human Igλ antibody (REF# 12-9990-42, eBioscience) (1:100) and PE anti-human Igκ antibody (REF# 12-9970-42, eBioscience) (1:100). Nonspecific binding was removed by washing with 0.05% Tween-20 in PBS. Cells were run on BD LSR II and data were analyzed with FlowJo (version 10.0.8).

Quantitation analysis methods were modified as follows: for each sample, mean fluorescence intensities (MFI) of PE channel (MFI^{PE}) within live GFP positive HEK293T cells ($HEK293T^{GFP+}$) and GFP negative HEK293T cells ($HEK293T^{GFP-}$) were calculated, then ΔMFI^{PE} was determined by subtracting MFI^{PE} of $HEK293T^{GFP-}$ from MFI^{PE} of $HEK293T^{GFP+}$. Cut-off value of ΔMFI^{PE} was determined as the mean of $\Delta MFI^{PE} + 3 \times$ standard deviation (SD) of control samples (35).

Cell-based assay (CBA) for autoantibody detection under microscope

A protocol described previously (36) was used. Briefly, HEK293T cells were cultured and transfected (as described above) on glass coverslip (REF# 354085, Corning) in a 12-well plate

(REF# 353043, Corning). After 24 hours, cells were fixed and permeabilized, and then incubated with human serum (1:20) or rabbit anti-RFX4 antibody (1:500) for 2 hours at room temperature (RT) in the dark, followed by Alexa Fluor® (AF) 555-conjugated anti-human IgG antibody (Cat# A-21433, ThermoFisher) (1:500) or AF555-conjugated anti-rabbit IgG antibody (Cat# A-21428, ThermoFisher) (1:500), respectively. All antibodies or sera were diluted in perm buffer and cells were washed twice with perm buffer after each incubation. Images were taken by EVOS® FL imaging system (Cat# AMF4300, ThermoFisher). Images were analyzed using ImageJ2.0.0 image processing software. An area in the region of interest was selected and all DAPI+GFP+ and DAPI+GFP+AF555+ cells were counted using the cell counter plugin in ImageJ.

Immunoprecipitation (IP)

HEK293T cells (equal aliquot) were cultured and transfected in 75cm² flasks as described above. After 24 hours, cells were harvested, washed with cold phosphate buffered saline (PBS) (Cat# 10010-049, Gibco) and lysed in RIPA buffer (Cat# 89900, ThermoFisher) supplemented with complete protease inhibitor (REF# 11873580001, Roche) on ice for 15 minutes. Debris was removed by centrifugation at 12,000rpm for 15 minutes. Supernatant was incubated with anti-tGFP-conjugated agarose beads (Cat# TA183081, OriGene) overnight at 4°C by end-to-end rotation. These beads were washed 3 times and boiled at 95°C for 10 minutes after adding 50 µl of 4× Laemmli buffer (Cat# 1610747, Bio-Rad). Supernatant was loaded to a precast gel (Cat# 4561093, Bio-Rad) for western blotting.

Western blotting

For western blotting protocol, see https://www.biorad.com/webroot/web/pdf/lsr/literature/Bulletin_2895.pdf. Briefly, proteins were transferred from gel to a PVDF membrane (Cat# IPVH00010, EMD Millipore) and then incubated with primary anti-RFX4 antibody (Cat# H00005992-B01P, Abnova) (1:2,000) or human serum (1:50) (200 μ l in 10 ml of blocking buffer) for 2 hours at RT, followed by secondary peroxidase-conjugated anti-mouse IgG antibody (Code# 715-035-150, Jackson ImmunoResearch) (1:5,000) or anti-human Ig λ (NBP1-73715, Novus) (1:5,000) and anti-human Ig κ (NB7466, Novus) (1:5,000), respectively. Non-specific binding was removed through extensive washing of 3*10 minutes with 0.05% Tween-20 in PBS. Chemiluminescent substrate (Prod# 32106, ThermoFisher) was applied to the blot and film (Cat# 28906838, GE Healthcare) was developed with a tablet processor (Item# SRX-101A, Konica).

