Copy number variations across the blood–brain barrier in multiple sclerosis

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Abstract

Objective: Multiple sclerosis (MS) is a neuroinflammatory disease where immune cells cross the blood–brain barrier (BBB) into the central nervous system (CNS). What predisposes these immune cells to cross the BBB is still unknown. Here, we examine the possibility that genomic rearrangements could predisposespecific immune cells in the peripheral blood to cross the BBB and form sub-populations of cells involved in the inflammatory process in the CNS. **Methods**: We compared copy number variations in paired peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) cells from MS patients. Thereafter, using next generation sequencing, we studied the T-cell receptor beta (TRB) locus rearrangements and profiled the αβ T cell repertoire in peripheral CD4$^+$ and CD8$^+$ T cells and in the CSF. **Results**: We identified deletions in the T-cell receptor alpha/delta (TRA/D), gamma (TRG), and TRB loci in CSF cells compared to PBMCs. Further characterization revealed diversity of the TRB locus which was used to describe the character and clonal expansion of T cells in the CNS. T-cell repertoire profiling from either side of the BBB concluded that the most frequent clones in the CSF samples are unique to an individual. Furthermore, we observed a difference in the proportion of expanded T-cell clones when comparing samples from MS patients in relapse and remission with opposite trends in CSF and peripheral blood. **Interpretation**: This study provides a characterization of the T cells in the CSF and might indicate a role of expanded clones in MS pathogenicity.

Introduction

Multiple sclerosis (MS), is a chronic demyelinating inflammatory disease of the central nervous system (CNS) and a complex disease involving both genetic and environmental factors. Genetic analysis of MS has made significant progress in the past years as a result of large international research collaborations. These studies have focused on genome wide association studies (GWAS) of samples from several thousand MS patients and healthy controls and have resulted in the identification of more than 200 MS associated genetic variants, known as single nucleotide polymorphisms, which can be significantly associated with MS susceptibility.[1–3] Currently the genetic variants associated with MS explain <1/3 of the total MS heritability.[3] Hence, the issue of "missing heritability" in

the field of MS genetics has been introduced[4] and there have been several suggestions on how to reveal this missing heritability including analyzing genetic pathways, studying rare genetic variants, and applying more sophisticated analysis methods and whole genome or exome sequencing initiatives.[5]

There has also been attempts to study other types of genetic variants, such as copy number variations (CNVs), which include insertions, duplications, and deletions of a DNA segment ranging from a couple of thousands to a few million base-pairs. Baranzini et al. have compared CNVs between the genomes of peripheral blood CD4$^+$ T cells from discordant monozygotic twins.[6] They identified a few CNVs but they were present in both the affected and unaffected twin. CNVs were also studied in a sub-population of MS patients characterized with early onset

of MS before the age of 18 years, using comparative genomic hybridization arrays and finding de novo CNVs.[7]

In the present study, we hypothesize that genetic variants within a subpopulation of immune cells could make these cells more prone to invade the CNS with an impact on the inflammatory process and subsequent consequences for the MS process. This would represent a form of somatic mosaicism as this subpopulation of immune cells will be genetically different from the majority of cells in the peripheral circulation within the same individual. The possibility of somatic mosaicism in complex diseases has generally not been taken into consideration. It is however important for the understanding of the etiology of other diseases such as cancer development which is one of the classical examples, but somatic mosaicism has been also established in a number of monogenetic disorders such as hemophilia A and neurofibromatosis type 1.[8] It has also attracted great interest in understanding the etiology of neuropsychiatric diseases such as schizophrenia.[9] Furthermore, the interest in somatic mosaicism in autoimmune diseases is gaining momentum.[10,11] To test our hypothesis, we aimed to identify CNVs between immune cells inside and outside the CNS in MS patients.

Materials and Methods

Samples collection

Paired peripheral blood (PB) and cerebrospinal fluid (CSF) samples were collected from MS patients with consent at the Neurology Clinic at Karolinska University Hospital, Sweden. Samples were also collected from patients with other neurological diseases (ONDs) and heathy controls (HC). These samples were collected as part of the Stockholm prospective assessment of MS (STOPMS) I (DNR 02–548, Stockholm) and II (DNR 2009/2107–31/2, Stockholm) projects. A total of 38 individuals (29 MS patients, six non-MS patients, and three HCs) were included in this study (Table 1). PB samples were collected in sodium citrate-containing cell preparation tubes (BD Vacutainer™ CPT™ Tube; BD Biosciences, Franklin Lakes, NJ, USA) and peripheral blood mononuclear cells (PBMCs) were isolated according to the manufacturer's protocol. Isolated PBMCs were frozen at −80°C in freezing medium (10% dimethyl sulfoxide in fetal bovine serum). CSF samples were centrifuged at 350 x g to isolate the CSF cells which were then frozen at −80°C.

