Enhanced influenza A H1N1 T cell epitope recognition and cross-reactivity to protein-O-mannosyltransferase 1 in Pandemrix-associated narcolepsy type 1

A. Vuorela1,13, T. L. Freitag2,3,13✉, K. Leskinen3, H. Pessa3, T. Härkönen1, I. Stracenski2, T. Kirjavainen4, P. Olsen5, O. Saarenpää-Heikkilä6, J. Ilonen7,8, M. Knip1,4,9, A. Vaheri10, M. Partinen1,11,12, P. Saavalainen3, S. Meri2,3 & O. Vaarala1

Narcolepsy type 1 (NT1) is a chronic neurological disorder having a strong association with HLA-DQB1*0602, thereby suggesting an immunological origin. Increased risk of NT1 has been reported among children or adolescents vaccinated with AS03 adjuvant-supplemented pandemic H1N1 influenza A vaccine, Pandemrix. Here we show that pediatric Pandemrix-associated NT1 patients have enhanced T-cell immunity against the viral epitopes, neuraminidase 175–189 (NA175–189) and nucleoprotein 214–228 (NP214–228), but also respond to a NA175–189-mimic, brain self-epitope, protein-O-mannosyltransferase 1 (POMT1 675–689). A pathogenic role of influenza virus-specific T-cells and T-cell cross-reactivity in NT1 are supported by the up-regulation of IFN-γ, perforin 1 and granzyme B, and by the converging selection of T-cell receptor TRAV10/TRAJ17 and TRAV10/TRAJ24 clonotypes, in response to stimulation either with peptide NA175–189 or POMT1675–689. Moreover, anti-POMT1 serum autoantibodies are increased in Pandemrix-vaccinated children or adolescents. These results thus identify POMT1 as a potential autoantigen recognized by T- and B-cells in NT1.
Identification of influenza A H1N1 virus T-cell epitopes in Pandemrix-associated NT1 patients. To dissect the influenza A (H1N1)-directed T-cell immunity, PBMC from a discovery cohort of well-characterized Finnish pediatric cases of Pandemrix-associated NT1, and from healthy age-matched, Pandemrix-vaccinated control children (Table 1, Supplementary Tables 3A, B), were stimulated with a panel of pre-selected 15-mer peptides from HA, NA or NP (Supplementary Table 2). Interestingly, two viral T-cell epitopes were differentially recognized and induced high IFN-γ and IL-2 responses in Pandemrix-associated NT1 patients in comparison to healthy vaccines. These epitopes were AWSASACHDGINWLTV (NA178-192) and KTRIAYRMCMNIIKQFQK (NP214-231). Epitopes AFAMERNAGSGIIIS189 (HA271-285) and MERNAG5GHIISDTTP (HA274-288), previously associated with NT1, were also recognized, but the IFN-γ and IL-2 responses were relatively low, and no significant differences between patients and healthy vaccines were observed (Fig. 3a–f, Supplementary Fig. 2A–F).

Next, the AWSASACHDGINWLTV (NA178-192) and KTRIAYRMCMNIIKQFQK (NP214-231) epitopes were mapped in a validation cohort of Finnish pediatric Pandemrix-associated NT1 patients, and from healthy age-matched, Pandemrix-vaccinated controls (Table 1, Supplementary Tables 3A, B). Stimulations with overlapping 15-mer peptides in T-cell assays showed that AWSASACHDGIN (NA178-189) and IAYRMCMNIIKQFQK (NP217-232) are T-cell core epitopes (Fig. 4a, d, Supplementary Fig. 3A, D). Interestingly, the AWSASACHDGIN (NA178-189) epitope includes WSASACHD (NA178-189), an influenza A (H1N1) B-cell epitope, predicted by phage display-mimotope variation analysis to react with sera from Pandemrix-associated NT1 patients.

Different T-cell responses to influenza A H1N1 epitopes in NT1 patients and HLA-DQB1*0602 positive or negative healthy vaccines. T-cell responses to the epitopes NA175-189, NA178-192, and NP214-228 were stronger in NT1 patients than in either HLA-DQB1*0602 positive or negative controls (combination of NT1 patient discovery and validation cohorts; Fig. 4b, c, e, Supplementary Fig. 3B, C, E). Homozygosity for HLA-DQB1*0602 did not appear to augment responses to these T-cell peptides beyond the levels reached in HLA-DQB1*0602 heterozygous NT1 patients. In contrast, T-cell responses against HA271-285 and HA274-288 were not increased in NT1 patients in comparison to controls, while responses against NP217-231 were significantly enhanced in NT1 patients only when compared to HLA-DQB1*0602 negative, but not positive controls (Supplementary Fig. 4A–D, Fig. 4f, Supplementary Fig. 3F). The results indicated that NT1 patients mounted unusually strong T-cell responses against NA175-192 and NP214-228 viral epitopes in response to Pandemrix vaccination, and that this was not simply a result of restriction by HLA molecules present on the DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0602 haplotype. These findings were consistent with our previous report of enhanced antibody responses in NT1 patients against viral proteins from Pandemrix, also in comparison to healthy vaccines carrying the HLA-DQB1*0602 allele.

Search for human protein sequences with homology to identified influenza A H1N1 T-cell epitopes from NA and NP. To identify possible cross-reactive self-epitopes, we performed basic local alignment of 12-mer peptides against human proteome (blastp). Sequence similarity searches for IAYRMCMNIIKQFQK (NP217-232) and ESYAWASACHD (NA175-186) produced top hits for syntrophin gamma-1 (YEMC88KILK, SNTG1338-3466 E-value 2.0, 75% query cover, 78% identity) and protein-O-
mannotransferase 1 (VAWYSSACH, POMT1 675–689; E-value 0.083, 75% query cover, 78% identity). Both proteins are known to be expressed in the human brain.

