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Short Communication

Shared T cell receptor chains in blood memory CD4⁺ T cells
of narcolepsy type 1 patients

Running head: Shared T cell receptors in narcolepsy

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ABSTRACT

Convergent evidence points to the involvement of T cells in the pathogenesis of narcolepsy type 1 (NT1). Here, we hypothesized that expanded disease-specific T cell clones could be detected in the blood of NT1 patients. We compared the TCR repertoire of circulating antigen-experienced CD4⁺ and CD8⁺ T cells from 13 recently diagnosed NT1 patients and 11 age-, sex-, and *HLA-DQB1*06:02*-matched healthy controls. We detected a bias in the usage of TRAV3 and TRAV8 families, with public CDR3 α motifs only present in CD4⁺ T cells from patients with NT1. These findings may offer a unique tool to identify disease-relevant antigens.

1. Introduction

Narcolepsy type 1 (NT1) is a rare, chronic, and disabling sleep disorder defined by excessive daytime sleepiness, sudden loss of muscle tone triggered by strong emotions, and fragmented nocturnal sleep [1]. Although pathological changes in narcolepsy include the almost complete and selective loss of orexin/hypocretin-producing neurons in the lateral hypothalamus [2, 3], considerable knowledge gaps persist regarding the precise mechanisms leading to damage of orexinergic neurons.

However, convincing genetic and epidemiological evidence support an immune-mediated, or even an autoimmune basis for NT1. First, the association between NT1 and the *HLA-DQB1*06:02* allele is strikingly strong (odds ratio > 250). Second, narcolepsy is associated with polymorphisms in other immune-relevant genes such as HLA class I, TCR α , TCR β , and perforin [4-8]. These findings point towards the importance of CD4⁺, but also CD8⁺, T cells in disease development. Third, consistent epidemiological studies indicate that vaccination against the 2009 pandemic H1N1 *influenza* virus using Pandemrix[®] was significantly associated with onset of NT1 within weeks after vaccination [9].

Efforts to identify NT1-specific autoantibodies have been either negative or inconclusive. Moreover, no data has proven that autoantibodies are causally involved in the demise of orexinergic neurons [10]. Recent evidence, however, supports the involvement of T cells in the pathogenesis of this sleep disorder. We recently showed that autoreactive cytotoxic CD8⁺ T cells contribute to immune-mediated tissue damage, using an experimental model of immune-mediated narcolepsy [11]. We also revealed the multifaceted activation of circulating CD4⁺ and CD8⁺ T cells, as well as quantitative and qualitative alterations in follicular helper T cells in narcoleptic patients [12, 13]. A recent study convincingly showed that the frequency of orexin-specific CD4⁺ T cells is elevated and their reactivity increased in people with NT1 compared to *HLA-DQB1*06:02*-positive healthy donors [14]. These findings strongly suggest the involvement of T-cell responses in the pathogenesis of NT1.

Given the genetic association with HLA and TCR polymorphisms, we hypothesized that disease-specific clonal expansion might be detected among blood T cells in recently diagnosed narcoleptic patients. These disease-specific T cell clones may offer a unique probe to identify disease-associated antigens. Based on this reasoning, we conducted a systematic analysis of the TCR repertoire of antigen-experienced CD4⁺ and CD8⁺ T cells in NT1 using next generation sequencing.

2. Materials and methods

2.1. Participants

Thirteen patients (Table 1) fulfilling the International Classification of Sleep Disorders criteria, third edition, for narcolepsy type 1 were enrolled in this study. They were all *HLA-*

*DQB1*06:02*⁺. Their blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated and cryopreserved within 24h of blood collection. Eleven *HLA-DQB1*06:02*⁻, age- and sex-matched healthy individuals served as controls. Each study participant gave written informed consent to take part in the research program, which was approved by an ethics committee.

2.2. Genotyping

HLA typing of narcoleptic patients and healthy controls was performed by PCR using sequence-specific oligonucleotides, and four-digit allele assignments were performed by Luminex xMAP technology, as previously described [12].

2.3. Surface staining and flow cytometry

Cryopreserved PBMCs were thawed and stained with the antibodies UCHT1 (CD3-V450), SK3 (CD4-PerCP-Cy5.5), SK1 (CD8-APC-H7), L48 (CD45RA-PE-Cy7), and 150503 (CCR7-PR), and sorted using a FACSARIA-SORP flow cytometer (BD Biosciences) to isolate the memory and effector CD4⁺ and CD8⁺ T cell populations, through exclusion of the CD45RA⁺CCR7⁺ naïve cells.