RFX4 peptide library

An overlapping 15-mer peptide (11-amino acid overlap) library covered 6 RFX4 variants. Each peptide was synthesized with >90% purity at GenScript and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 5 mM.

Tetramer DQ0602-peptide staining and Single cell TCR sequencing (TCRseq)

The full protocol was described previously (78, 79). In brief, biotinylated DQ0602 was loaded with peptide at pH 5.2 and incubated with PE-conjugated streptavidin. Peripheral blood mononuclear cells (PBMCs) were stained with concentrated and purified tetramer DQ0602-peptide and antibody cocktail, followed by Fluorescence-activated cell sorting (FACS)

(Supplemental Fig. 4). Tetramer DQ0602-peptide positive CD4⁺ T cells were single-cell sorted into 96-well plates for single cell TCR sequencing.

Luciferase activity

TCR $\alpha\beta$ chains tagged with GFP were cloned to N103 vector (a gift from Dr. Mark Davis) by IDT (Integrated DNA Technologies, Inc.). Jurkat 76 (J76)-NFATRE-luc cells (a gift from Dr. Mark Davis) were transfected with TCR using the Lipofectamine[®] 3000 reagent (Cat# L3000015, Invitrogen). TCR positive cells were sorted using anti-human TCR $\alpha\beta$ antibody (Cat# 306702, BioLegend). In 200 μ l of complete RPMI medium (RPMI (Cat# 61870-036, Gibco) supplemented with 10% FBS and 1% Pen/Strep), 0.1 million of J76-TCR cells were co-cultured with 0.1 million of RM3-DQ0602 (80) cells at the presence of 10 μ M of pHA₂₇₃₋₂₈₇, NP₁₇₋₃₁, HCRT_{54-66-NH₂}, HCRT_{86-97-NH₂}, or RFX4-43, or 5 μ M of RFX4-86, RFX4-95, or RFX4-60 or 0.2 μ l DMSO for 8 hours. Luciferase activity was detected with Nano-Glo Luciferase Assay Kit (Cat# N1130, Promega).

Statistical analysis

For anti-RFX4 autoantibody analysis and luminescence of J76-TCR, values were expressed as mean $\pm$ SD and mean $\pm$ Standard Error of the Mean (SEM), respectively. Statistical comparisons were calculated with two-tailed *t*-tests. Data were plotted using GraphPad Prism 5 and R. P-value < 0.05 was considered statistically significant.

Results

Autoantibodies against RFX4 are absent or very rare in cases and controls

To test for the presence of autoantibodies directed against RFX4 in T1N cases, RFX4_v1, RFX4_v3 and RFX4_v4 tagged with green fluorescent protein (GFP) were expressed in human embryonic kidney (HEK) 293T cells. Mean fluorescence intensities (MFI) of R-phycoerythrin (PE) were recorded using quantitative flow cytometry-based assay after staining HEK293T cells with sera of 39 post-Pandemrix® T1N (PP-N) cases, 18 post-Pandemrix® controls (PP-C), 47 recent early onset T1N (EO-N) cases, and 70 other controls (O-C), and secondary PE-conjugated anti-human immunoglobulin (Ig). Comparing $\Delta\text{MFI}^{\text{PE}}$ determined by subtracting MFI^{PE} of HEK293T^{GFP-} from MFI^{PE} of HEK293T^{GFP+}, no significant difference was observed between patients and controls for either v1, v3, or v4, however, anti-RFX4_v3 autoantibody level in PP-N cases were significantly higher compared to patients without vaccination and vaccinated controls (p-value = 0.018 and 0.006, respectively) (Fig. 1A). Out of 174 subjects, autoantibody levels of anti-RFX4_v1, v3 and v4 above cut-off were detected in one (O-C), three (2 PP-N and 1 O-C) and two (1 PP-N and 1 O-C) subjects, respectively (Fig. 1A and Dataset 3), thus suggesting anti-RFX4 autoantibodies are rare. Furthermore, two PP-N patients and one O-C showed positive correlations between PE and GFP signals for RFX4_v3, but not for v1 or v4 (Fig. 1B), which was confirmed by cell-based immunostaining assay (CBA) (Fig. 1C) and using western blotting (Fig. 1D), therefore validating results attained using high sensitive flow cytometry-based assay. Taken together, these findings show that autoantibodies against RFX4 are very rare, and are unlikely to mediate pathology in T1N, as already suggested by previous studies (35-37).