Discovery of a putative influenza A H1N1 virus cross-reactive T-cell self-epitope in Pandemrix-associated NT1 patients. To test immunological cross-reactivity, we performed T-cell assays with overlapping 15-mer peptides covering the two possible self-epitopes identified by blastp in SNTG1 and POMT1 (Supplementary Table 2). Interestingly, increased IFN-γ responses to peptide IFALVVAWYSSACH (POMT1 675–689) were demonstrated in NT1 patients (p < 0.01, Fig. 5a; Supplementary Fig. 5A). This suggested that POMT1 could represent an autoantigen in NT1. As a first step, influenza A (H1N1) virus HA, NA, and NP peptide T cell recognition was tested with spleen cells from Pandemrix-vaccinated HLA-DQ6.2 mice, restimulated with pools of 5 peptides each (15-mers). Pools that stimulated IFN-γ or IL-2 expression were broken up, and single peptides tested either with spleen cells from additional Pandemrix-vaccinated HLA-DQ6.2 mice, or PBMC from Pandemrix-vaccinated HLA-DQB1*0602 positive individuals. As a second step, recognition of single peptides was then tested with PBMC from pediatric Pandemrix-associated NT1 patients, and healthy Pandemrix-vaccinated controls. As a third step, influenza A (H1N1) T cell peptides that showed increased stimulation of IFN-γ or IL-2 secretion in patients vs. controls were validated and mapped. Cross-reactive T cell self-epitopes were predicted by BLAST against human proteome, and recognition tested with PBMC from pediatric Pandemrix-associated NT1 patients, and healthy Pandemrix-vaccinated controls.

Transcriptomic analyses of PBMC stimulated with influenza A H1N1 NA175–189 or human POMT1 675–689 indicate activation of T helper 1-cell and cytotoxic T-cell responses with cross-reactivity in Pandemrix-associated NT1. To further characterize T-cell responses against the epitopes from influenza A (H1N1) virus NA and human POMT1, and to identify putative pathogenic pathways in NT1, we studied the gene expression levels of viral NA175–189 or human POMT1 675–689-stimulated PBMC from Pandemrix-associated NT1 patients and Pandemrix-vaccinated healthy controls. In RNA sequencing, stimulation induced a clear response in NT1 patients versus healthy controls. Altogether 74 genes were differentially expressed, either in response to NA175–189 (49 genes), or POMT1 675–689 (28 genes; overlap of 3 genes, **p < 0.01; Fig. 6, Supplementary Fig. 6, Supplementary Data 2). The results further demonstrated that the gene expression profiles of PBMC from NT1 patients responding to these two peptides were closely matched (Fig. 6), and that no genes were differentially expressed in PBMC from NT1 patients stimulated with either one of the peptides (p-level < 0.05). These findings confirmed the correlation analyses for IFN-γ responses, and supported T-cell cross-reactivity between NA and POMT1. The list of 74 differentially expressed genes contained several genes closely associated with cytotoxic T-cell function, e.g., IFN-γ, chemokines/chemokine ligands known to be associated with IFN-γ, perforin 1, and granzyme B. A focused view onto T-cell effector molecules also showed that T-cells from individual NT1 patients responded similarly to stimulation either with viral NA175–189 or human POMT1 675–689, consistent with cross-reactivity of specific, influenza A (H1N1) virus-directed T-cells with the autoantigen POMT1 in NT1 (Fig. 7). This was exemplified most clearly by two strong responders, NT1 patients P003 and P015 (Figs. 6, 7), both characterized clinically by rapid onset of disease with the development of excessive daytime sleepiness and cataplexy within 4 weeks from vaccination with Pandemrix.
In addition, RNA sequencing of FACS-sorted, NA_{175–189}-stimulated CD4+ and CD8+ T-cell populations from a single pediatric, Pandemrix-associated NT1 patient (P003) revealed upregulation of IFN-γ, perforin 1, granzyme B, chemokine (C–C motif) ligand 4, and chemokine (C motif) ligand in CD8+ T-cells, while the gene expression profile of CD4+ T-cells from the same patient was characterized mainly by IFN-γ (Fig. 6; FACS gating and sort strategy shown in Supplementary Fig. 7). Overall, gene expression in the NA_{175–189}-stimulated CD8+ T-cell sample matched the profile obtained from NA_{175–189}-stimulated PBMC, suggesting that peptide NA_{175–189} might be presented by both HLA class I and II. This finding was also consistent with the notion that cytotoxic T-cells are a key effector cell population mediating immunity against influenza A virus^{20}. 

Influenza A (H1N1) virus T-cell epitope screen in HLA-DQ6.2 mice

| Stimulation index | IFNg (mRNA) | IFNg (supernatant) | IL-2 (supernatant) |
|------------------|-------------|---------------------|-------------------|
|                  |             |                     |                   |
| HA               |             |                     |                   |
| NA               |             |                     |                   |
| NP               |             |                     |                   |
T-cell receptor sequencing demonstrates converging selection of influence A HINI NA175-189 and human POMT1675-689-reactive clonotypes in Pandemrix-associated NT1. To characterize T-cell responses against the putative cross-reactive epitopes at the clonal level, we sequenced TCR α-chains (TRA) and β-chains (TRB) from RNA of viral NA175-189 or human POMT1675-689-stimulated PBMC from Pandemrix-associated NT1 patients and Pandemrix-vaccinated healthy controls. TRA variable (TRAV), TRA joining (TRAJ), TRB variable (TRBV), TRB diversity (TRBD), and TRB joining (TRBJ) gene segment usage did not differ significantly between peptide-stimulated and medium control samples, neither in patients nor controls (Supplementary Data 3). We identified TRA clonotypes that were upregulated in ≥3 NT1 patient samples, stimulated either with NA175-189 or POMT1675-689 peptides (Fig. 8). Among these public TRA clonotypes, six clonotypes significantly increased in abundance both when stimulated with NA175-189 or POMT1675-689 (proportion of false positives <0.05), further supporting T-cell cross-reactivity. Most strikingly, clonotypes CVVVSIAKAGNKLITF-TRAV10/TRAJ17 and CVVVSAMTDWSKGKQF-TRAV10/TRAJ24 were upregulated in 3 of 8 (3 of 8) NA175-189-stimulated, and 5 of 10 (4 of 10) POMT1675-689-stimulated samples. In NT1 patients P003 and P015, characterized by T helper 1 (Th1)-cell/cytotoxic T-cell gene expression signatures in response to stimulation with NA175-189 or POMT1675-689, both clonotypes were upregulated in response to either of the two peptides. Three additional clonotypes were found, significantly more abundant after stimulation with at least one of the two peptides, and with high similarity to either of the two clonotypes above: CVVVIITKAAGNKLITF-TRAV10/TRAJ17, significantly upregulated in POMT1675-689-stimulated samples, including P003; CVVSVSGMTDSWSKGKQF-TRAV10/TRAJ24, upregulated in NA175-189-stimulated samples, including P015; and CVVVSGMTDSWSKGKQF-TRAV10/TRAJ24, upregulated in both NA175-189 and POMT1675-689-stimulated samples, including both P003 and P015. These results demonstrated converging selection of NA175-189- and/or POMT1675-689-reactive T-cell clones using TRAV10/TRAJ17 or TRAV10/TRAJ24 gene segments in Pandemrix-associated NT1 patients.