2.4 RNA extraction, cDNA synthesis, and high-throughput T-cell receptor sequencing

Total RNA was extracted from the sorted cells using PureLink kits (Thermo Fisher). To correct for PCR-biases and -errors we used unique molecular identifiers (UMI) [15]. cDNA was synthesized using the SmartScribe Kit (Clontech), treated with fresh uracil-DNA glycosylase (NEB), and amplified by two rounds of nested PCRs, 27 cycles each. Adaptors and barcodes were added by using Nextera XT Index Kit v2 (Illumina).

Next generation sequencing (NGS) libraries were analyzed by Illumina HiSeq, 2x150bp paired end sequencing. Raw UMI-barcoded TCR sequencing data were processed using the MIGEC pipeline [15]. After barcode-based error correction, resulting TCR repertoires were suitable for post-analysis using the VDJtools software [16]. Hierarchical clustering of samples was performed based on the Euclidean distance between V-segment frequency as derived from VDJtools software, weighted for clonotype size.

3. Results

Our cohort included 13 NT1 patients with a short range of disease durations (mean=44.2 months) and 11 tightly age-, sex-, and HLA-DQB1-matched healthy donors (**Table 1**). Five patients had been vaccinated with Pandemrix[®] 2 to 6 months prior to disease onset. Antigen-experienced CD4⁺ and CD8⁺ T cells from healthy donors and narcoleptic patients were individually sorted by flow cytometry from PBMCs to a median of 99% purity. Transcripts

from CD4⁺ and CD8⁺ T cells were labeled with UMIs that allow reliable quantification of mRNA molecules and exclude cross-contaminations, subjected to RT-PCR, and the TCR repertoire of both subpopulations was analyzed by NGS. To identify shared clones between individuals, we performed hierarchical clustering of distinct combinations of TCR V-regions with defined complementarity determining region (CDR)-3 sequences.

Clonal linkage and hierarchical clustering of identical CDR3 sequences to particular TRAV and TRBV families of CD4⁺ and CD8⁺ cells are shown as heat maps in **Fig. 1A-D**. Analysis of TCR α chain sequences from CD4⁺ T cells revealed several expanded 'public' clones that were shared between all individuals (boxed in black, lower panel) (**Fig. 1A**). Public clones are often detected in subjects with identical HLA alleles [17]. Strikingly, we observed an additional strong bias to TRAV3 and TRAV8 families and corresponding CDR3-motifs of the TCR α chains (boxed in red) exclusively in both vaccinated and non-vaccinated NT1 patients but not in HLA-matched healthy subjects. Of note, the TRAV3 and TRAV8 families are structurally related, as evidenced by the phylogenetic tree of all TRAV-regions (**Suppl. Fig. 1**). The CD4 TCR β chain sequencing also identified public clones (lower panel, boxed in black) (**Fig. 1B**). Differing from the CD4 TCR α chains, there was, however, no preponderance of certain V-families in this case. No sequences specific to NT1 patients were found for both chains of CD8⁺ T cells (**Fig. 1C and D**). Finally, as expected, in all subjects we identified public TRAV1-2 and TRBV20 chains, which are characteristic for mucosal associated invariant T (MAIT) cells [18].

To identify specific recognition motifs, we analyzed TRAV3 and TRAV8 chains that had been detected only in three or more narcoleptic patients (**Fig. 2**). The amino acid sequences of CDR1 and CDR2 of the TCR α chains were highly homologous (**Fig. 2A**, columns 4 and 5), consistent with their phylogenetic relation (**Suppl. Fig. 1**). The CDR3 of the TCR α chains also exhibited striking similarities (**Fig. 2A**, column 6). All but one of the TRAV3 chains displayed the prominent charged amino acids R and D in positions 107 and 108. This TRAV3-CAVRD motif was present in 9 of 13 patients, but in none of the 11 controls ($p=0.0006$; Fisher exact test). Positions 109 and 110 did not show particular motifs, but from 111 to 113, we found predominantly hydrophilic amino acids, which were mostly small (G, A, S, T) or in rare cases bulky (N, Q, K, R). A similar pattern of S to R substitution at position 107 was seen within TRAV8-4 chains; the resulting TRAV8-4-CAVSD/E motif being present in 6 patients but absent in controls ($p=0.016$; Fisher exact test). Four of the five public narcolepsy-specific TRAV8-3 chains showed a different pattern present in 5 patients ($p=0.041$; Fisher exact test). The small amino acids G and A were at positions 107 and 108, at 109 and 110 no specific residues were found, but again 111 to 113 were predominantly hydrophilic residues. The motifs in the CDR3 of the TCR α chain hint towards common or

similar peptides recognized by the different TRAV3, TRAV 8-4, and TRAV 8-3 chains. Notably, all 3 motifs were present in 4 narcoleptic patients, including 2 post-Pandemrix[®] ones.