CD4⁺, CD8⁺, and γ/δ T cells isolation

CD4⁺ T cells and CD8⁺ T cells were isolated from the PBMCs on an autoMACS separator by positive selection using the CD4⁺ and CD8⁺ MicroBeads (Miltenyi Biotec,

Bergisch Gladbach, Germany), respectively. For γ/δ T cells isolation PBMCs were stained with brilliant Violet 421™ anti-human CD3 Antibody (BioLegend, San Diego, CA, USA) and anti-TCRγ/δ-PE (REA591; Miltenyi Biotec, Bergisch Gladbach, Germany) and cells were sorted on BD influx (BD Biosciences, Franklin Lakes, NJ, USA).

DNA extraction

DNA was extracted from paired PBMCs, CD4⁺, CD8⁺, γ/δ T, and CSF cells simultaneously using QIAamp DNA mini kit (Qiagen, Düsseldorf, Germany). Extracting enough DNA from the CSF cells was the bottleneck for including these samples in this study. The amount of DNA used for each analysis is mentioned in its respective methods sections.

CNV detection

The CytoScan HD Array (Affymetrix, Santa Clara, CA, USA) at the Array and Analysis Core Facility at Uppsala University was used for CNV comparison in the paired PBMCs and CSF cells samples. A minimum yield of 140 ng DNA was used. The CytoScan HD Array is specifically designed for CNV detection. It contains approximately 2.7 million markers covering all OMIM and RefSeq genes, with intragenic and intergenic markers spacing of 880 and 1737 base-pairs, respectively.[12] The intensities from each probe was normalized to a reference panel using the chromosome analysis suite Software, calculating the log R ratio. CNVs between the PB and CSF were identified using the Nexus Copy Number software (BioDiscovery Inc, Hawthorne, CA, USA) and a threshold of a minimum five consecutive probes for calling a CNV was used.

Validation of the CNV

Further validation of the CNV regions identified by the array was performed using Taqman copy number assays on Quantstudio 7 flex real time PCR system (Applied Biosystems, Waltham, MA, USA). An approximate 2 ng of DNA per reaction was used. Taqman copy number assays for the human T-cell receptor (TCR) gamma (TRG) (Hs07530615_cn, Hs03646230_cn, and Hs04980855_cn), TCR beta (TRB) (Hs04330161_cn, Hs04329666_cn, Hs03643995_cn, and Hs07530853_cn), and TCR alpha (TRA) (Hs03308605_cn and Hs03094858_cn) regions were used. From the CT values of the real-time PCR run, the copy numbers of the target genes was calculated using PBMCs for each individual as a calibrator on the CopyCaller™ Software (Applied Biosystems, Waltham, MA, USA).

Table 1. Demographics of the subjects included in the study.

ID	Sex	Age at sampling	Diagnosis	Treatment status	CSF-mononuclear cells, ×10⁶/L	Method
14-036	M	39	RRMS, remission	Not treated	10	CytoScan HD Array
14-087	F	31	RRMS, remission	Not treated	22	CytoScan HD Array, MiSeq
14-250	F	22	RRMS, relapse	Not treated	14	CytoScan HD Array, MiSeq
14-265	F	35	RRMS, remission	Not treated	8	CytoScan HD Array
15-138	M	24	HC	NA	4	CytoScan HD Array
15-237	M	22	HC	NA	2	CytoScan HD Array
14-131	F	31	RRMS	Not treated	8	CytoScan HD Array
14-155	F	42	RRMS, remission	Not treated	8	CytoScan HD Array, MiSeq
09-073	M	36	PPMS	Not treated	4	CytoScan HD Array
07-98	M	33	RRMS	Not treated	47	Taqman
07-381	F	30	RRMS	Not treated	5.8	Taqman
07-564	M	34	RRMS	Not treated	8.6	Taqman
08-454	F	19	RRMS	Not treated	12.8	Taqman
11-439	F	32	RRMS	Not treated	8.3	Taqman
12-447	F	40	RRMS	Not treated	9.8	Taqman
12-449	F	26	RRMS	Not treated	14.8	Taqman
14-111	M	33	RRMS	Not treated	2	Taqman
15-213	M	21	HC	NA	4	Taqman
17-8608	F	41	RRMS, relapse	Not treated	2.9	Taqman
17-8805	F	38	RRMS, remission	Rituximab	6	Taqman
17-8813	M	43	RIS	Not treated	2.8	Taqman
17-8823	F	40	RRMS, relapse	Not treated	3	Taqman, MiSeq
17-8832	F	28	RRMS, remission	Fingolimod	5	Taqman
17-8838	F	44	RRMS, remission	Not treated	10	Taqman, MiSeq
17-8839	F	33	Brain tumor + CIS, relapse	Not treated	11	Taqman
17-8847	F	42	OND[1]	NA	2	Taqman
17-8848	F	55	RRMS, relapse	Not treated	2	Taqman
14-003	F	43	OND[2]	NA	12	Taqman
14-205	F	53	OND[3]	NA	33	Taqman
14-272	M	21	OND[4]	NA	131	Taqman
17-8803	M	63	PTSD (headache)	NA	2	Taqman
17-8809	F	73	OND[5]	NA	3	Taqman
17-8801	M	30	RRMS, relapse	Not treated	44.7	MiSeq
18-8856	F	40	RRMS, remission	Not treated	4	MiSeq
17-465	M	28	RRMS, relapse	Not treated	42.2	MiSeq
14-137	F	32	RRMS, remission	Not treated	2	MiSeq
16-098	F	35	RRMS, remission	Not treated	8	MiSeq
16-223	F	32	RRMS, relapse	Not treated	<1	MiSeq