A complete list of both public and private clonotypes using TRAV10/TRAJ17 and TRAV10/TRAJ24, identified in NT1 patients or healthy controls, is provided in Fig. 9a, b. The results demonstrate that in addition to the identified public clonotypes using TRAV10/TRAJ17 (CVVVSIAKAGNKLITF, CVVVTIKAGNKLITF) and TRAV10/TRAJ24 (CVVVSAMTDWSKGKQF, CVVVSAMTDWSKGKQF, CVVVSAMTDWSKGKQF), several highly similar clonotypes were present in peptide-stimulated samples from various NT1 patients, including CVVVSMKIAGNKLITF-TRAV10/TRAJ17, CVVVSMTDWSKGKQF-TRAV10/TRAJ24, and CVVSSLTDSWSKGKQF-TRAV10/TRAJ24. The identified public clonotypes were not exclusive to NT1 patients, but occasionally seen in healthy controls, too. In particular, the HLA-DQB1*0602-negative healthy control HC27 shared several of the clonotypes upregulated in NT1 patient samples stimulated with NA175-189 or POMT1675-689. However, in contrast to NT1 patients, HC27 did not show an increase in expression of inflammatory cytokines/chemokines in response to peptide stimulation (compare Fig. 4a, b; 5a; 7). Therefore, these TRAV10–TRAJ17 and TRAV10–TRAJ24 clonotypes might have contributed to an inflammatory reaction in NT1 patients, but their functional phenotype could be effectively regulated when present in healthy controls.

Applying the same search criteria as used for TRA, we also identified a smaller number of TRB clonotypes that were upregulated in ≥3 NT1 patient samples, stimulated either with NA175-189 or POMT1675-689 peptides (Supplementary Fig. 8). Among these public TRB clonotypes, again one clonotype significantly increased in abundance in both NA175-189- or POMT1675-689-stimulated patient samples, further supporting T-cell cross-reactivity. This clonotype was CASSEAGQGAYEQF-TRBV6-1/TRAJ24–2 (upregulated in P003, P015, and P023).

Discovery of POMT1 autoantibodies in Pandemrix-vaccinated children. Finally, we studied levels of plasma antibodies to POMT1 by radioimmunoassay (RIA), a sensitive and specific liquid-phase method for the detection of autoantibodies recognizing conformational epitopes. Autoantibodies to POMT1 were increased in Pandemrix-vaccinated children (both in NT1 patients and healthy controls) in comparison to unvaccinated control children, suggesting that Pandemrix induced autoimmunity to POMT1 (**p < 0.0001, Fig. 10; Table 1).

Discussion

Genetic studies strongly implicate HLA-DQ6.2-restricted CD4+ T-cells in the pathogenesis of NT1. A central role for CD4+ T-cells is seen in many autoimmune disorders, e.g., in celiac disease and type 1 diabetes. Both disorders show exceptionally strong HLA class II associations, to be surpassed still in NT1. HLA class II-associated autoimmune diseases are considered antigen driven (e.g., by wheat gliadin in celiac disease), although tissue pathology is not mediated by CD4+ T-cells only. Other immune cells are involved in tissue destruction, and antigen spreading leads to broad autoimmunity. In Pandemrix-associated NT1, we hypothesized that CD4+ T-cells primed by influenza A (H1N1) virus epitopes are disease drivers, cross-react with CNS auto-antigens, and orchestrate an immune-mediated attack against the hypocretin neuronal network. Therefore, we focused our study on influenza A (H1N1) T-cell epitopes that are recognized in the context of HLA-DQ6.2, although HLA-DQ6.2 peptide binding was not directly tested. Due to limitations in the availability of blood samples from pediatric, Pandemrix-associated NT1 patients, we started to search for dominant T-cell epitopes using Pandemrix-primed mice that expressed HLA-DQ6.2 as the only MHC class II allele, and Pandemrix-vaccinated, HLA-DQB1*0602-positive individuals. Choosing this strategy, the
Table 1 Clinical information on PBMC and plasma sample donors.

|                      | NT1 patients (Discovery cohort) | NT1 patients (Validation cohort) |
|----------------------|---------------------------------|-----------------------------------|
|                      | Healthy unvaccinated controls   | Healthy vaccinated controls       |
|                      | Number of subjects              | 20                                |
|                      | Vaccinated with Pandemrix (%)   | 100                               |
|                      | Age at vaccination (years), mean| 11.5 (4.1–6.4)                    |
|                      | (range)                         | 11.4 (7.7–23.1)                   |
|                      | 1078                             | 550                               |
|                      | Time between vaccination (days), | 689                               |
|                      | median                           | 689 (52.3)                        |
|                      | HLA-DQB1*06:02 genotype, N (%)   | 28 (100)                          |
|                      | Gender, female, (%)              | 18 (64.3)                         |

**PBMCTable 1 Clinical information on PBMC and plasma sample donors.**

selected T-cell peptides were enriched for HLA-DQ6.2-binding, but likely included both MHC class II- and/or class I-binding peptides.