To assess the possible contribution of Pandemrix[®] vaccination in the selection of the TCR CDR3 motifs in the narcoleptic patients, we have specifically assessed the presence of these motifs and compared the TCR sequences in vaccinated vs. non-vaccinated patients. No Pandemrix[®]-associated public clones or motifs were found.

4. Discussion

Using high-throughput sequencing of the TCR repertoire of blood antigen-experienced T cells obtained from NT1 patients and matched controls, we found CD4⁺ T cell TCR α chains shared by different narcoleptic patients but not expressed in healthy controls. These public sequences were grouped in disease-specific motifs present in both Pandemrix[®]-vaccinated and non-vaccinated NT1 subgroups, in patients sampled within 3 years of disease onset or later, and in patients with CSF orexin levels > 10 pg/mL or below 10 pg/mL.

By hierarchical clustering of the TCR repertoire we revealed clones that use the same V-segments together with distinct CDR3 sequences. If we had focused on V-regions alone, we would have revealed many *HLA-DQB1*06:02*-specific TCRs, irrespective of their relation to NT1. When we filtered for chains that were specifically detected in narcoleptic patients, we obtained a limited set of 11 TRAV3 and 11 TRAV8 chains. The TRA CDR1 and CDR2 are clonally related and highly homologous in accordance with the canonical structure of TCR/HLA/peptides complexes, where CDR1 and CDR2 interact predominantly with the alpha-helices of the HLA molecules, while CDR3s recognize the antigenic peptides [19]. Although CDR3s of narcolepsy-specific TCR chains display stretches of identity or high homology, some other stretches are quite divergent. This is expected, due to the known degeneracy of TCR-HLA/peptide recognition [20]. Indeed, many similar TCR molecules can recognize a single HLA-peptide complex and, conversely, a given TCR can recognize a large number of slightly different HLA-peptide complexes. As these homologous TCR α chains were found only in NT1 patients, we assume that the TCRs made up by these chains might recognize a narcolepsy-relevant antigen.

We found that some TCR α chains were shared by a subgroup of NT1 patients, but that they were not expressed by healthy controls. An obvious question is why NT1-specific chains were not detected in more patients. A technical reason could be that the use of UMI-based high-fidelity sequencing protocol decreases the sensitivity, although the advantages of this approach preponderate, because analysis becomes quantitative and cross-contaminations are prevented. Another reason could be that NT1-related clones are likely

highly diluted in blood drawn months after disease onset (and after Pandemrix® vaccination when performed). Moreover, considering the volume of the hypothalamus, the number of NT1-specific T cells was probably very low even at the acute stage of the disease. Further, most activated T cells do not become memory cells but rather die when their cognate antigen disappears. Thus, the number of NT1-specific clones was *a priori* very low.

Based on the genetic associations, it is generally assumed that T cells play a major role in the pathogenesis of NT1. A key role for CD4⁺ T cells is inferred from the striking association with *HLA-DQB1*06:02* [21]. Moreover, recent evidence revealed a heightened CD4⁺ T cell reactivity of narcoleptic patients against orexin peptides as compared to HLA-matched controls [14, 22]. In one study, TCRβ sequencing of autoreactive CD4⁺ T cell clones showed no bias in TRBV usage [14]. In the second study, *in vitro* expanded short-term CD4⁺ T cell lines specific for C-amidated orexin peptides presented by HLA-DQ602 also showed large TCR gene segment usage [22]. The peptide sequences recognized by CD4 T cells in orexin-A (HGAG**NHAAGIL**TL-NH2) and orexin-B (ASGN**HAAGIL**TM-NH2) share important homology (in bold). However, enrichment in TRAV2, TRAJ24, TRBV4-2 and TRBV15 usage was notable, both in NT1 patients and healthy controls. In our study, which investigated *ex vivo* TCR usage among polyclonal CD4⁺ T cells, these gene segments were not differentially expressed between NT1 patients and controls. Whether this CD4 T cell reactivity to orexin is causally involved in the disease process or is a mere consequence of it is currently unknown [23].