Detection of RFX4 antigen specific CD4+ T cells

To detect antigen-specific CD4+ T cells, peripheral blood mononuclear cells (PBMCs) of 3 EO-N, 7 PP-N, 5 PP-C and 1 O-C were cultured with Pandemrix® or a single RFX4 peptide and then stained with tetramer DQ0602, loaded by 32 strong and 6 weak RFX4 peptides (Table I). Sporadic pattern of Pandemrix® (Supplemental Fig. 3) stimulation suggested no cross reactivity between Pandemrix® and RFX4. Notably, stimulation with the three different peptides, RFX4-60, -86 and -95, resulted in clearly separated populations of specific CD4+ T cells in four patients and two sibling controls of cases (Fig. 2A), suggesting T cell response engagement with RFX4 in these narcoleptic cases

T cell reactivity to RFX4-86 presented by DQ0602 was detected in both 2 PP-N (021 and 948) and 2 PP-C subjects (5688 and 901) while recognition of RFX4-95 was only found in 2 PP-N patients (021 and 900) (Fig. 2A). T cell reactivity to RFX4-60 was solely detected in one EO-N patient (848) with a tail-like pattern (Fig. 2A). Notably, patient 021 reacted to both RFX4-86 and RFX4-96, which are shared in all 6 RFX4-variants (Table I). Altogether, these findings reveal RFX4-specific CD4+ T cells in both post-Pandemrix® and early-onset narcolepsy patients, therefore suggesting involvement of RFX4 in T cell immunity of narcolepsy.

RFX4 antigen specific single CD4+ T cell TCR sequencing

In the next step, our aim was to determine the identity of RFX4-specific CD4+ T cells. Antigen specific CD4+CD8- T cells of 7 positive reactions to the three peptides RFX4-86, -95, and -60 in 6 subjects were single-cell sorted into 96-well plates for TCR α and β sequencing (i.e. 7 x 96 cells

in total) (Dataset 4). For each peptide-subject reaction, more than 65 out of 96 (68%) cells were recovered with pairing TCR $\alpha\beta$, except for RFX4-86/5688 (twin control of case, 40 cells, 42%) and RFX4-60/848 (PP-N, 12 cells, 13%) (Fig. 2B, Dataset 4). One TCR $\alpha\beta$ pair in each RFX4 peptide-subject reaction was enriched at least 43% (≥ 7 counts) (Fig. 2B). For example, two TCR $\alpha\beta$ pairs enriched in PP-N patient 021 reacted to either RFX4-86 (73% and 22%) or RFX4-95 (46% and 38%), while a single TCR $\alpha\beta$ pair was enriched in RFX4-86 PP-N patient 948 (79%), RFX4-86 O-C 5688 (58%) and PP-C 901 (43%), RFX4-95 PP-N 900 (89%) and RFX4-60 PP-N 848 (58%) (Fig. 2B). Notably, RFX4-86 TRBV4-2, which is associated with T1N(13), was also enriched in one patient (79%) and one control (58%) (Fig. 2B).

Next, the usage of TCR V gene segments in patients and controls was analyzed. TRBV28 (34%) and TRBV4-2 (32%) were the two most frequently used V gene segments in patients and controls respectively (Fig. 2C). Notably, TRBV4-2, which has previously been associated with T1N (13), was also frequently used in patients (19%). Contrastingly, however, frequently used TRAV genes showed significantly more diversification between patients and controls (Fig. 2C).