We found that T-cell responses against two NA- and NP-derived epitopes were enhanced in children and adolescents with Pandemrix-associated NT1 in comparison to Pandemrix-vaccinated controls. Further, our study provided evidence for influenza A (H1N1) virus-directed T- and B-cell cross-reactivity with human POMT1. Homology of a viral NA peptide sequence and a human POMT1 sequence was suggested by basic local alignment. We could show that IFN-γ secretion in response to NA_{175}–189/NA_{178}–192 and POMT1_{689}–689 stimulation was correlated, and that T cell cytokine/chemokine gene expression patterns were closely matched. This was exemplified by two strong responders, patients P003 and P015, who both developed clinical narcolepsy with cataplexy within one month from Pandemrix vaccination. TCR repertoire analyses in NT1 patients revealed increased abundance of seven public clonotypes in response to stimulation both with NA_{175}–189 and POMT1_{689}–689 in NT1 patients. In addition, vaccination with Pandemrix induced anti-POMT1 autoantibodies in children. These results were consistent with cross-reactivity of specific, virus-directed T-cells and antibodies with the autoantigen POMT1 in Pandemrix-associated NT1.

The reasons remain unknown for the strongly enhanced T-cell reactivity to different influenza A (H1N1) protein fragments demonstrated in this study in patients who developed NT1 after Pandemrix vaccination. Our earlier study showed that viral proteins contained in the Pandemrix antigen suspension formed complexes, which may have contributed to the enhanced reactivity to both NA- and NP-derived epitopes.

Specific HLA class I alleles, most consistently HLA-A*11:01, account for additional genetic risk contributions in NT1, independent from HLA-DQB1*06:02.

Cytotoxic CD8+ T cells, likely acting in concert with CD4+ Th1-cells, are frequently implicated in the pathogenesis of autoimmune diseases, including the neurological disorders multiple sclerosis and NT1.

In this study of Pandemrix-associated NT1, cytokine and chemokine gene expression by PBMC in response to stimulation with influenza A (H1N1) NA_{175}–189 or human POMT1_{689}–689 matched closely, and revealed a cytotoxic T cell signature, characterized by IFN-γ, IFN-γ–associated genes, perforin 1, and granzyme B. This phenotype was consistent with CD8+ T-cells, but also unconventional, cytotoxic CD4+ T-cells, previously observed in influenza A infection.

In FACS-sorted T cell fractions, available for study from one Pandemrix-associated NT1 patient only, perforin 1 and granzyme B expression was upregulated in NA_{175}–189-stimulated CD8+ T-cells, but not in CD4+ T cells. Thus, NA_{175}–189 peptide is most likely binding to both HLA class I and II molecules.

Six TRA clonotypes, and one TRB clonotype, significantly increased in abundance in samples from Pandemrix-associated NT1 patients both when stimulated with NA_{175}–189, or with POMT1_{689}–689. Strikingly, public TRA clonotypes CVVSAILKAAGNKLIF-TRA10/TRA17 and CVVSAAMTDDSWGKFQF-TRA10/TRAJ24 were upregulated in 37.5% of NA_{175}–189-stimulated, and 40–50% of POMT1_{689}–689-stimulated samples. In addition, we identified several public TRA clonotypes with high similarity to these two dominant clonotypes in samples from NT1 patients. These results suggest converging selection in different NT1 patients of NA_{175}–189 and/or POMT1_{689}–689-reactive T-cell
Identification of influenza A (H1N1) virus T-cell epitopes in Pandemrix-associated NT1 patients

Fig. 3 Identification of influenza A H1N1 virus T-cell epitopes in Pandemrix-associated NT1 patients. PBMC from pediatric Pandemrix-associated NT1 patients (NT1) or pediatric Pandemrix-vaccinated healthy controls (C) were stimulated in culture with single 15-mer peptides from influenza (A/ reassortant/NYMC X-179A (California/07/2009 × NYMC X-157)(H1N1)) vaccine virus hemagglutinin (HA), neuraminidase (NA) or nucleoprotein (NP) (discovery cohort). Recombinant neuraminidase (rNA) and nucleoprotein (rNP) were used as positive controls. The secretion of IFN-γ (a, c, e) or IL-2 (b, d, f) was measured by FMIA (protein). Results are expressed as the ratio between cytokine concentrations measured in peptide-stimulated and negative control samples (stimulation index). Statistical comparisons between groups were performed, using Kruskal-Wallis and Dunn’s multiple comparisons tests.
clones using TRAV10/TRAJ17 or TRAV10/TRAJ24 gene segments. This indicates that these clonotypes are likely involved in the pathogenesis of NT1.

However, the identified public clonotypes were not exclusive to NT1 patients. Occasionally, they were seen in Pandemrix-vaccinated healthy controls, too. Interestingly, a HLA-DQB1*0602-negative healthy control shared both dominant TRAV10–TRAJ17 and TRAV10–TRAJ24 clonotypes, indicating that T-cells expressing these clonotypes were probably not HLA-DQ6.2-restricted. Therefore, TRAV10–TRAJ17 and TRAV10–TRAJ24 clonotypes might have contributed to an inflammatory reaction in NT1 patients, likely driven by CD8+ T-cells recognizing NA175–189 and putative

Mapping of identified influenza A (H1N1) virus T cell epitopes NA178-189 and NP217-228 in Pandemrix-associated NT1 patients