Accumulating data suggest a potential role for CD8⁺ T cells as final effectors of the selective neuronal damage. Indeed, HLA studies revealed independent associations with HLA class I alleles [24, 25]. Furthermore, heterozygosity for a loss-of-function polymorphism within the *Perforin* gene protects from NT1 [8]. This is further supported by a mouse model of autoimmune narcolepsy in which cytotoxic CD8⁺ T cells, but not Th1 CD4⁺ T cells, could kill orexin-neurons [11] and by the analysis of post-mortem hypothalamic CD8⁺ T cell infiltration in a case of secondary narcolepsy [26]. A plausible scenario is that autoreactive CD4⁺ T cells could initiate the disease process and that autoreactive CD8⁺ T cells would then be needed for the execution of tissue damage [21]. However, without knowing the exact immune mechanisms leading to orexin-neuron destruction, it is very challenging to design rational immune-targeting therapies for patients with NT1 [23]. In fact, current therapy in narcolepsy is limited to symptomatic management.

Our study did not detect disease-specific expansion within the CD8⁺ T cell compartment. However, we identified 22 TCRα sequences shared by at least 3 narcoleptic patients and not by *HLA-DQB1*06:02*⁺ healthy controls. These sequences could be further grouped in three clear CDR3 motifs significantly associated with NT1, although none of the motifs was detected in all patients studied.

5. Conclusion

Our study identified a bias in the usage of TRAV3 and TRAV8 families among circulating antigen-experienced CD4⁺ T cells from patients with NT1, resulting in patient-specific CDR3 α motifs. These CDR3 sequences may offer a unique tool, not only to identify through bioinformatics tools and functional studies the nature of NT1-relevant antigens, but also to develop novel blood-based biomarkers for NT1.

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Author Contributions

Study concept and design: E.B., X-H.N., Y.D., K.D., R.S.L. Data acquisition and analysis: all authors. Drafting the manuscript or figures: E.B., X-H.N., K.D., R.S.L. All authors critically reviewed and approved the final manuscript.

Potential Conflict of Interest

Unrelated to the current study, R.S.L. has received grant support from GSK to study the relationship between Pandemrix[®] vaccination and narcolepsy type 1 using a mouse model.

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Figure legends

Figure 1. Clonal linkage and clustering of identical CDR3 sequences to particular TRAV and TRBV families. The studied individuals are listed according to their status (top right): vaccinated narcolepsy (NAR) patients are shown in blue, non-vaccinated narcoleptic patients in green, and healthy donors in beige. Each horizontal line in the heat map indicates a particular combination of TCR variable regions with a distinct CDR3-sequence. The color code is shown at the top left: a red color indicates that a distinct CDR3-sequence is linked to a certain V-family at high frequency. Blue color indicates that no such link is observed. The designated IMGT-numbers of the V-families are listed at the right. Clustering of V-families and distinct CDR3-sequences is shown on the left side. Public TRV-CDR3 combinations that are found in all subjects irrespective of their status are boxed in black. TRV-CDR3 combinations that were found preferentially in NAR patients are boxed in red. **(A)** TRA-chains of CD4⁺ T cells. TRAV3 and members of the TRAV8 family are the only chains that were predominantly present in narcoleptic patients (boxed in red). **(B)** TRB-chains of CD4⁺ T cells. **(C)** TRA-chains of CD8⁺ T cells. The public TRBV1-2 chain (boxed in black) belongs to MAIT cells. **(D)** TRB-chains of CD8⁺ T cells. The public TRBV20 chain (boxed in black) belongs to MAIT cells.

Figure 2. Amino acid sequences of the narcolepsy-specific TCR α chains used by antigen-experienced CD4⁺ T cells. The figure displays sequences of TCR α chains that are only found in narcoleptic patients in the hierarchical clustering analysis (Fig. 1) and that are shared by three or more patients. The first column lists the frequencies of detection (freq). The second column shows how often the chains were detected in narcoleptic patients. Sequences were included only if they were absent in healthy subjects. The third column lists the IMGT families. Columns four to six provide the amino acid sequences of the CDR1, CDR2, and CDR3 regions using the single letter code. The color code is according to RasMol. We show amino acids 27 to 38 for CDR1, 56 to 65 for CDR2, and from the conserved Cys104 to Phe118 for CDR3. Amino acids 107 to 116 may interact with peptide-MHC complexes. Numbering is according to IMGT: <http://www.imgt.org/IMGTScientificChart/Numbering/IMGTIGVLSuperfamily.html>.

