Research Paper

Prostaglandin D2 Receptor DP1 Antibodies Predict Vaccine-induced and Spontaneous Narcolepsy Type 1: Large-scale Study of Antibody Profiling

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ABSTRACT

Background: Neuropathological fi ndings support an autoimmune etiology as an underlying factor for loss of orexin-producing neurons in spontaneous narcolepsy type 1 (narcolepsy with cataplexy; sNT1) as well as in Pandemrix in fl uenza vaccine-induced narcolepsy type 1 (Pdmx-NT1). The precise molecular target or antigens for the immune response have, however, remained elusive.

Methods: Here we have performed a comprehensive antigenic repertoire analysis of sera using the next-generation phage display method - mimotope variation analysis (MVA). Samples from 64 children and adolescents were analyzed: 10 with Pdmx-NT1, 6 with sNT1, 16 Pandemrix-vaccinated, 16 H1N1 infected, and 16 unvaccinated healthy individuals. The diagnosis of NT1 was defined by the American Academy of Sleep Medicine international criteria of sleep disorders v3.

Findings: Our data showed that although the immunoprofi les toward vaccination were generally similar in study groups, there were also striking differences in immunoprofi les between sNT1 and Pdmx-NT1 groups as compared with controls. Prominent immune response was observed to a peptide epitope derived from prostaglandin D2 receptor (DP1), as well as peptides homologous to B cell lymphoma 6 protein. Further validation confi rmed that these can act as true antigenic targets in discriminating NT1 diseased along with a novel epitope of hemagglutinin of H1N1 to delineate exposure to H1N1.

Interpretation: We propose that DP1 is a novel molecular target of autoimmune response and presents a potential diagnostic biomarker for NT1. DP1 is involved in the regulation of non-rapid eye movement (NREM) sleep and thus alterations in its functions could contribute to the disturbed sleep regulation in NT1 that warrants further studies. Together our results also show that MVA is a helpful method for fi nding novel peptide antigens to classify human autoimmune diseases, possibly facilitating the design of better therapies.

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1. Introduction

Narcolepsy type 1 (NT1) is a chronic neurological disease characterized by irresistible daytime sleepiness, disturbed nocturnal sleep, and cataplexy associated with the inadequate function of the hypothalamus (Peyron et al., 2000; Thannickal et al., 2000; Partinen et al., 2014). The major neuropathological features of NT1 are loss of orexinergic neurons and an increased gliosis in the posterior hypothalamic nuclei (Partinen et al., 2014). Increased levels of pro-inflammatory cytokines have been associated with (spontaneously occurring) idiopathic (sNT1) and Pandemrix vaccine-induced narcolepsy (Pdmx-NT1) close to disease onset (Lecendreux et al., 2015). Pandemrix (Pdmx) is an in fl uenza vaccine used during the H1N1 2009 swine in fl uenza A(H1N1) pandemic and was distributed to over 30 million people in EU/EEA countries.
during the A(H1N1) outbreak. As of January 2015, >1300 cases of vaccine-associated NT1 had been reported to the European Medicines Agency. Epidemiologic and clinical studies conducted in different countries including Finland, Sweden, Ireland, England, Norway, and France have confirmed the association of NT1 in children and adolescents with the AS03-adjuvanted Pdmx (Partinen et al., 2014; Sarkanen et al., 2017). Subsequently, wild-type influenza A(H1N1) infections in China were associated with narcolepsy (Han et al., 2013, 2011). Along with the pandemic A(H1N1) infection, seasonality and post-infectious priming by upper respiratory tract viruses and streptococci have been suggested as triggers of autoimmune response that leads to NT1 in genetically susceptible individuals (Aran et al., 2009; Longstreth Jr et al., 2009).