Frequent usage of TRBV and TRAV/TRBV pairings was distinct between RFX4-86, -95, and -60 (Fig. 2C and D). In RFX4-86, TRBV4-2 (36%) most frequently paired to TRAV2 (52 counts, 20%) in patients and TRAV38-2/DV-8 (23 counts, 9%) in controls, while for RFX4-60, TRBV14 (58%) paired to TRAV29/DV5 (7 counts, 58%) in patients (in controls, no pairing of more than 5 cells could be identified). In RFX4-95, TRBV28 (69%) paired to TRAV19 (74 counts, 49%) and TRAV3 (26 counts, 17%) in patients (Fig. 2D). Taken together, these findings show that TCR is

341 enriched in tetramer DQ0602 positive CD4+ T cells and V gene segments are partially similar
342 between patients and controls, however, differ significantly between RFX4-86, -95 and -60.

343

344 **TRBV4-2 is involved in RFX4 narcolepsy**

345 In a last step, our aim was to probe the reactivity of specific TCRs to RFX4-86, RFX4-95 and
346 RFX4-60. Therefore, enriched TCR $\alpha\beta$ s (≥ 7 clones, Table II) were expressed in Jurkat 76 (J76)-
347 NFATRE-luc cells, which lack native TCRs and subsequently co-cultured with antigen presenting
348 cells (APCs) RM3-DQ0602. Luciferase activity showed that TCR162
349 (TRBV28_CASSSSGQGGNSPLHF_TRBJ1-6/TRAV19_CALSGDVYGGSQGNLIF_TRAJ42),
350 TCR166 (TRBV2_CASSGQGSYEQYF_TRBJ2-7/TRAV9-2_CALSSLWGEKLTIF_TRAJ48)
351 and TCR170 (TRBV28_CASSFSGTRTGELFF_TRBJ2-2/TRAV12-
352 2_CAVMPNDYKLSF_TRAJ20) significantly reacted to RFX4-95. Four TCRs showed reactivity
353 against RFX4-86, namely TCR163 (TRBV12-3_CASSLRGLNYGYTF_TRBJ1-
354 2/TRAV41_CAVLNRDDKIIF_TRAJ30), TCR165 (TRBV4-2_CASSQEGGKETQYF_TRBJ2-
355 5/TRAV6_CALDRGTDKLIF_TRAJ34), TCR172 (TRBV5-6_CASSLGPRTPKGQYF_TRBJ2-
356 7/TRAV39_CAVENNNDMRF_TRAJ43) and TCR173 (TRBV4-
357 2_CASSQDVRQGGYGYTF_TRBJ1-2/TRAV29/DV5_CAASDQAGTALIF_TRAJ15). Lastly,
358 TCR174 (TRBV14_CASSLQGRRGDTQYF_BJ2-
359 3/TRAV29/DV5_CAASAGAGSYQLTF_TRAJ7) was shown to react against RFX4-60 (Fig. 3).
360 As outlined earlier, TRBV4-2 is among a number of specific TCR segments of interest in the
361 context of narcolepsy, as genetic QTL locations modulating its usage are strongly associated with
362 this condition (13). Notably, two out of four (50%) TCRs reactive against RFX4-86 (TCR165 and
363 TCR173) shared TRBV4-2, hence suggesting causality and cardinally implicating T cell reactivity

against RFX4-86 via TRBV4-2 in some cases of narcolepsy. Notably, no cross-reaction between RFX4 and HCRT/Pandemrix[®] was detected, suggesting these TCRs are RFX4 specific (Fig. 3). TCR activation by RFX4 further suggests RFX4 is involved in T1N.

Discussion

The autoimmune hypothesis of narcolepsy arose more than thirty years ago based on the strong association of HLA-DQ0602 of the disorder, and although initially, evidence in support of this hypothesis was hard to demonstrate, recent data now suggest T cell involvement in the destruction of HCRT cells. In this work, we investigate whether RFX4, which is highly enriched in HCRT cells, might be a relevant autoantigen in T1N. This was done by testing for the presence of autoantibody and/or of DQ0602 restricted T cell reactivity against it.