CSF, cerebrospinal fluid; RRMS, relapsing remitting MS; PPMS, primary progressive MS; HC, heathy controls; RIS, radiologically isolated syndrome; CIS, clinically isolated syndrome; PTSD, post-traumatic stress disorder; OND, other neurological disease.

[1] Tension headache.
[2] Demyelinating disease.
[3] SLE and aseptic meningitis.
[4] Idiopathic intrathecal hypertension.
[5] Herpes encephalitis.

TRB locus sequencing

We investigated the clonality of the T cells by studying the TRB locus rearrangements using next generation sequencing. Library preparation was performed with the LymphoTrack® TRB assay- MiSeq® kit (72250009; Invivoscribe, San Diego, CA, USA), where primers in the kit target the conserved V_β and J_β regions of the TRB locus. The amount of DNA used per library PCR reaction from the CD4⁺, CD8⁺, and CSF samples, was on average 47.9, 53.2, and 38.3 ng, respectively. Paired-end 2 × 250 sequencing was done using the MiSeq Reagent Kit v2 (MS-102–2003, Illumina, San Diego, CA, USA) on the Illumina MiSeq platform at the Bioinformatics and

Expression Analysis facility at Karolinska Institutet. Eight samples were run per flow cell, including positive and negative controls. The generated FASTAQ data were processed using the MiXCR software to assemble the clonotypes and provide the highly variable CDR3 sequence.[13] The software VDJtools was used for further analysis of the TCR repertoire.[14]

CDR3 sequences annotation

To identify the specificity of the detected T-cell clones we searched in the publicly available VDJdb database[15] for matching TCR with previously known antigen specificity using the software VDJmatch version 1.3.1.[16]

Statistical analysis

Comparison of the copy number of the target genes between MS and non-MS patients was done using Wilcoxon rank-sum test. From the TRB locus sequencing, a unique CDR3 nucleotide sequence represents a unique T-cell clone. The frequency of a clone is defined as its sequence count compared to the total count of all sequences in a sample. Clones with a frequency ≥0.1% were considered as expanded and clones with a frequency <0.1% as non-expanded. Wilcoxon rank-sum test was also used to compare the proportions of expanded clones between independent groups. Statistical analysis and graphs were done using R software version 3.3.2.[17]

Results

Whole genome CNVs screening

In the initial screen we used the cytoscan HD array to search for CNV between paired CSF and PBMCs samples from six relapsing remitting MS (RRMS) patients, one primary progressive MS patient and two HC. We could detect CNVs in three regions on chromosomes 14, 7q, and 7p consistent with the TRA/D, TRG, and TRB loci. These CNVs where present in 8/9, 7/9, and 5/9 samples for TRA/D, TRG, and TRB, respectively and indicated deletions in all three regions when comparing CSF to PB (Fig. 1). The extent of the deleted regions for the different samples could be mapped using the array data. The deletions were larger in the TRA/D locus with a median length of ≈ 296 kb, while for TRG and TRB were ≈61 and 92 kb, respectively.