Genome-wide association studies have revealed a strong association of narcolepsy with the T-cell receptor alpha locus (Hallmayer et al., 2009) and especially with Major Histocompatibility Complex (MHC) class II DQB1*06:02 alleles (Bonvalet et al., 2017; Tafi et al., 2014). DQB1*06:02 is present in approximately 30% of Finnish and Swedish populations (Bomfim et al., 2017). In Finland, all patients with Pdmx-NT1 have been positive for DQB1*06:02 (Partinen et al., 2014). The latter immune haplotype is also strongly associated with the Pdmx-NT1 in Sweden (Bomfim et al., 2017). In another series of 522 patients with narcolepsy and cataplexy from different countries, only 9 patients (1.7%) with low levels of orexin (OX) in cerebrospinal fluid (CSF) were DQB1*06:02 negative (Han et al., 2014). It was also suggested that cross-reactive epitopes to Pdmx vaccine antigens may exist in NT1 disease as a significant proportion of HLA-DQB1*0602-positive Finns diagnosed with NT1 and with a history of H1N1 vaccination were immunoreactive to OX receptors (Ahmed et al., 2015). However, it still is unclear whether OX-positive neurons and/or their neighboring cells express OX receptors that could be targets for the immune response in NT1 (Valko et al., 2013; Vassalli et al., 2015). The antibody levels to viral nucleoprotein (NP), a Pdmx vaccine antigen, were increased in NT1-diseased carrying the HLA DQB1*06:02 allele (Vaarala et al., 2014), whereas the role of this and other circulating (including intrathecal) autoantibodies in NT1 pathogenesis is not fully understood (see list of previously identified antigens in Table S1). Although NT1-related autoantibodies are found in some patients, the clinical response to intravascular immunoglobulin (IVIG) has been hard to predict (see list of previously identified autoantibodies in patients affected by NT1 and in controls). We had access to the clinical cohorts composed of 16 NT1 (sNT1 (n = 6) and Pdmx-NT1 (n = 10)) cases, where all NT1-diseased subjects carried the HLA DQB1*06:02 allele, and apart from 2 sNT1 patients, all had been vaccinated with Pdmx. For reference, we used three well-defined control groups: 16 Pandemrix-vaccinated healthy controls (Pdmx-HC), 16 H1N1-infected Finnish subjects (H1N1-HC), and 16 healthy Estonian donors (HC – healthy controls) (Table 1). Our data revealed complex patterns of immune response in all patient groups including novel epitope sequences present in sera of Pdmx-NT1 and H1N1-HC. One such peptide epitope was identified as belonging to the prostaglandin D2 receptor (DP1) that together with its ligand prostaglandin D2 (PGD2) is involved in sleep regulation in humans and experimental animal models (see ref. in Urade and Hayashi (2011)).

### 2. Materials and Methods

#### 2.1. Vaccines

Pandemrix vaccine is derived from X-179A, a reassortant of hemagglutinin (HA), neuraminidase (NA) and polymerase acidic protein (PA) of A/California/07/2009 and X-157 H3N2 in a PR8 backbone (Jacob et al., 2015; Nicolson et al., 2012; Robertson et al., 2011). The vaccine composition can be found summarized by the European Medicines Agency and GlaxoSmithKline plc (European Medicines Agency, 2009).

#### 2.2. Study Population

The present study comprises a total of 64 individuals (Table 1). Altogether, 16 serum samples of H1N1-infected military servicemen (H1N1-HC), 16 serum samples of age/sex-matched Pandemrix-vaccinated healthy controls (Pdmx-HC) were kindly provided by National Institute of Health and Welfare, Finland. 16 serum samples of age/sex-matched Pandemrix-vaccinated healthy controls (Pdmx-HC), 16 serum samples of H1N1-infected military servicemen (H1N1-HC), and 16 healthy Estonian donors (HC – healthy controls) (Table 1). Our data revealed complex patterns of immune response in all patient groups including novel epitope sequences present in sera of Pdmx-NT1 and H1N1-HC. One such peptide epitope was identified as belonging to the prostaglandin D2 receptor (DP1) that together with its ligand prostaglandin D2 (PGD2) is involved in sleep regulation in humans and experimental animal models (see ref. in Urade and Hayashi (2011)).

| Characteristics | Narcolepsy (NT1) patients | Healthy controls (HC) |
|-----------------|---------------------------|-----------------------|
| Group size (n)  | 10                        | 16                    |
| Gender (female/male) | 5/5                      | 12/2*                 |
| Pandemrix vaccination | 11/2009-1/2010          | 11/2009-1/2010*       |
| Sample collection | 2011                      | 2011                  |
| Median age at onset (y) | 13                       | 2011                  |
| Median age at sampling (y) | 14                       | 2011                  |
| Unambiguous cataplexy | 10/10 (100%)             | 10/6                  |
| MSLT mean SL (range) | 2.6 (0-7.5)              | 21                    |
| SOREMPs mean (range) | 3.7 (2-5)                | NA                    |
| HLA DQB1*0602 (%) | 6/6 (100%)               | NA                    |
| CSF-orexin < 150 pg/mL (lower 1/3 limit in Finland) | 5/5 (100%) | NA |

HC - healthy control, H1N1-HC – H1N1 infected, Pdmx-HC – Pandemrix-vaccinated, NT1 - narcolepsy type 1 (including 10 Pdmx-induced NT1 samples (Pdmx-NT1) and 6 sporadic NT1 (sNT1) samples), NA – not available, SL – sleep latency, MSLT – Multiple sleep latency test, SOREMPs – Sleep onset REM periods as defined by the American Academy of Sleep Medicine.

* Gender of two Pdmx-HC is unknown.

b Four out of 6 sNT1 patients were vaccinated after they had been diagnosed with NT1.

### Table 1

Description of samples studied.