Whether or not autoantibodies are involved in the pathophysiology of narcolepsy is controversial. Studies of autoantibodies directed against hypocretin and hypocretin receptors in narcolepsy patients have rendered mixed results (35-38). Other groups have studied reactivity to other proteins expressed in HCRT cells, such as TRIB2, with similarly mixed results (31, 81). Our results show that autoantibodies against RFX4 are rare in T1N patients, with anti-RFX4_v3 autoantibody-like reactivity being significantly increased solely in two patients (1%), both vaccinated with Pandemrix®. None of these patients were positive for anti-HCRTR2 autoantibodies (37). For anti-RFX4_v1/v4 autoantibodies, $\Delta\text{MFI}^{\text{PE}}$ of subject(s) above cut-off could not be repeated and dot plots showed no obvious correlation between PE and GFP channels (Fig. 1), suggesting that anti-RFX4_v1/v4 autoantibody levels in these subjects were too low to be detected. Taken together, these findings support previous data from our lab and others (34), supporting that autoantibodies are unlikely to be involved in the pathophysiology of narcolepsy. Further to this, our findings validate that high throughput flow cytometry-based autoantibody detection assay as a method for primary autoantibody screening is reliable, and not solely for surface antigens.

408

409 Next, we probed the presence of antigen specific CD4+ T cells by co-culturing PBMCs from early-
410 onset and post-Pandemrix® T1N patients with Pandemrix® or a single RFX4 peptide. Although
411 tetramer DQ0602 positive CD4+ T cells were not clearly clustered when stimulated with
412 Pandemrix®, they were observed sporadically in both patients and controls. For example, cells
413 reactive against RFX4-36, -76, and -172 were identified in an early-onset patient (165), PP-N
414 patient (900), and PP-C (023), respectively (Supplemental Fig. 3). The proportions of CD4+ T
415 cells recognized by tetramer pDQ0602 in CD3+ T cell populations were very small (generally
416 lower than 0.085%, most <0.05%), except for one control (901), which also had high background
417 (peptide NC, 0.12%) (Supplemental Fig. 3).

418

419 In probing for the presence of RFX4-antigen specific CD4+ T cells, we detected reactivity against
420 RFX4-60, -86 and -95 in one EO-N, three PP-N patients and two controls. We therefore sought to
421 determine the identity of these cells further and conducted single cell TCR sequencing of reactive
422 cells. Analysis of frequently used TRAV/TRBV pairings revealed that although V gene segments
423 are partially similar between patients and controls, they differ significantly between RFX4-86, -95
424 and -60. To probe the reactivity of specific TCRs to these three peptides, we expressed enriched
425 TCRαβs in Jurkat 76 (J76)-NFATRE-luc cells co-cultured with RM3-DQ0602. Most notably, 50%
426 of TCRs reactive against RFX4-86 shared TRBV4-2, which has been strongly implicated in
427 narcolepsy pathophysiology by our lab before through genetic analysis (13).

428

429 Although the autoimmune etiology of T1N was recently solidified, the exact pathophysiology
430 underlying this disorder still remains elusive. Our findings suggest that future work should be

dedicated to investigating the exact role of RFX4 in T1N and how reactivity to this protein may impact HCRT neuronal death. Competing binding assay and DQ0603 tetramer staining assays, although laborious, were found to be feasible and reliable for screening autoantigen reactivity in T1N, so that these may now be extended to the study of more antigens and patients using new technologies that have higher throughput. Such additional experiments could for example use 10x compatible single cell analysis with DNA barcode labelling and pooling of tetramer reactivity for both strong and weak RFX4 to DQ0602. Single cell TCR sequencing and phenotyping of more RFX4 specific CD4+ and CD8+ T cells could elucidate the role of RFX4 in pathophysiology of narcolepsy.

In conclusion, we provide preliminary evidence for the involvement of RFX4 in T1N in some subjects through both anti-RFX4 autoantibody detection and tetramer DQ0602-strong RFX4 binders staining. Most notable was the enrichment of TRBV4-2, which we have previously shown to be genetically associated with T1N (13) and involved in T cell recognition of HCRT-NH2 in this disorder (46) , hence suggesting causality. We propose an involvement of T cell reactivity against specific RFX4 peptides in the pathophysiology of some T1N cases.