Validation of the identified CNVs

In order to confirm and further explore the CNVs across the TCR regions we analyzed a further 12 paired CSF and PBMCs DNA samples from eight RRMS, three OND patients, and one HC using TaqMan analysis with probes mapping across the TCR regions which had been identified in the previous screen (Table 2). The results from the TaqMan analysis confirmed the deletions in the CSF cells across the TRA/D and TRG regions where deletions could
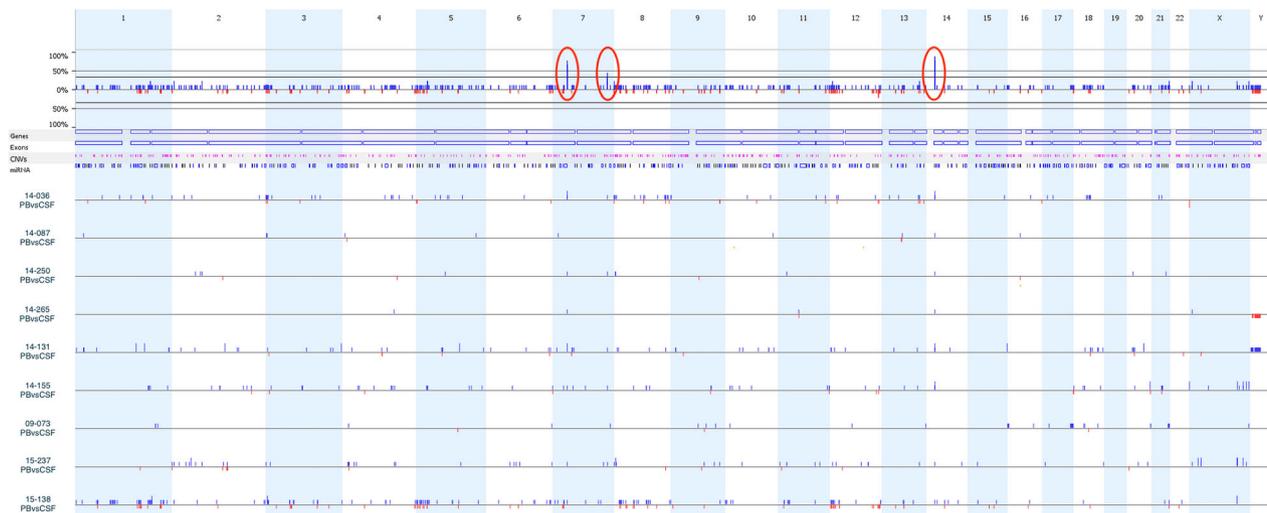

Figure 1. Whole genome CNVs between PB and CSF cells from the seven MS patients (14-036, 14-087, 14-250, 14-265, 14-131, 14-155, and 09-073) and two HC (15-237 and 15-138). The upper panel shows the CNV frequency plot for all nine individuals with blue upward bars or red downward bars indicating more copy numbers in the PB or more copy numbers in the CSF cells respectively, with the chromosome numbers indicated at the top of the image. The panel below shows the annotation tracks for genes, exons, CNVs & miRNA according to a reference database. The lower panel shows the CNV between PB and CSF for each individual. CNV, copy number variation; PB, peripheral blood; CSF, cerebrospinal fluid; HC, heathy controls.

Table 2. Taqman copy number assays used for the CNV validation.

	Taqman_assay id	Chromosome	Position	Gene
TRG	Hs07530615_cn	chr7	38248115	TCRGC2
	Hs03646230_cn	chr7	38278966	5319 bp from TRGJP1
	Hs04980855_cn	chr7	38369439	TRG-AS1
TRB	Hs04330161_cn	chr7	142657429	216 bp from TRBV24-1
	Hs04329666_cn	chr7	142738548	1658 bp from TRBV29-1
	Hs03643995_cn	chr7	142765864	PRSS2
	Hs07530853_cn	chr7	142806573	TRBC2/TRBV30
TRA/D	Hs03308605_cn	chr14	22300377	3677 bp from TRAV39
	Hs03094858_cn	chr14	22409503	12,868 bps from TRDV2

CNV, copy number variation; TRG, T-cell receptor gamma; TRA/D, T-cell receptor alpha/delta; TRB, T-cell receptor beta.

be identified in 11/12 and 9/12 of the samples, respectively (Fig. 2A). The results for the TRB locus revealed a more complex structure where the extent of the deletion varied between samples. Even though the number of non-MS individuals was low, we compared the deletions in the TCR regions between the eight RRMS and four non-MS (three OND patients and one HC). Only deletions in the TRB locus of CSF cells were significantly different in RRMS than non-MS ($p = 0.03$), with RRMS having less copy numbers, that is, more deletions, in the TRB locus (Fig. 2B).

CNVs in paired CD4^{+}, CD8^{+} T, and CSF cells

For the purpose of further study of TCR deletions in different types of T cells we compared CD4^{+} and CD8^{+} cells to CSF cell samples from six RRMS, one clinically isolated syndrome, one radiologically isolated syndrome, one SPMS, and two OND patients. The results from this analysis were in agreement with the previous observation that TRA/D and TRG deletions were consistent across the analyzed regions and that TRB displayed a more complex structure with variations in the extent of deleted regions within CSF samples from different individuals as well as from different types of T cells within one individual (Fig. 3).