H1N1-HC – H1N1 infected, Pdmx-HC – Pandemrix-vaccinated, NT1 - narcolepsy type 1 (including 10 Pdmx-induced NT1 samples (Pdmx-NT1) and 6 sporadic NT1 (sNT1) samples), NA – not available, SL – sleep latency, MSLT – Multiple sleep latency test, SOREMPs – Sleep onset REM periods as defined by the American Academy of Sleep Medicine.
2.3. Ethical Permissions

The patients have participated in the NARPANord narcolepsy study (Academy of Finland, grant nr. 260603), and they have given a written informed consent. The serum samples of H1N1-infected military servicemen and serum samples of the Pdmx-vaccinated healthy controls were provided by the National Institute of Health and Welfare, Finland. The ethical permissions were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, Finland.

2.4. Mimotope-Variation Analysis

For qualitative and quantitative characterization of humoral immune response from sera samples, we used an in-house developed mimotope-variation analysis (MVA) method. Fig. 1A provides an overview of the process. In brief, a random 12-mer peptide phage library consisting of a 36-bp random insert region and a constant region. We sequenced per sample. In order to compensate for the different numbers of reads per sample normalization of read counts was performed. All samples were trimmed to 3 million reads (RPM units). The resulting data was represented as a cross-table where each row corresponded to a different 12mer peptide, each column corresponded to a different sample, and each cell showed the read count of the peptide in the respective sample measured in RPM-units. According to the manufacturer (NEB), naive library contained up to $10^9$ different sequences. For reasoned cost purposes, the estimated outcome of sequence data represented 0.1% of the initial library input containing up to $2.8 \times 10^6$ different peptide sequences per sample. Complete analysis of sequence diversities obtained by MVA remains out of the scope of the current study.

2.6. Clustering Workflow

The main assumption was that every obtained peptide sequence mimics the target of an antibody. The sequence reads of one sample often included many copies of the same peptide sequence. The read counts of a peptide could range from 1 to thousands. To reveal recognition patterns (epitope motifs) which were enriched in the cases compared to controls, we used SPEXS2 software (https://github.com/egonelbre/spexs2; (Vilo, 2002, Brazma et al., 1998)). For clustering the peptides with motifs and generating mimotope regular expression and sequence logos, the “motiffree” tool was used (Kruup, 2013). The Multiple EM for Motif Elicitation (MEME-MAST) algorithm (Bailey and Elkan, 1994; Bailey and Gribskov, 1998) was used to align peptides to proteins. For cell epitope mapping IEDB 3.0 database was used (Vita et al., 2015).

2.7. Statistical Analysis

All statistical analyses (ANOVA, t-Test, correlation analyses, Chi-square test) were done using MedCalc software (MedCalc Statistical Software version 17.0.4 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2017)). For visualization of peptide abundance across samples, peptide frequency values were converted to heatmap images (Tagged Image File) with Excel Visual Basic for Applications (VBA) scripts. For visualization of selected peptide set alignment profile on proteins of interest Excel VBA script was used. The protein sequence was scanned with every peptide and at every position where the peptide aligned with it in at least four perfectly matching positions, one was added with its frequency. For random reference profile, amino acid sequence of each peptide was randomized and scanned using the same rules over the target sequence.

2.8. Influenza Virus Serology

Levels of influenza-specific IgG antibodies were determined by the enzyme-linked immunosorbent Vir-ELISA anti-H1N1/H3N2 IgG assay (Influenza virus type A IgG ELISA test system, Euroimmun), carried out in accordance with the manufacturer’s specifications. Absorbance was measured at 450 nm with SpectraMax Paradigm.

2.9. Peptide ELISPOT

For peptide ELISPOT the following peptides were designed:

| Peptide | Sequence |
|---------|----------|
| #1      | RVLAPALDSWGTTGCGDYYKDDD[LYS(BIOTIN)] |
| #2      | LPKFSAPASGPGGDDYKDDD[LYS(BIOTIN)] |
| #3      | ESTRYQLWLHPQGDDYKDDD[LYS(BIOTIN)] |

For peptide ELISPOT control peptide was:

| Peptide | Sequence |
|---------|----------|
| Control | AVLAAALASWGTGCGDDYKDDD[LYS(BIOTIN)] |
In brief, 110 pg biotin-conjugated peptides were printed on nitrocellulose coated slides (10485323, Whatman) by SpotBot® 4 (Arrayit). For primary antibody human precleared serum (1:100) was used, for secondary antibody rabbit anti-human IgG (H&L) (HRP) (Abcam) was used. All incubations were done for 1 h at room temperature. Results were scanned using Ettan Digelager (GE Healthcare Life Sciences) and images calculated using ImageQuant software version 8.1 (GE Healthcare Life Sciences).