Table 1. Summary of the clinical and biological characteristics of the narcoleptic patients and matched healthy donors included in this study

Healthy donors	
<i>N</i>	11
Age (years)	26 (13-47)
Sex (male/female)	5/6
<i>HLA-DQB1*0602</i> positive	11
Narcolepsy type 1 patients	
<i>N</i>	13
Age (years)	27 (13-47)
Sex (male/female)	7/6
<i>HLA-DQB1*06:02</i> positive	13
Prior Pandemrix® vaccination	5
Disease duration (months)	44.2 (8-93)
CSF orexin levels < 110 pg/mL and/or Cataplexy (<i>N</i> / <i>N</i> tested)	13/13

Supplementary figure 1. Phylogenetic tree of all TRAV families calculated and built using the IMGT/PhyloGene software. IMGT gene and allele name, IMGT/LIGM-DB accession number, and species Latin names are displayed systematically for sequences coming from the IMGT/PhyloGene database.

Figure 1A.

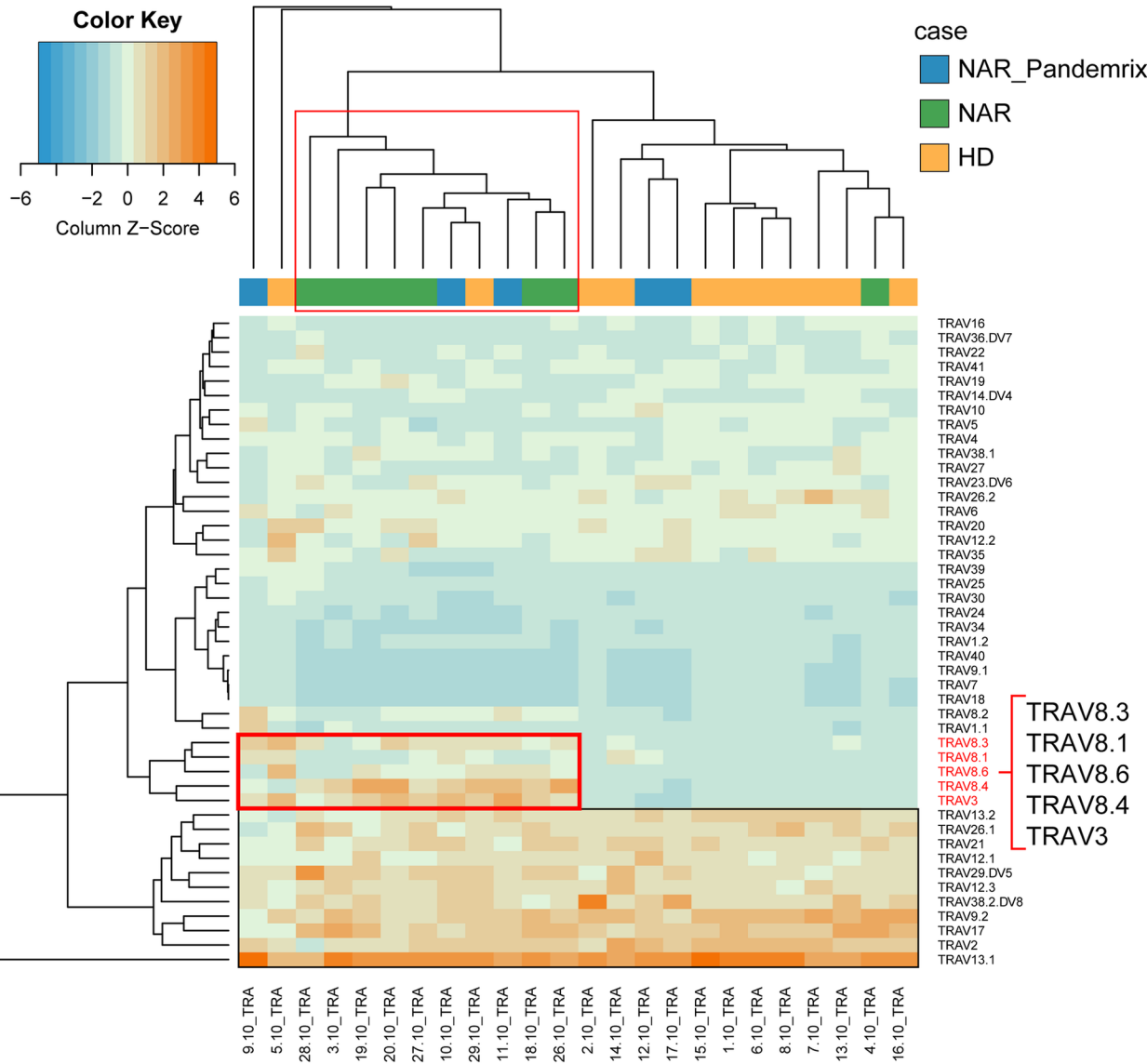


Figure 1B.

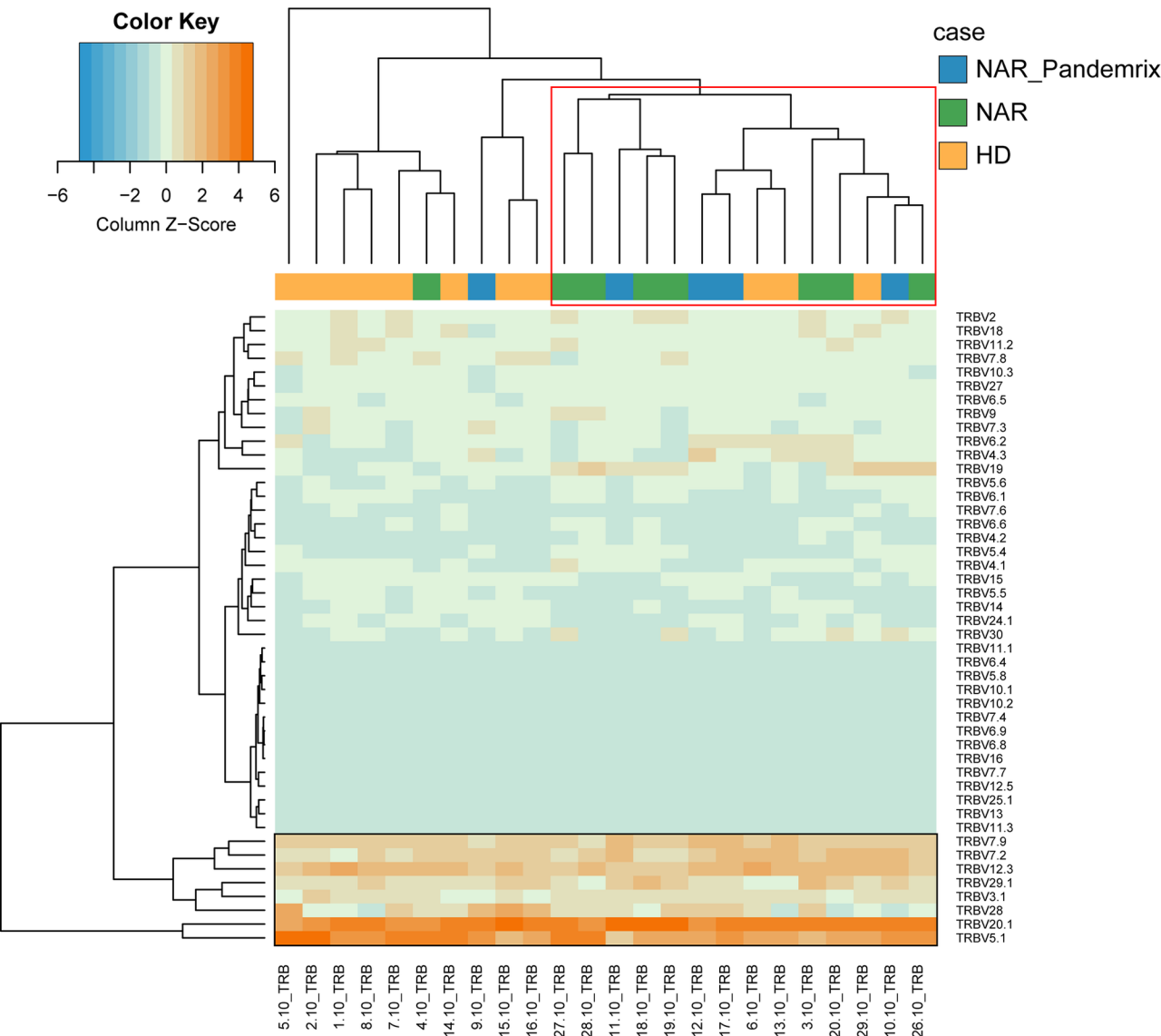


Figure 1C.