CNVs in paired γδ T and CSF cells

Because the initial screen revealed less deletions of TRB compared to the TRA/D and TRG regions in CSF samples we wanted to examine the possibility of γδ T cells constituting a larger proportion of CSF cells as compared to PB. We therefore purified γδ T cells from PB and

compared the CNV pattern in these cells with CSF samples and CD4^{+} and CD8^{+} cells. We did not detect any similarities between the CNV pattern of CSF and γδ T cells (Fig. 4). As expected, the γδ T cells demonstrated deletions in the TRG region. The position of our TRA probes did not allow specific detection of TRD deletions. However, we did detect deletions of TRB region in all the γδ T cells samples that we analyzed (Fig. 4). The extent of the TRB deletions varied between the γδ T cells samples.

Clonality in MS patients

Based on that the TRB locus displayed a more complex structure with inter-individual and intra-variations between CSF, and CD4^{+} and CD8^{+} T cells, we studied the TRB locus rearrangements and profiled the αβ T cell repertoire in peripheral CD4^{+} and CD8^{+} T cells and in the CSF. The average number of unique TRB sequences in the studied MS patients were 8206 ($\pm$1820), 6265 ($\pm$2810), and 6383 ($\pm$2021) in CD4^{+} T cells, CD8^{+} T cells and in CSF cells, respectively (Table 3). Each unique CDR3 or TRB nucleotide sequence, as a result of TRB locus rearrangements, is considered a unique clone, and when using a threshold for clonal expansion of 0.1% in all three compartments, most of their frequencies was under 0.1% (Fig. 5). CD4^{+} T cells were the most diverse compared to CD8^{+} T cells and CSF cells, which displayed comparable number of unique clones (Fig. 6).

Intra- and interindividual overlap of expanded clones