2.10. Cancer Cells, Human Mesenchymal Stem Cells (hMSC) and Post-Mortem Tissues

Immortalized glioblastoma multiforme cells (human glioma cells - hGC) (kind gift of Prof. Aavo-Valdur Mikelsaar, Estonia), human neuroblastoma cell line Kelly (ATCC) and human mesenchymal stem cells (hMSC) (isolated from human subcutaneous adipose tissue as described (Jaager and Neuman, 2011)) were grown in Dulbecco’s modified Eagle's medium (DMEM (PAA)) containing 10% fetal bovine serum (PAA), 1 mg mL⁻¹ penicillin (PAA) and 0.1 mg mL⁻¹ streptomycin (PAA). All cells were cultured at 37 °C in 5% CO₂. The identity of hMSC was confirmed by using cell morphology and flow cytometry methods for analysis of cell surface markers: CD73+/CD90+/CD105+/CD45⁻/CD34⁻ (Kauts et al., 2013). For treatments, hMSCs were grown with media containing IL-1α (1 ng/mL), IFNγ (2 ng/mL) for 8 h, or PGD2 (10 μM) for 1 h.

Human post-mortem tissues were procured from the North-Estonian Regional Hospital, Tallinn, Estonia. All experiments with human tissues were done with the approval of the local ethical committee (license no. 2234, date of issue 09.12.2010).

![Diagram of workflow](image)

Fig. 1. Humoral immune response studied using the mimotope-variation analysis (MVA) method. A. Schematic drawing of the workflow in MVA. MVA is a high-throughput random peptide phage display analysis. A random peptide display library (PhD12) was used which contained 10⁹ different 12-mer peptide sequences introduced to the N-terminus of the phage major coat protein p11 (NEB). For MVA, sample-specific IgG proteins (antibodies, Human IgG fraction) present in human sera of interest are allowed to interact with the phage-displayed peptides and the IgG–phage complexes were captured to protein G magnetic beads, while the unbound phages were washed away (Peptide library display). Captured phages were lysed and DNA amplified with primer sequences containing a tag with a unique barcode sequence and the final amplicons were pooled for NGS analysis (HTS sequencing). The primer set homologous to the M13KE vector sequences that flank the random peptide coding sequence was used to amplify a 50-bp fragment. Data analysis to classify peptides that were specific to Pmdx-infected, -vaccinated and NT1-diseased individuals was carried out by comparing the profiles of peptides (mimotopes) from diseased to those from non-diseased (Peptide profile analysis). On average, MVA generated 1.8 million peptide sequences with unique structure (divergence) totaling 2.8 million peptide sequences in abundance (total abundance; number of reads) per sample. Altogether, a peptide data set with ~14% of the peptide sequences were shared between samples, out of these ~8.5% were common to 2 samples, ~5% to 3 samples. The right pie (red) displays the distribution of shared 16,844 peptide sequences out of which ~60.7% were common to 2 samples, 35.7% to 3 samples, 0.5% were detected in all unique peptides; ~0.3% were detected in ~10 samples and 3.6% were seen in >10 samples. The four pie charts (below) exemplify the peptide profile structures in different clinical cohorts. The size of each pie piece is proportional to the number of unique peptides common to one or more samples of a clinical cohort. Blue - represents unique peptides, red - the most shared. C. Individual variation in peptide divergence is characteristic to all immunoprofiles. Top 2500 peptides were analyzed to assess the range of individual peptide variation across study cohorts. Blue dots mark peptide divergence in a single sample. As indicated, between one to two thousand peptides were individual-specific, whilst the most common peptides (shared by >10 individuals) ranged in divergence from tens to 350 across samples. Range of unique peptide variations was similar across all study samples. D. Heat map image of a random fragment of MVA profile encompassing 400 peptides across study samples. Peptide profiles were individual-specific with a highly varying abundance. Each column represents the peptide profile of a single individual, and each line represents a peptide with a unique primary structure. Abundance is presented as counts in logarithmic scale (in log); black colour depicts peptides captured at higher abundance, and white those at lower abundance. Shown are peptide profiles that were common to 3–10 individuals. Abbreviations: Abundance – peptide frequency; Divergence – all unique peptides; HC - healthy control; H1N1-HE - H1N1 infected; Pmdx-HC - Pandemrix-vaccinated; NT1 - narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).
2.11. Immunofluorescence and Western Blot Analysis

For immunofluorescence analysis, cells grown on glass inserts were fixed using 4% PFA (Scharlau) for 15 min and blocking of the unspecific reactivity was done with 5% BSA. The antibodies used included: anti-DP1 (Abnova; 1:500), precleared human sera (1:400), and the secondary Alexa Fluor 488 and 647 (Invitrogen, 1:2000). For epitope blocking peptide #1 was used in final concentration 6.6 μg/mL. Hoechst 33342 (Invitrogen) was used to detect cell nuclei. Imaging was done using Nikon Eclipse 80i microscope.