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Figure legends

Fig. 1. Anti-RFX4 autoantibody detection. (A) Sera were screened using HEK293T cells expressing GFP tagged RFX4 variants and PE-conjugated anti-human Ig λ / κ antibody. Analysis was performed between groups of post-Pandemrix[®] T1N (PP-N) patients, post-Pandemrix[®] controls (PP-C), recent early onset T1N (EO-N) patients, and other controls (O-C) for RFX4_v1, RFX4_v3, and RFX4_v4. Each dot corresponds to one subject. $\Delta\text{MFI}^{\text{PE}} = (\text{MFI}^{\text{PE}} \text{ of HEK293T}^{\text{GFP+}}) - (\text{MFI}^{\text{PE}} \text{ of HEK293T}^{\text{GFP-}})$. Cut-off value of $\Delta\text{MFI}^{\text{PE}}$, which is denoted by dotted line, was determined as the mean of $\Delta\text{MFI}^{\text{PE}} + 3 \times$ standard deviation (SD) of control samples. Subject IDs above cut-off value and statistical significance are shown. *, $P < 0.05$; **, $P < 0.01$. It was independently repeated once. (B) FACS dot plots of three subjects in anti-RFX4 autoantibody detection. HEK293T cells expressing RFX4_v1, v3 and v4-GFP were incubated with human serum (1:20) or positive rabbit anti-RFX4 antibody, followed by PE anti-human Ig λ / κ antibody (1:100) or PE anti-rabbit IgG antibody (1:100), respectively. Representative dot plots of 3 subjects and positive antibody (1:500) with % of each quadrant population in live single cells are shown. Ab, antibody. It was independently repeated once. (C) Anti-RFX4_v3 autoantibody detection using cell-based immunostaining and observation under microscope. HEK293T cells expressing GFP-tagged RFX4_v1, v3, and v4 were stained with human serum (1:20) or rabbit anti-RFX4 antibody (Ab) (1:500), followed by AF555-conjugated anti-human IgG antibody (1:500) or AF555-conjugated anti-rabbit IgG antibody (1:500), respectively. 3 subjects with $\Delta\text{MFI}^{\text{PE}}$ above cut-off value of RFX4_v3 were tested. Bar=200 μm . It was performed once. (D) Anti-RFX4 autoantibody detection using western blotting. HEK293T cells expressing GFP-tagged RFX4_v1, v3, v4 and vector were lysed and anti-tGFP-conjugated agarose beads were incubated with supernatant for Co-IP. Positive antibody and three subjects with $\Delta\text{MFI}^{\text{PE}}$ above cut-off value were

tested. RFX4_v1, RFX4_v3 and RFX4_v4 are indicated in positive antibody staining. V, vector. Cell lysates were loaded into two 10-lane gels and proteins were transferred to two polyvinylidene difluoride (PVDF) membranes. Each membrane was cut into two pieces for incubation with different sera and secondary antibodies. All four pieces of membranes were developed in one cassette for the same exposure. Different contrast was applied to each piece after cropping with Illustrator (Version 16.0.0, Adobe®). The full-length gel is included in the supplementary files (Supplemental Fig. 5). It was performed once.

Fig. 2. RFX4 antigen specific CD4+ T cell receptor repertoire. (A) FACS dot plots of tetramer DQ0602-RFX4 staining, PBMCs were stimulated with 6.25 μ M RFX4 peptide in a 96-well plate (1-2.5 $\times$ 10⁶ cells/ml) for 10 days at 37°C, 5% CO₂. 20 IU/ml IL2 was supplemented from day 8 to day 10. Cultured cells were incubated with PE-labelled tetramer DQ0602-RFX4 peptide for 90 minutes at 37°C, 5% CO₂, followed by staining with BV421 anti-CD3, FITC anti-CD4 and AF700 anti-CD8 antibodies on ice. Live single CD3+ T cells with percentage of each quadrant population are shown. EO-N, early onset patient; PP-N, post-Pandemrix® patient; PP-C, post-Pandemrix® control; O-C, other controls. It was independently repeated at least once. (B) Paired TCR enrichment. Tetramer DQ0602 positive CD4+CD8- T cells were single-cell sorted directly into 96-well plates, followed by 3 nested PCR reactions and TCR sequencing pipeline. TCR $\alpha\beta$ counts in each subject-peptide reaction. Each slice represents a unique TCR $\alpha\beta$ clone. The number of single cells for each unique clone is shown in the slices. Unnumbered slices represent a unique clone that was observed only once. The total number of single cells (n) for each subject-peptide reaction is displayed on x-axis below subject. (C) Usage of V-gene segments in patient and control paired TCR repertoire. Single cells obtained from all patients or controls respectively, using the