We wanted to study expanded clones in the CSF with a potential role in the neuroinflammatory process by comparing the occurrence of expanded clones in the CSF and the periphery. CSF samples had an average of 70.4 expanded clones, which could also be found (as expanded or non-expanded clones) in 39 and 43% of the paired CD4^{+} and CD8^{+} samples, respectively (Table 3).

Table 4 presents the five most frequent clones in the CSF and their frequency in the periphery if present in the CD4^{+} and CD8^{+} compartments.

When comparing the nucleotide sequence of the expanded clones between different individuals (interindividual comparison) there seems to be no overlap of the expanded clones. However, an interindividual comparison on the basis of the amino acid sequence shows an overlap of five clones between different pairs of individuals. CSF samples from patients 14–087 and 14–250 had two clones that were shared with another patient (Table 5).
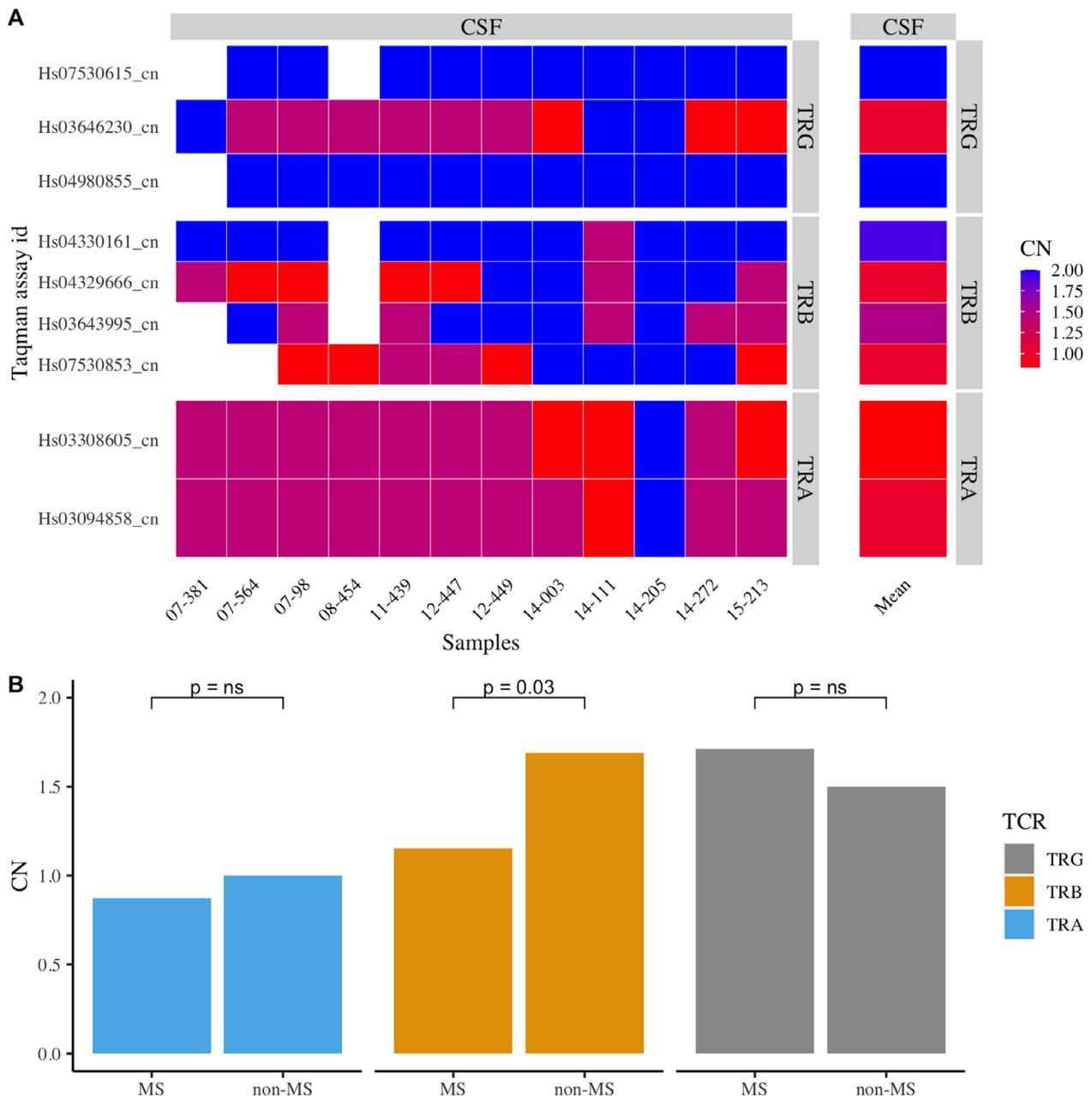
Figure 2. Validation of CNVs in CSF cells using Taqman copy number assays targeting genes in the TRG, TRB, and TRA regions. CSF cells are compared to PBMCs and PBMCs in each individual was used as a calibrator in the CopyCaller™ Software (Applied Biosystems) to calculate the CN of the target genes, that is, for each target gene the CN in CSF cells is calculated in comparison to CN in the PBMCs. (A) Showing the individual variations in copy numbers of the target genes. (B) Comparing the copy numbers of the target genes of MS ($n = 8$) to non-MS ($n = 4$). Wilcoxon rank-sum test was used for the statistical testing and generating the p values presented in the figure. ns, not significant; CNVs, copy number variations; CSF, cerebrospinal fluid; CN, copy number; TRG, T-cell receptor gamma; TRA, T-cell receptor alpha; TRB, T-cell receptor beta; PBMCs, peripheral blood mononuclear cells; MS, multiple sclerosis.