Sequences (RLPALDWSGT and DYKKDDDK (flag)) were inserted at the N-terminus of the pII of the M13Ke phage by in vitro mutagenesis PCR using primers s1 5′ GTGGATAGTGAGGGCAACCGTGAGGTTTCG GCCGAAACC3′, as1 5′ GCCGGAGCTGATACAGGTTGGAGGAATGTA AAAACCGTAC3′, s2 5′ GTGGATAGTGAGGGCAACCGTGAGGTTTCG GCCGAAACC3′, as2 5′ GCCGGAGCTGATACAGGTTGGAGGAATGTA AAAACCGTAC3′, as3 5′ ATCGTTTAATATACCGCTGCCGTTTTAT3′, s3 5′ ATCTTTTAATATACCGCTGCCGTTTTAT3′, s4 5′ ATCTTTTAATATACCGCTGCCGTTTTAT3′, as4 5′ ATCTTTTAATATACCGCTGCCGTTTTAT3′. PCR reactions were carried out with phusion Hot Start II High-Fidelity DNA Polymerase (ThermoScientific). Constructs were verified by sequencing, 30 μg of protein lysate or 1 x 10^13 phage particles were resolved on 10% SDS–polyacrylamide gels and transferred onto PVDF membranes (Amersham) for 1.5 h using BioRad wet blotter in standard Towbin buffer. The membrane was blocked with 5% nonfat milk (AppliChem), incubated overnight with the following primary antibodies: anti-DP1 (St. John Laboratory, 1:1000), anti-GAPDH (Sigma, 1:10,000), precleared human sera (1:500). The epitope blocking peptide #1 was used in final concentration 6.6 μg/mL. The membrane was incubated for 1 h at room temperature with the secondary anti-mouse, anti-rabbit, or anti-human IgG antibodies (Abcam; dilution 1:10,000), The ECLEmto kit (Ansham) was used for detection of immunoblotted target proteins.

2.12. RNA Extraction, RT-PCR and qRT-PCR

Total RNA from human brain parts was extracted using RNAWiz (Ambion) as recommended by the manufacturer. Total RNA from cells was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA from cells was reverse transcribed using SuperScript III first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The resulting cDNAs were used as templates for subsequent RT-PCR reactions. RT-PCRs were carried out using FIREPol® DNA polymerase (Solis Biodyne), 40 amplification cycles and an annealing temperature of 58 °C. Amplification of the housekeeping gene GAPDH was performed for 25 cycles using FIREPol® DNA polymerase (Solis Biodyne) and used as an internal control. Used primer sequences: PTGDR sense 5′ ATGAAAGTCGAGCTTCTAC3′, PTGDR antisense 5′ CATGAAGAAGCCAAGCTG3′, GAPDH sense 5′ GAAGGTGAAGGTCGGAGT3′, GAPDH antisense 5′ GATGATGATAAAGGTGG3′, the sera to protein fragments representing the four major antigens of H1N1 infection and Pdmx-vaccination using type A influenza ELISA (Quantum) diagnostic tests. High-titer responses to influenza A virus major antigens (including H1N1) were evident for both Pdmx-vaccinated and H1N1 naturally infected individuals (Fig. 2A). The humoral response to seasonal flu (A/H1N1 and A/H3N2) was relatively weaker in NT1-diseased as compared with Pdmx-NC HC individuals as determined by using a commercial ELISA test (p < 0.001). This was in slight contrast to earlier findings reporting that Pdmx-NT1 patients had higher median levels of anti-H1N1 antibodies than controls (Lind et al., 2014), and may reflect the characteristics of the samples collected (Table S1). We next assessed the reactivity of the sera to protein fragments representing the four major antigens of H1N1 virus proteome (strain A/California/7/2009). MVA data analyses of Top2500 peptide data set revealed 4 antigenic regions for hemagglutinin (H1N1/HA, C4RUW8), 5 for neuraminidase (H1N1/NA, C3W6G3), 3 for nucleoprotein (H1N1/NP, B4URE0), and 6 for polymerase acidic protein (H1N1/PA, IP6THC5), some of which corresponded to known immunogenic epitopes from IEDB (http://www.iedb.org/; Fig. 2B). Statistically distinct coverage profiles with different peaks on H1N1 HA, NA, NP, and PA antigens were obtained from analysis of Top2500 peptide data sets of H1N1-NC, Pdmx-NC and NT1 samples (Fig. 2C). Data showed that the most commonly shared epitopes raised by the anti-Pdmx/anti-H1N1 immune response were found in the C-terminal region of H1N1/HA (C4RUW8) located between amino acids 521 to 531 (Fig. 2B–C), directly before a proven T cell-antigenic region in HA between amino acids 527–541 of A/California/04/2009 (H1N1, (Schaden et al., 2011)). About 700 peptides from the total peptide dataset clustering to motif with sequence consensus E[ST]R.[QM] were highly abundant in H1N1-NC, and relatively infrequent in Pdmx-NC and NT1 samples as compared with HCs (Fig. 2D).