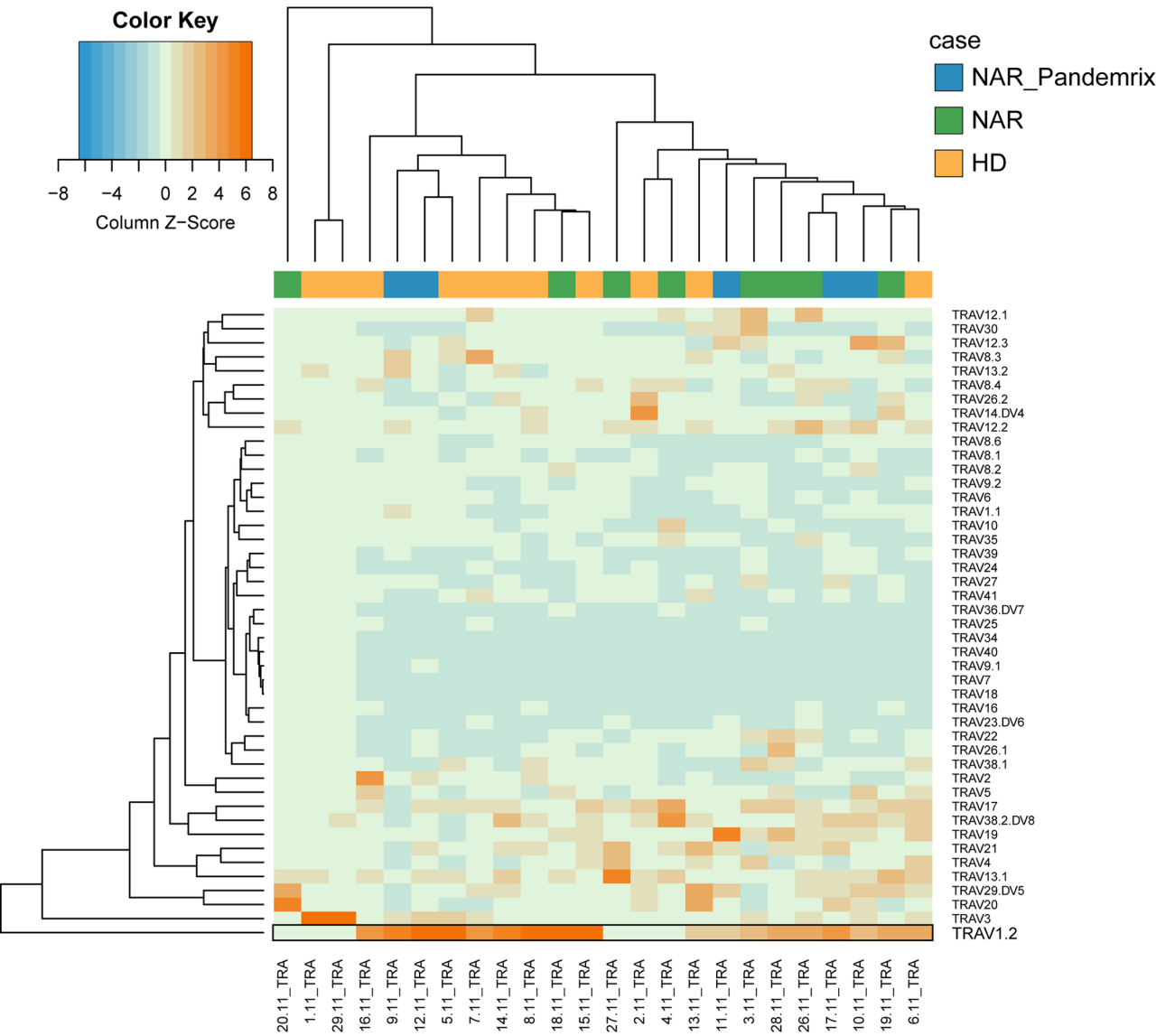


Figure 1D.