Fig. 4 Mapping of identified influenza A H1N1 virus T-cell epitopes in Pandemrix-associated NT1 patients. a, d PBMC from pediatric Pandemrix-associated NT1 patients (NT1; validation cohort) or pediatric Pandemrix-vaccinated healthy controls (C) were stimulated in culture with overlapping 15-mer peptides from influenza (A/reassortant/NYMC X-179A (California/07/2009 × NYMC X-157)(H1N1)) vaccine virus neuraminidase (NA) or nucleoprotein (NP), as indicated. b, c, e, f PBMC from NT1 patients (invariably HLA-DQB1*0602 positive; discovery and validation cohorts combined; HLA-DQB1*0602 homozygous NT1 patients marked with red dots) or HLA-DQB1*0602 positive (C/DQ6+) or negative (C/DQ6−) healthy controls were stimulated with single NA- or NP-derived peptides. The secretion of IFN-γ was measured by FMIA (protein). Results are expressed as the ratio between cytokine concentrations measured in peptide-stimulated and negative control samples (stimulation index). Statistical comparisons between groups were performed, using Kruskal-Wallis and Dunn’s multiple comparisons tests.
cross-reactive POMT1675-689 (auto-) antigens, while the same clonotypes appeared effectively regulated when present in healthy controls. Use in future studies of single cell technology may allow the direct functional characterization of T-cell clonotypes in NT1 patients and controls.

Of special interest is the identification of CVVSAMTDDSW GKFQF-TRAJ10-TRAJ24 as a dominant public clonotype, and of additional, highly similar TRAV10–TRAJ24 clonotypes, upregulated in NT1 patients in our study, two encoded for phenylalanine (F), and one for leucine (L). All TRAJ24 (and 3 of 4 TRAJ17) clonotypes, previously isolated from NT1 patients using HLA-DQ6.2-HCRT tetramers. To our knowledge, this is the first step of O-mannosyl glycan synthesis. In humans, O-mannose-linked glycosylation is observed mainly in brain, peripheral nerve, and muscle glycoproteins. The best-known substrate of POMT1/POMT2 is α-dystroglycan, mediating the binding of extracellular matrix components such as laminin to the dystrophin complex. Congenital defects in POMT1 are a cause of muscular dystrophy-dystroglycanopathy types A1, B1, and C. Some forms of muscular dystrophies, most clearly myotonic dystrophy type 1, are associated with rapid eye movement (REM) sleep dysregulation. It is currently unknown whether POMT1 or its substrates are involved in neuronal sleep regulation, but this possibility could be explored in experimental studies.

Among the identified T-cell epitopes, IAYERMCNILKG (NP217-226, Immune Epitope Database ID 145824), derived from the NYMC X-157 vaccine strain, is shared between different influenza A virus strains. In contrast, ESVAWSASACHD (NA175_186; ID 97390) and MERNAGSGIISDTP (HA274-285; ID 188723) are carried mainly by influenza A (H1N1) virus strains. A recent study by Luo et al. demonstrated an increased frequency of T-cell-specific for HA273-287 in NT1 patients in comparison to HLA-DQ81*0602 positive controls. However, a report by Schinkelshoek et al. found no significant increase in T cell reactivity against HA273-287 in NT1 patients in comparison to HLA-DQ81*0602 positive controls. Peptides HA271-285 and HA274-288 were recognized by patients with NT1 and healthy vaccinees in our study cohort, confirming HA273-287 as an influenza A (H1N1) T-cell epitope. Yet again, we did not see a clear-cut difference between NT1 patients and healthy vaccinees, neither in comparison to HLA-DQ81*0602 positive or negative controls, questioning the role of HA273-287-reactivity in the pathogenesis of NT1.

Using HLA-DQ6.2 tetramers, the study by Luo et al. demonstrated the presence of CD4+ T-cells specific for C-terminally amidated, human HCRT54-66 and HCRT54-76 peptides, but not for native HCRT peptides, in NT1 patients and controls. Furthermore, TCR sequencing analyses suggested cross-reactivity with HA. The recent study by Jiang et al. also using HLA-DQ6.2 tetramers, detected CD4+ T cells specific for native HCRT54-66 and HCRT57-100 peptides in NT1 patients and controls. In addition, Latorre et al. reported increased frequencies of predominantly HLA-DR-restricted CD4+ T cell clones recognizing much more diverse peptide epitopes of HCRT in NT1 patients. These clones did not show reactivity with HA. Notably, T-cell reactivity to HCRT was also detected in type 2 narcolepsy, which is not associated with HCRT-deficiency. Thus, the current literature on T cell reactivity to HCRT in NT1 remains conflicting. In many autoimmune diseases, autoimmunity is directed against multiple autoantigens expressed in the target tissue. The results of our study do not exclude a role of autoimmunity to HCRT, but identify with POMT1 another autoantigen in NT1.

POMT1 is a glycosyltransferase anchored in the endoplasmic reticulum, catalyzing in complex with POMT2 the first step of O-mannosyl glycan synthesis. In humans, O-mannose-linked glycosylation is observed mainly in brain, peripheral nerve, and muscle glycoproteins. The best-known substrate of POMT1/POMT2 is α-dystroglycan, mediating the binding of extracellular matrix components such as laminin to the dystrophin complex.
**Fig. 6 Gene expression profiling by RNA-seq of peptide-stimulated PBMC from Pandemrix-associated NT1 patients.** Hierarchically clustered heatmap of the gene expression levels (z-score scaling generated with the Heatmapper program) in all tested PBMC samples for 74 genes showing significant differences in expression (\(^{**}p < 0.01\)) between NT1 patients and controls either after stimulation with NA\(_{175-189}\) (49 genes) or POM\(_{T1675-689}\) peptides (28 genes; overlap of 3). Differentially expressed genes were identified using edgeR, based on a test analogous to Fisher’s exact test. The paired method was used for comparisons, calculating 2-sided \(p\)-values and adjusting for multiple testing using BH correction. If significance was reached (\(^{*}p < 0.05\)), log2 fold changes between NT1 patients and controls are shown on the right. Data derived from FACS-sorted CD4+ and CD8+ cells available from one NT1 patient were added for comparison.
In this study, T-cell reactivity with POMT1 was detected in Pandemrix-associated NT1 patients. Samples from unvaccinated NT1 patients were not available for comparisons. Currently, it remains unknown whether T cell reactivity against POMT1 can be observed in NT1 patients not exposed to influenza A (H1N1) vaccination or infection. Other questions to be addressed by future studies include the identities of the HLA alleles involved in the restriction of the dominant public TRAV10–TRAJ17, TRAV10–TRAJ24, and TRBV6–1/TRBJ2–7 clonotypes described, their function, antigenic peptide specificity and cross-reactivity, and their value for NT1 diagnosis.