same TRAV or TRBV gene, were grouped into one category. Each slice represents a unique TRAV or TRBV gene (see adjacent legend) and the percentage of cells using it. TRAVs and TRBVs used by less than 3% of cells are represented by slices without color and number. (D) TRBV and corresponding TRAV/TRBV pairings to each RFX4-86/95/60 peptide. Each slice represents a unique TRBV gene or TRAV/TRBV pairing. Slices show TRBV or TRAV genes used by more than 5 single cells and the same TRBV gene is represented by the same color. In the TRAV/TRBV pairing bar, the top digit describes the particular TRAV gene and the bottom number the total number of single cells using each respective gene (and whether they were isolated from patients P or controls C). Diverse TRBV and TRAV/TRBV pairings expressed by fewer than 5 single cells are represented by bars without color and annotation. TCR sequencing was performed once.

Fig. 3. Activation of TCR signaling by RFX4 antigens presented by DQ0602. Jurkat 76-NFATRE-luc cells were transfected with TCR $\alpha\beta$ and then co-cultured with RM3-DQ0602 presented with 10 μ M of pHA₂₇₃₋₂₈₇, NP₁₇₋₃₁, HCRT_{54-66-NH2}, HCRT_{86-97-NH2}, or RFX4-43, or 5 μ M of RFX4-86, RFX4-95, or RFX4-60, or 0.2 μ l DMSO for 8 hours. Luciferase activity was detected in a plate (n=3). Luminescence fold to RFX4-43 was shown. ***, p<0.001; **, p<0.01; *, p<0.05. It was performed at least once.

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Figures

Figure 1

Anti-RFX4 autoantibody detection. (A) Sera were screened using HEK293T cells expressing GFP tagged RFX4 variants and PE-conjugated anti-human Ig λ / κ antibody. Analysis was performed between groups of post-Pandemrix® T1N (PP-N) patients, post-Pandemrix® controls (PP-C), recent early onset T1N (EO-N)

patients, and other controls (O-C) for RFX4_v1, RFX4_v3, and RFX4_v4. Each dot corresponds to one subject. $\Delta\text{MFIPE} = (\text{MFIPE of HEK293TGFP}+) - (\text{MFIPE of HEK293TGFP}-)$. Cut-off value of ΔMFIPE , which is denoted by dotted line, was determined as the mean of $\Delta\text{MFIPE} + 3 \times \text{standard deviation (SD)}$ of control samples. Subject IDs above cut-off value and statistical significance are shown. *, $P < 0.05$; **, $P < 0.01$. It was independently repeated once. (B) FACS dot plots of three subjects in anti-RFX4 autoantibody detection. HEK293T cells expressing RFX4_v1, v3 and v4-GFP were incubated with human serum (1:20) or positive rabbit anti-RFX4 antibody, followed by PE anti-human Ig λ / κ antibody (1:100) or PE anti-rabbit IgG antibody (1:100), respectively. Representative dot plots of 3 subjects and positive antibody (1:500) with % of each quadrant population in live single cells are shown. Ab, antibody. It was independently repeated once. (C) Anti-RFX4_v3 autoantibody detection using cell-based immunostaining and observation under microscope. HEK293T cells expressing GFP-tagged RFX4_v1, v3, and v4 were stained with human serum (1:20) or rabbit anti-RFX4 antibody (Ab) (1:500), followed by AF555-conjugated anti-human IgG antibody (1:500) or AF555-conjugated anti-rabbit IgG antibody (1:500), respectively. 3 subjects with ΔMFIPE above cut-off value of RFX4_v3 were tested. Bar=200 μm . It was performed once. (D) Anti-RFX4 autoantibody detection using western blotting. HEK293T cells expressing GFP-tagged RFX4_v1, v3, v4 and vector were lysed and anti-tGFP-conjugated agarose beads were incubated with supernatant for Co-IP. Positive antibody and three subjects with ΔMFIPE above cut-off value were tested. RFX4_v1, RFX4_v3 and RFX4_v4 are indicated in positive antibody staining. V, vector. Cell lysates were loaded into two 10-lane gels and proteins were transferred to two polyvinylidene difluoride (PVDF) membranes. Each membrane was cut into two pieces for incubation with different sera and secondary antibodies. All four pieces of membranes were developed in one cassette for the same exposure. Different contrast was applied to each piece after cropping with Illustrator (Version 16.0.0, Adobe®). The full-length gel is included in the supplementary files (Supplemental Fig. 5). It was performed once.