3. Examination of Identified NT1-specific Autoantigens in MVA Dataset

Next we determined peptides that were different between the clinical study groups to examine whether they were consistent with the prior knowledge of Pdmx-NT1-specific immunogenic epitopes. For the study, we used an exhaustive sequence pattern search (SPExS - https://github.com/egonelbre/spexs2; (Vilo, 2002, Brazma et al., 1998)) gene ontology analysis, combined with the interrogation of the presence of known autoantigens previously identified in Pdmx-NT1 disease (Table S1(Ahmed et al., 2015, Bergman et al., 2014, Cvetkovic-Lopes et al., 2010, De La Herran-Arita et al., 2013, Haggmark-Manberg et al., 2016, Katzav et al., 2013, Zandian et al., 2017)). Thus, we were able to confirm statistically significant patterns of epitope recognition in the samples. Particularly, we identified epitopes resembling those in the N-termini of OX (4/16) and OX1R/2R (2/16; 4/16), in mitogen-activated protein kinase 7 (MAP3K7) (amino acids 318–328; 3/16) and in 5′–nucleotidase cytosolic IA (NT5C1A) (amino acids 35–48; 2/16), as well as in B-cell lymphoma 6 protein (BCL6), encompassing amino acids 279–288 in 6 out of 16 sera samples of NT1 diseased (Fig. 3, Table S1). According to MVA data, none of the previously identified antigens was prominently detected across NT1 diseased and were also common also to HC if less stringent statistical power criteria were used (Fig. 3). In contrast, we found no evidence of stratifying peptides with consensus sequences mimicking tribbles pseudokinase 2 (TRIB2), neureptope glutamic acid–isoleucine/α-melanocyte-stimulating hormone (NEI/aMSH), or others that were reported by earlier studies.
In analyses of the peptides that were unique among the disease groups, we observed that the Top2500 dataset contained ~1300 peptides with a high enrichment in NT1 (Fig. S1A–B). The most abundant peptide having the sequence RVLAPALDSWGT showed a high sequence homology within the second extracellular loop region in the human prostan glandin D2 receptor DP1 (Q132358). This region in DP1 is predicted to function in ligand recognition (Avlani et al., 2007; Nagata et al., 2017) and is not conserved in mouse and rat (Fig. 4A). Extraction of all peptides from the total dataset having the highest homology to RVLAPALDSWGT and to DP1 revealed a set of 4428 unique peptides containing the RxxxPxxD (RPD) consensus sequence that discriminated the NT1 samples from controls (p < .001, ANOVA; Fig. 4B–C). We then also determined that the 2157 RPD-containing peptides out of 4428 (Fig. 4B) had a high sequence homology to DP1 protein where the bona fide immunodominant epitope with sequence RVLAPALD represented amino acids 94 to 101 in DP1 (Fig. 4D).

To validate the data, we employed different methods and measured the serologic response to peptides carrying the RPD consensus sequence using sera samples of the study (Fig. 5–6). In line with previous reports (Table S1). These data allowed concluding that apart from the BCL6 related subset, peptides corresponding to previously identified autoantigens had relatively little discriminative power, suggesting also that these antigens were either rare or recognized promiscuously in patient groups with a clinical and ethnical heterogeneous background.

3.4. A Defined Set of Peptides Derived From DP1 Acts as Antigenic Epitopes in NT1

(IEBD database (www.iedb.org/), four MHC-I binding epitope regions of DP1 encompassing amino acids 132–311 (ID: 637966), 195–311 (ID: 697995) encoded another extracellular domain of DP1 (Q132358), which was not present in both seasonal infection carriers and Pdmx-vaccinated individuals. The graphs show antigen-specific profiles of overall peptide abundance where the number of peptides were counted for each amino acid position for the following proteins: hemagglutinin (H1N1/HA, C4RUW8), neuraminidase (H1N1/NA, C3W6G3), nucleoprotein (H1N1/NP, B4URE0) and polymerase acidic protein (H1N1/PA_I6THC5). The latter (303–311) encoded another extracellular domain of DP1 (Q132358), which was not present in both seasonal infection carriers and Pdmx-vaccinated individuals. The graphs show antigen-specific profiles of overall peptide abundance where the number of peptides were counted for each amino acid position for the following proteins: hemagglutinin (H1N1/HA, C4RUW8), neuraminidase (H1N1/NA, C3W6G3), nucleoprotein (H1N1/NP, B4URE0) and polymerase acidic protein (H1N1/PA_I6THC5).
(Urade and Hayaishi, 2011), we observed a broad expression of DP1 mRNA across different regions of human brain, in human glioma (hGC), and normal mesenchymal stem cells (hMSCs) (Fig. 5A). Treatments of hMSCs with the ligand prostaglandin D2 increased DP1 expression, whilst the pro-inflammatory cytokines interleukin 1β and interferon γ (IL-1β and IFN-γ) either slightly increased or decreased its expression, respectively (Fig. 5B). In contrast, PGD2 strongly reduced IL-1β and IFN-γ expression suggesting that these cells recapitulate the intact PGD2-DP1 signaling pathway by inducing anti-inflammatory responses in the studied cells (Fig. 5B). Performing phage Western blot assay we confirmed that MVA predicted DP1-positive Pdmx-NT1 sera showed IgG reactivity to phages that displayed RVLAPALDSWGT peptides (RVLAPALD-pIII, Fig. 5C). No specific reactivity was detected using DP1-negative sera (Fig. 5C). This peptide target specificity was further confirmed by Western blot analysis using Pdmx-NT1 sera where the interactions between human IgGs and antigen expressing phages were blocked by RVLAPALDSWGT synthetic peptides (Fig. 5C, Fig. S2). Immunoblot analysis using commercial anti-DP1 polyclonal sera and clinical sera of Pdmx-NT1, confirmed the presence of DP1 expression in hGC_1 and not in hGC_2 glial cells and also here specific blocking effects to the seroreactivity in the presence of RVLAPALDSWGT peptides were confirmed (Fig. 5D), but not in case of control peptides or irrelevant sera (Fig. 5C, Fig. S2). Immunocytochemical analyses showed that DP1 was predominantly localized on the cell surface of hMSC and hGC cells, and more importantly, was equally well-detected by immunocytochemistry using commercial anti-DP1 polyclonal sera and Pdmx-NT1 clinical sera (Fig. 5E). Furthermore, the synthetic peptide RVLAPALDSWGT competed for the binding of anti-DP1 antibodies present in sera of Pdmx-NT1 diseased (Fig. 5E). Data combined from Western blot and immunocytochemistry analysis suggested that peptide RVLAPALDSWGT could embed a structural as well as a linear epitope given that upon competition it interfered with DP1-specific serorecognition of globular as well as denatured epitopes (Fig. 5D and E).