In summary, this study provides evidence for an influenza A (H1N1) virus directed, POMT1 cross-reactive T-cell response in NT1, in the context of HLA-DQ6.2. We identified two dominant T-cell epitopes of influenza A (H1N1) NA and (NYMC X-157 vaccine strain) NP virus proteins in Pandemrix-associated NT1 patients. Patients mounted a vigorous Th1-cell/cytotoxic T-cell response against these epitopes and showed T-cell reactivity against a self-epitope in POMT1, an enzyme expressed in brain. The POMT1 epitope is a peptide mimic of a dominant T-cell epitope of influenza A (H1N1) NA in Pandemrix-associated NT1. IFN-γ, perforin 1, granzyme B, and chemokine gene expression in response to stimulation with T-cell peptides NA_{175–189} or POMT1_{675–689} was closely matched in individual NT1 patients. TCR repertoire analyses demonstrated converging selection of TRAV10/TRAJ17 and TRAV10/TRAJ24 clonotypes in NT1, upregulated in response to either NA_{175–189} or POMT1_{675–689} stimulation, thus supporting T-cell cross-reactivity and a pathogenic role of these clonotypes in NT1. Moreover, vaccinees had elevated antibody levels against human POMT1, suggesting that Pandemrix vaccination triggered an autoimmune response to POMT1. The results of this unbiased search identify POMT1 as an autoantigen recognized by T and B cells in NT1.

**Methods**

**Vaccine.** The 2-component vaccine Pandemrix, containing inactivated influenza A/California/7/2009 (H1N1) split virus (3.75 μg per 0.5 ml emulsion), and AS03 adjuvant (10.69 mg squalene, 11.86 mg DL-alpha-tocopherol and 4.86 mg polysorbate 80 per 0.5 ml emulsion), was obtained from GlaxoSmithKline Biologicals, Rixensart, Belgium.

**Mice.** Homozygous HLA-DQA1*0102, DQB1*0602, human CD4-transgenic Ab0 NOD mice (“HLA-DQ6.2 mice”; stock number 006023) were purchased from Jackson Laboratories, Bar Harbor, ME, USA, and bred at the University of Helsinki Laboratory Animal Centre. All mice were housed under specific pathogen-free conditions, under a 14–10 h light–dark cycle, at 22 ± 2 °C and 50–60% humidity. Pandemrix vaccine was injected under isoflurane anesthesia into the right thigh.
Fig. 8 TCR α chain sequencing of peptide-stimulated PBMCs from Pandemrix-associated NT1 patients. TCR α chains (TRA) were sequenced from the same RNA samples as used for RNA sequencing. P: patient; HC: healthy control. Displayed are TRA clonotypes that were upregulated in ≥3 NT1 patient samples, based on fold changes between peptide-(NA175–189 or POMT1675–689-) treated and medium control samples from the same participant in data normalized by downsampling (heatmap). Statistical significance on the group level (NT1 patients only; Rank products test; proportion of false positives <0.05) is indicated on the left side bar.
Peptides and recombinant proteins. 15-mer peptides covering hemagglutinin (GenBank entry ACP41953.1) and neuraminidase (YP_009118627.1) of influenza (A/California/07/2009 (H1N1)), and nucleoprotein (ADE29096.1) of influenza (A/reassortant/NYMC X-179A (California/07/2009 × NYMC X-157)(H1N1)) with 12 amino acid overlap were produced. 2 × 5 15-mer peptides with 12 amino acid overlap were used for RNA sequencing (compare Fig. 8). P: patient; HC: healthy control. Heatmaps present clone fractions, i.e. the proportions of clones using TRAV10-TRAJ17 (a) and TRAV10-TRAJ24 (b) of all clones in total data. Clones are clustered based on CDR3 sequence.
Fig. 10 Autoantibodies in Pandemrix-associated NT1. Plasma from pediatric Pandemrix-associated NT1 patients (NT1) or pediatric Pandemrix-vaccinated or unvaccinated healthy controls were analyzed by POMT1 radioimmunoassay. Radioactivity is expressed in relative units (RU). The medians (line in box), 75 and 25% quartiles (upper and lower box boundaries) and maxima/minima (upper and lower whiskers) of each group are displayed. Statistical comparisons between groups were performed, using Kruskal–Wallis and Dunn’s multiple comparisons tests.

Mouse spleen cell stimulation. Spleen cells from 1 to 2 individual mice were pooled, and seeded in triplicates in 96-well plates at 2 × 10^5 cells/well in RPMI 1640 medium (200 μl volume) containing heat-inactivated fetal calf serum (10%), penicillin/streptomycin, glutamine, and HEPES (25 mM), at 37 °C and 5% CO2. Cells were stimulated for 6 days with 15-mer peptides (10 μg/mL resulting in DMSO concentration 0.1%), recombinant influenza A (H1N1) (A/California/07/2009) hemagglutinin (cat11085-V08H), (A/California/04/2009) neuraminidase (cat11058-VNAHC or cat11058-V01H), and (A/California/07/2009) or (A/Porto Rico/34/Mount Sinai) nucleoprotein (cat40205-V08B and cat11675-V08B) purchased from Sino Biological, Beijing, China. Endotoxin-free, recombinant human protein-O-mannosyltransferase 1 and synthrophin gamma 1 were from Origin, Rockville, Maryland, and endotoxin-free ovalbumin from Hyglos, Bernried, Germany.