Figure 2

RFX4 antigen specific CD4⁺ T cell receptor repertoire. (A) FACS dot plots of tetramer DQ0602-RFX4 staining, PBMCs were stimulated with 6.25 μ M RFX4 peptide in a 96-well plate ($1-2.5 \times 10^6$ cells/ml) for 10 days at 37°C, 5% CO₂. 20 IU/ml IL2 was supplemented from day 8 to day 10. Cultured cells were incubated with PE-labelled tetramer DQ0602-RFX4 peptide for 90 minutes at 37°C, 5% CO₂, followed by staining with BV421 anti-CD3, FITC anti-CD4 and AF700 anti-CD8 antibodies on ice. Live single CD3⁺ T

cells with percentage of each quadrant population are shown. EO-N, early onset patient; PP-N, post-Pandemrix® patient; PP-C, post-Pandemrix® control; O-C, other controls. It was independently repeated at least once. (B) Paired TCR enrichment. Tetramer DQ0602 positive CD4+CD8- T cells were single-cell sorted directly into 96-well plates, followed by 3 nested PCR reactions and TCR sequencing pipeline. TCRαβ counts in each subject-peptide reaction. Each slice represents a unique TCRαβ clone. The number of single cells for each unique clone is shown in the slices. Unnumbered slices represent a unique clone that was observed only once. The total number of single cells (n) for each subject-peptide reaction is displayed on x-axis below subject. (C) Usage of V-gene segments in patient and control paired TCR repertoire. Single cells obtained from all patients or controls respectively, using the same TRAV or TRBV gene, were grouped into one category. Each slice represents a unique TRAV or TRBV gene (see adjacent legend) and the percentage of cells using it. TRAVs and TRBVs used by less than 3% of cells are represented by slices without color and number. (D) TRBV and corresponding TRAV/TRBV pairings to each RFX4-86/95/60 peptide. Each slice represents a unique TRBV gene or TRAV/TRBV pairing. Slices show TRBV or TRAV genes used by more than 5 single cells and the same TRBV gene is represented by the same color. In the TRAV/TRBV pairing bar, the top digit describes the particular TRAV gene and the bottom number the total number of single cells using each respective gene (and whether they were isolated from patients P or controls C). Diverse TRBV and TRAV/TRBV pairings expressed by fewer than 5 single cells are represented by bars without color and annotation. TCR sequencing was performed once.

Figure 3

Activation of TCR signaling by RFX4 antigens presented by DQ0602. Jurkat 76-NFATRE-luc cells were transfected with TCR $\alpha\beta$ and then co-cultured with RM3-DQ0602 presented with 10 μ M of pHA₂₇₃₋₂₈₇, NP₁₇₋₃₁, HCRT_{54-66-NH2}, HCRT_{86-97-NH2}, or RFX4-43, or 5 μ M of RFX4-86, RFX4-95, or RFX4-60, or 0.2 μ l DMSO for 8 hours. Luciferase activity was detected in a plate (n=3). Luminescence fold to RFX4-43 was shown. ***, p<0.001; **, p<0.01; *, p<0.05. It was performed at least once.

Supplementary Files

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