Relapse and T-cell clonality

To investigate whether the clonality of the T cells in CSF, CD4^{+}, and CD8^{+} cell compartments is related to the MS patient being under relapse or remission at the time of sampling, we compared the proportion of the expanded T-cell clones in each separate compartment in patients under relapse (for each CD4^{+} and CD8^{+} cell compartments $n = 4$ and for CSF $n = 5$) and in remission (for each CD4^{+} and CD8^{+} cell compartments $n = 4$ and for
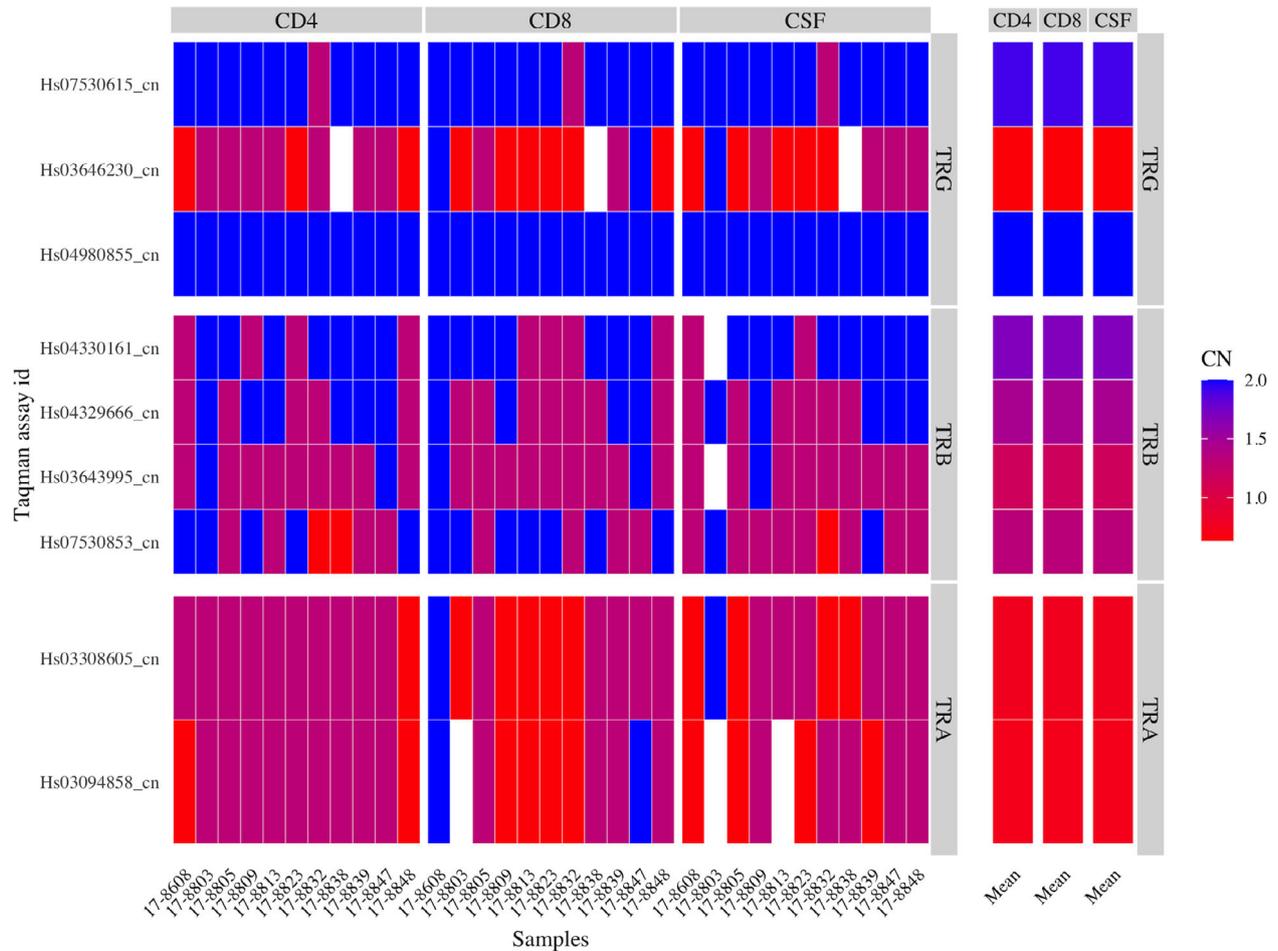
Figure 3. CNVs in the TCR regions in paired CD4$^+$, CD8$^+$ T, and CSF cells. The PBMCs in each individual was used as a calibrator in the CopyCaller™ Software (Applied Biosystems) to calculate the CN of the target genes, that is, for each target gene the CN in the CD4$^+$ and CD8$^+$ T cells is calculated in comparison to the CN in the PBMCs. CNVs, copy number variations; CSF, cerebrospinal fluid; TCR, T-cell receptor; CN, copy number; PBMCs, peripheral blood mononuclear cells.

CSF $n = 6$). The proportion of expanded T-cell clones was higher in patients under relapse than in remission in the CSF cells, while showing the opposite in the periphery in both CD8$^+$ cells and CD4$^+$ cells although the observed differences were not statistically significant (Fig. 7).

T-cell clone specificity

Taking advantage of publicly available databases for TCR sequences[15] and their known targets, we performed an in silico investigation of the specificity of the T-cell clones. We used the CDR3 sequences of our identified T-cell clones provided by MIXCR and searched for matches in the VDJdb database. The CDR3 sequences and their target antigens matches with moderate and high confidence scores included clones specific for antigens presented by different viruses such as Epstein–Barr virus (EBV),

cytomegalovirus, hepatitis C virus, yellow fever virus, influenza A, HIV, and Dengue virus. From 332 matching clones, nine were expanded, of which five clones were targeting three EBV antigens; EBNA3A, EBNA3B, and BMLF1. One of the clones targeting EBNA3A was expanded in all three compartments of patient 17-8838 and the same patient had another expanded clone targeting EBNA3B present in the CD8$^+$ and CSF cells. In addition, another clone targeting a different epitope of EBNA3A antigen, RPPIFIRRL, was present in the CSF of patients 14-87 and 14-250 (Table S1).