Patient and healthy control donor material. The T cell study included 28 pediatric Pandemrix-associated NT1 patients (20 in the discovery cohort, and 14 in the validation cohort; 6 patients were included in both cohorts), and 33 healthy Pandemrix-vaccinated control children or adolescents (Table 1, Supplementary Table 3A, B). If results from different experiments were pooled for analysis, and two results from a single patient were available, only the result obtained with the sample drawn earlier was used. PBMC from Pandemrix-vaccinated HLA-DQB1*0602-positive individuals (sleep clinic patients without a diagnosis of NT1) were also used for influenza A (H1N1) virus T cell epitope screening (Supplementary Table 1). The plasma study included 37 pediatric Pandemrix-associated NT1 patients, 57 healthy Pandemrix-vaccinated control children or adolescents, and 130 healthy control children or adolescents that had not received Pandemrix vaccine (Table 1). NT1 patients were diagnosed in outpatient clinics at Finnish university hospitals, by pediatric neurologists, pediatricians or neurologists with expertise in sleep medicine. All NT1 patients were diagnosed based on criteria defined in the third edition of the International Classification of Sleep Disorders (IL-2 were measured in cell culture supernatants using human or mouse Milliplex MAP Kits (HCYTGMAG-60K, MCYTGMAG-70K, Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. Quantification was performed with a Magpix instrument and xPONENT 4.2 or 4.3 software (Luminex Corp., Austin, TX), or Bio-Plex 200® System and Bio-Plex Manager software version 5.0 (BIO-RAD Laboratories, Hercules, CA, USA). The concentration of each cytokine was determined from an 8-point standard curve using five parameter logistic regression. The samples below minimum detectable concentration (MinDC) were given an arbitrary value of 50% of MinDC. Each sample was compared to an unstimulated sample.

Basic local alignment of protein sequences. Basic local alignment of protein sequences was performed using the web-based BLASTp program search tool (version 2.2.32+) at the National Centre for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/).
RNA extraction from human PBMC and sorted CD4+ and CD8+ T-cells. RNA was prepared from stimulated PBMCs cultured at ‐70 °C in an RLT buffer (Qiagen), using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer’s instructions. Cells were homogenized with QIAshredder columns (Qiagen). After extraction, RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA samples were stored at ‐70 °C. RNA was captured from stimulated CD4+ or CD8+ T-cell cultures at ‐20 °C in an RNAlater (Thermo Fisher Scientific) after FACS sorting, according to a published protocol37. Briefly, samples were mixed with lysis buffer (0.3% (v/v) Triton X100, 20 mM DTT, 2 mM dNTPs) in ratio 1:1 and vortexed. Magnetic Dynabeads (M‐270 Streptavidin, Thermo Fisher Scientific) coated with RNA using polyT‐tailed Indexing Oligonucleotides (Illumina) were added to each well. After 5 min of incubation, the magnetic beads were separated from the supernatant and washed twice with 6X SSC buffer. Subsequently, the beads were combined with RT mix as described later.

RNA Sequencing. The in‐house 3′ bulk RNA‐sequencing “3B‐seq” method was modified from the single cell Drop‐seq protocol38,39, as described37. Briefly, 10 ng of RNA was mixed with indexing oligonucleotides (Integrated DNA Technologies, Coralville, IA) and after 5 min of incubation, the RNA was combined with RT mix (1× Maxima RT buffer, 1 mmol/L dNTPs, 10 U/μL Maxima H‐RNaseHase (all Thermo Fisher Scientific), 1 U/μL RNase inhibitor (Lucigen, Middleton, WI), and 2.5 μmol/L Template Switch Oligo (TSO; Integrated DNA Technologies, Coralville, IA)). Primers are listed in Supplementary Table 4. Samples were incubated in a T100 thermocycler (Bio‐Rad) for 30 min at 22 °C and 90 min at 52 °C. The constructed complementary DNA (cDNA) was amplified by PCR using Maxima H‐Long HotStart ReadyMix (Innova Biosystems, Wilmington, MA), and 0.8 μmol/L SMART PCR primer. The samples were thermocycled in a T100 thermocycler (Bio‐Rad) as follows: 95 °C for 3 min; then 4 cycles of 98 °C for 20 s, 65 °C for 45 s, 72 °C for 3 min; then 16 cycles of 98 °C for 20 s, 67 °C for 20 s, 72 °C for 3 min; then 1 cycle of 98 °C for 10 s, 65 °C for 15 s, 72 °C for the final extension step of 5 min at 72 °C. An aliquot of this PCR product was used for TCR sequencing (below). The PCR products were pooled together in sets of 12 samples containing different indexing oligos and purified with 0.6× HiSeq PreP PCR reagent (MACBIO, Gaitersburg, USA) according to the manufacturer’s instructions. They were eluted in 10 μL of molecular grade water. The 3′‐end complementary DNA fragments for sequencing were prepared using the Nextera XT (Illumina) tagmentation reaction. The reaction was performed according to the manufacturer’s instructions, except for the use of S5 SMART primer, which replaced the S5xx Nextera primer. Each set of 12 samples that was pooled after the PCR reaction was tagmented with a different Nextera index (Supplementary Table 4). Subsequently, the samples were PCR amplified as follows: 95 °C for 30 seconds, 11 cycles of 95 °C for 10 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, with the final extension step of 5 minutes at 72 °C. Samples were purified twice using 0.6× and 1× 0.8× HiSeq PreP PCR reagent and eluted in 10 μL of molecular‐grade water. The concentration of the libraries was assessed by using a Qubit 2 fluorometer (Invitrogen) and the Qubit DNA HS Assay Kit (Thermo Fisher Scientific). The quality of the sequencing libraries was assessed by using the LabChip GXII Touch HT electrophoresis system and quantified with a LabChip GXII Touch HT electrophoresis instrument and quantitative PCR. Libraries were sequenced with a MiSeq Reagent Kit v3 (600‐cycle) using the 300 + 300 bp paired‐end protocol, a custom read 1 primer (DS‐CustomHead_1STO) and 10% PhiX on an Illumina MiSeq instrument at Department of Virology, University of Helsinki, Finland.