We next studied whether the peptides identified here could be developed to an ELISPOT assay to discriminate sera in different disease groups. ELISPOT analysis data showed that peptides containing H1N1/HA-specific sequence ESTRYQL (peptide_3) discriminated between naturally H1N1 infected and healthy samples with no earlier H1N1 infection (ANOVA p < .001, Fig. 6A). RVLAPALD (epitope on DP1) and KAPSAS (epitope on BCL6) (peptide_1 and _2) peptides that were selected upon MVA data, correctly assigned upon ELISPOT analysis the NT1 group from HC samples (ANOVA p < .001, Fig. 6B). Combined ELISPOT analysis using all 3 peptides, could correctly classify 11 out of 16 NT1 (specifically - 7 Pdmx–NT1 and 3 NT1) samples across all controls (p < .001 Chi-squared test, Fig. 6C). Notably, majority of the DP1 and BCL6-peptide-positive NT1 samples had undetectable OX findings from the related CSFs (with average values of 6.1 pg/mL), whereas those 4 that were negative by our ELISPOT measurements, had OX levels in respective CSF samples still low but in detectable range (with average values of 77 pg/mL (Fig. 6C and see Materials and methods)). Unlike the IgG response, the IgM levels in response to the tested peptides were low or absent in all studied individuals (data not shown). These findings confirmed that peptides carrying the epitope motifs identified in the study could be used in ELISPOT analysis to develop a novel multibiomarker diagnostic assay for NT1.

4. Discussion

Despite extensive research using biomarker and neurophysiological approaches, known heterogeneity among NT1 diseased is not always consistent with serological marker-based subtype classification schemes. Using an unbiased analysis of serum samples from single individuals, we detected a high variance in humoral immune response profiles, both in healthy and diseased people. We found that variance in immunoprofiles representing multifactorial heterogeneity of NT1 clearly determined distinct disease-specific serological profiles. We focused our analysis on peptides specific to Pdmx-immunized and –NT1 diseased subjects, which encompassed vaccine antigens and autoantigens in order to have a full coverage of potential triggers of the disease. Our results show that patients with NT1 exhibit a specific immune response to epitopes of receptor DP1. This finding highlights the importance of the PGD2-DP1 pathway in the functioning of sleep-wake homeostasis as suggested by the role of DP1 in slow-wave sleep (Terao et al., 1998). However, the precise mechanism by which PGD2-DP1 signaling may influence orexinergic neurons and immune regulation in NT1 requires further studies. In addition, using MVA-based immunoprofiling, we discovered epitopes, such as those of the protein BCL6 specific for Pdmx-NT1 and sNT1 patient group. This underscores the complexity of NT1 with different molecular targets and pathways involved and contributing to the immune response. Enhanced inflammation due to immune system malfunction has been detected in human narcoleptics in the regions of OX cell loss (Bassetti et al., 2010; John et al., 2013; Nishino, 2011; Thannickal et al., 2000, 2003,
but the exact factors or mediators leading to the ultimate death of OX neurons are yet unknown.