TCR analysis. TCR sequencing reads were mapped and clonotypes assembled with MiXCR v3.0.12,14,15. The NTA_173‐190 stimulated sample from HC19 did not produce any new clonotypes and was excluded. Low‐count clones that had an identical CDR3 nucleotide sequence to a high‐count clone (over 500‐fold difference) were filtered out as probable cross‐sample contaminants. Non‐productive clones were also filtered out. To normalize the data, counts were downsampled pairwise using VDJtools v1.2.1 such that each NA173‐190‐treated and medium‐treated sample from the same patient was downsampled to the same total count. The same was done separately for the POMT1L275S‐treated samples. Further processing was done using R statistical software v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). Gene usage differences between peptide and media‐treated patient samples were examined using the Immunarch package v0.5.5 (Zenodo/CERN, Geneva, Switzerland) and Wilcoxon test with the paired method. P values were corrected for multiple testing with the Benjamini‐Hochberg method. For clone‐level analysis, low‐count clones were filtered out by requiring each clone to have count >10 in at least one sample. One pseudocount was added, the counts were log2 transformed and fold change between the peptide‐treated sample and medium control from the same patient were calculated for each clone (pairwise samples). Statistical significance was tested with Rank Products test implemented in the RankProd package v3.1206,47, using the paired method. Clones were hierarchically clustered based on the Levenshtein edit distance of their CDR3 sequences.

Plasma POMT1 antibody radioimmunoassay. The full‐length human POMT1 (amino acids 401–675) was cloned and sequenced into the pET30 vector (GenScript, Piscataway, NJ, USA) under the control of the Sp6 promoter, and multiplied in Invitrogen subcloning efficiency DH5a competent cells (ThermoFisher, Waltham, MA, USA), according to the manufacturer’s instructions. 35S‐methionine‐labeled POMT1 was produced in viro transcription and translation of the purified plasmid, using the TNT Ccoupled Reticulocyte Lysate System according to the manufacturer’s instructions (Promega, Madison, WI, USA) in the presence of 35S‐methionine (Perkin Elmer, Waltham, MA, USA). Unincorporated 35S‐methionine was removed by gel chromatography on NAP‐5 columns (GE Healthcare, Chicago, IL, USA). Plasma samples were analyzed for POMT1 (auto)‐antibodies by RIA, as described for GAD autoantibodies21. Briefly, 2 μl of plasma was incubated overnight at 4 °C in 96‐well plates with 20,000 cpm of 35S‐methionine‐labeled POMT1, diluted in 50 μL of TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween‐20, pH 7.4). Antibody‐antigen complexes were precipitated with protein A‐Sepharose, and unbound label was removed by washing several times with TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween‐20, pH 7.4). The bound radioactivity was measured with a liquid scintillation counter (1450 MicroBeta TriLux, Perkin Elmer Life Sciences, Turku, Finland), and the results were expressed in relative units (RU), based on a serial dilution in‐house standard curve.

Statistics. Statistical comparisons between groups were performed using R, with a version of 4.0.5 (Graph Pad Software, San Diego, CA), and Dunn’s multiple comparisons test. Correlation analyses were performed by calculating Spearman’s rank correlation coefficient. Additional details on the statistics used in the analysis of RNA and TCR sequencing data are included in the sections above.

Study approval. NT1 patients were Finnish participants in the NARPANORD narcolepsy study. Healthy control children and adolescents were recruited via the Finnish Diabetes Registry. Pandemrix‐vaccinated HLA‐DQB1*0602‐positive individuals without a diagnosis of NT1 were recruited at the Helsinki Sleep Clinic, Vätalmed Research Center, Helsinki, Finland. All participants or their guardians had written, informed consent. Ethical permissions for clinical studies were obtained from the Ethics Committee of the Hospital District of Helsinki and Uusimaa, Finland. All animal procedures were approved by the Board for Animal Research (ELLA), Southern Finnish State Administrative Agency (ESAVI/1064/04.10.03/2012).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The sequencing data have been deposited to the EMBL EBI European Genome‐phenome Archive (EGA) under the accession number EGAS0000104886 (RNA sequencing data, Fig. 6; TCR sequencing data, Fig. 7). Upon request and subject to a material transfer agreement, data submitted to EGA will be made available by the corresponding data access committee. Source data are provided with this paper.
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Author contributions

A.Vuorela, T.L.F., S.M., and O.V. planned the study and experiments. T.K., P.O., O.S.-H., M.K., A.Vaheri, and M.P. provided annotated patient and/or control samples. A.Vuorela, T.L.F., K.L., H.P., T.H., and I.S. performed experiments. A.Vuorela, T.L.F., K.L., H.P., T.H., P.S., and J.I. analyzed data. A.Vuorela, T.L.F., P.S., J.I., and O.V. interpreted results. T.L.F. drafted and A.Vuorela, K.L., H.P., P.S., J.I., S.M., and O.V. edited the manuscript.

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Competing interests

O.V. is an employee of Orion Pharma since June 2019 and was an employee of AstraZeneca/Medimmune from August 2014 to June 2019. This research was started before she joined AstraZeneca, and neither Orion Pharma nor AstraZeneca has a role in this study. M.P. is a member of the Medical Advisory Board of Bioprojet and UCBB Pharma. He has been involved in clinical trials on narcolepsy with Bioprojet, Jazz Pharmaceuticals, and UCB Pharma. These roles are not related to this research project. I.S. is an employee of fansPharmaceuticals. This research was started before she joined Janssen, and Janssen has no role in this study. P.S. is the CEO and board chair of SCellex, which has no role in this study. K.L. and H.P. are employees of SCellex. The other authors have no competing interests to declare.

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