Here we show that the DP1 receptor is linked to NT1 by acting as a possible antigen in the disease process. Prostaglandins play a key role in the inflammatory response and their synthesis is significantly increased after tissue injury and cell stress (Ricciotti and Fitzgerald, 2011). PGD2 is a major eicosanoid both in the Central Nervous System (CNS) and peripheral tissues with a role in inflammation as well as homeostasis (Jowsey et al., 2001; Vijay et al., 2017). PGD2 is abundantly produced by mast cells and Th2 cells, and among a wide range of
other body cells (see ref. in Farhat et al. (2011)). PGD2 elicits its downstream effects by activating DP1 and DP2 receptors with opposing effects on cyclic AMP (cAMP) production, and/or phosphoinositol turnover and intracellular Ca2+ mobilization (Liang et al., 2005). In the brain, PGD2 regulates sleep, body temperature, and nociception and its levels exhibit marked changes in different neuropathologies (reviewed in (Liang et al., 2005; Mohri et al., 2006; Urade and Hayaishi, 2011)). The microglial PGD2-DP1 pathway is also known to mediate neuronal damage through microglial activation (Bate et al., 2004; Vijay et al., 2017).

Among cells expressing DP1, mast cells (MCs) can release histamine and other factors that affect sleep and the immune response in the brain. Accumulating evidence shows that MCs play a role in the regulation of sleep and behavior (Chikihisa et al., 2013). MCs are most abundant in young individuals under the age of 19, after which their counts decline with age (Porzionato et al., 2004; Turygin et al., 2005). Most significantly, the maturation of MCs is influenced by PGD2 and the receptor DP1 (Taketomi et al., 2013). In addition to histamine, DP1 signaling may also influence the levels of adenosine that is known to regulate sleep (Urade and Hayaishi, 2011). The precise role of MCs and their released factors such as histamine in NT1 warrants further studies.

In conclusion, the present study shows that anti-DP1 antibodies are autoimmune agents in the course of NT1 prompting more studies on the role of PDG2-DP1 signaling in OX-signaling and in the disease. Currently DP1-selective agonist/antagonist therapies are considered in treating autoimmune disorders such as asthma (Maicas et al., 2012; Santini et al., 2016; Santus and Radovanovic, 2016). Our data also indicate that, depending on the antibody concentrations and affinities, anti-DP1 antibodies may modify the function of pharmaceutical compounds targeting PGD2-DP1–signaling pathways (Narumiyu and Fitzgerald, 2001) that need to be taken into account in clinical studies.

Supporting evidence that the humoral response in the CNS is derived from peripheral tissue antigens is provided by the findings that sera from NT1 diseased can bind brain and muscle structures (Ahmed et al., 2014; Smith et al., 2004). There is a plethora of data that genetic or experimental alterations of the OX system are associated with NT1, however, OXs are not restricted to the CNS and together with their receptors OX1R and OX2R are widely expressed in peripheral tissues (see ref. in (Voisin et al., 2003)). BCL6, another NT1 antigen, is a master regulator required in mature B-cells during germinal center (GC) reaction (Ref in Pei et al. (2017)). NRXN1-α has been isolated from brain and heart tissues suggesting a role also in heart development (Nagase et al., 1998). TRIB2 is present in many
cell populations both in and outside the nervous system, including the immune cells (Eder et al., 2008; Sung et al., 2006). Gangliosides (anti-GM3) are abundant in the brain, but in extraneural tissues, relatively high concentrations of ganglio-series GMs were found in bone marrow, erythrocytes, intestine, liver, spleen, testis, kidney, and in embryonic stem cells (Kolter, 2012). NEI-MCH has mostly been detected in peripheral organs (Viale et al., 1997). NT5C1A is highly abundant in skeletal muscle tissue (Hunsucker et al., 2001). GLS2 is expressed specifically in the liver, but also in extrahepatic tissues, like the brain, pancreas, cells of the immune system (ref in Martin-Ruflan et al. (2012)). However, it remains elusive what pathogenic roles these antibodies against the above-mentioned proteins may exert within the periphery.

Our data of immunoprofiling support the existence of immune defects in multiple pathways associating NT1 to a) DP1 and PDG2/histamine associated disorders, b) BCL6 and the chronic status of latent herpesviruses (such as EBV), c) orexin/OX1/2R-related dysfunctions, d) stress and inflammation-associated mitogen-activated pathways (such as MAP3K7, also known as transforming growth factor (TGF)-β-activated kinase 1 (TAK1)), and e) adenosine-deficiency linked dysfunctions (involving NT5C1A) (Table S1, Fig. 7). Together these results provide a comprehensive map of potential molecular targets contributing to NT1 that can be of help in designing future strategies for the diagnostics and treatment of the disease. More broadly, our study demonstrates the usefulness of MVA as a method for disease classification and for the discovery of novel biomarkers that can be applicable to any human disease.

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Conflicts of Interest

AV, OV, MP, KP, TN, and KP are co-applicants on a pending patent application related to diagnosing of narcolepsy (WO 2017/203106). All other authors declare no competing interests.
Author Contributions

HS, AP, TN, MP, AV, and KP contributed to the design of the study. MP, OV, and AV were members of NARPANord consortium, MP was the chairman. HS, AP, AK, SP, MJ, DL, and KP contributed to the development and implementation of the data analysis plan. JV and PA were responsible for data management and pattern recognition analysis of the MVA data. All authors were involved in data interpretation, drafting, review, and approval of the report, and the decision to submit for publication.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.01.043.
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