Discussion

In the current study we wanted to explore the possibility of somatic mosaicism displayed as sub-populations of immune cells with genomic variation within the CNS in
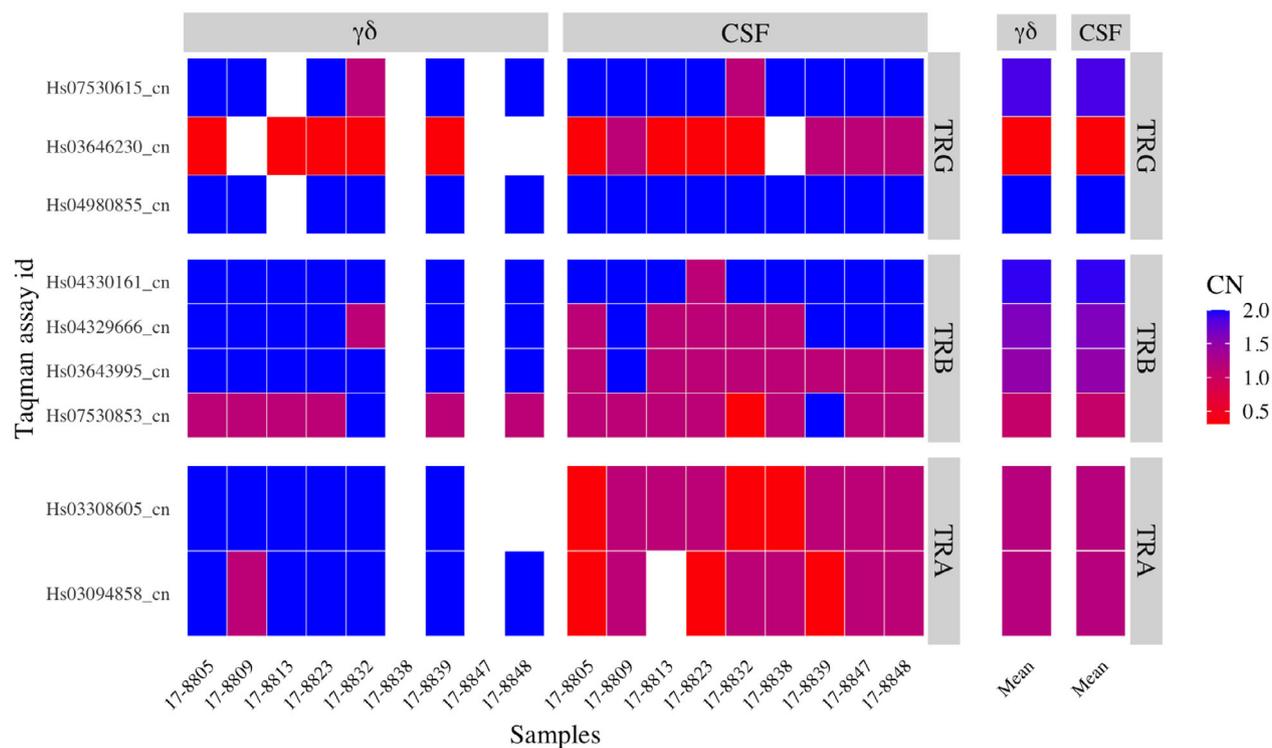
Figure 4. CNVs in the TCR regions in paired γδ T and CSF cells. PBMCs in each individual was used as a calibrator in the CopyCaller™ Software (Applied Biosystems) to calculate the CN of the target genes, that is, for each target gene the CN in the CSF and γ/δ T cells is calculated in comparison to the CN in the PBMCs. CNVs, copy number variations; CSF, cerebrospinal fluid; TCR, T-cell receptor; CN, copy number; PBMCs, peripheral blood mononuclear cells.

Table 3. Showing the total number of clones and number of expanded clones with a frequency of ≥0.1% per sample.

Patient id	CD4 cells		CD8 cells		CSF cells		CD4-CSF	CSF/CD4 (%)	CD8-CSF	CSF/CD8 (%)
	Total no. of clones	No. of expanded clones	Total no. of clones	No. of expanded clones	Total no. of clones	No. of expanded clones				
18-8856	9802	14	7336	51	5006	124	54	44	43	35
17-465	9349	16	8026	42	7275	30	10	33	13	43
17-8801	10,550	32	4784	128	6610	88	43	49	43	49
17-8823	6653	30	5379	65	3930	130	45	35	49	38
17-8838	8087	15	5362	80	10,595	24	9	38	14	58
16-098	7560	27	3306	68	7702	58	23	40	17	29
16-223	8714	3	12,005	39	7564	35	8	23	16	46
14-137	4931	67	3918	80	6480	49	26	53	23	47
14-155	NA	NA	NA	NA	4654	65	NA	NA	NA	NA
14-250	NA	NA	NA	NA	3555	126	NA	NA	NA	NA
14-87	NA	NA	NA	NA	6844	45	NA	NA	NA	NA
Average	8206	25.5	6265	69.1	6383	70.4	27.25	39	27.25	43

Overlap between expanded CSF clones and CD4^{+} and CD8^{+} T cells clones. CSF, cerebrospinal fluid; NA, not available; CD4-CSF, number of expanded CSF clones overlapping with CD4^{+} T-cell clones; CSF/CD4, percentage of expanded CSF clones overlapping with CD4^{+} T-cell clones; CD8-CSF, number of expanded CSF clones overlapping with CD8^{+} T-cell clones; CSF/CD8, percentage of expanded CSF clones overlapping with CD8^{+} T-cell clones.

MS. In order to explore this hypothesis, we performed a CNV comparison between CSF and PBMC samples from MS patients as well as OND patients and HC. The aim was to identify genomic regions which were over- or under-represented in the CSF samples which could be an indication of sub-groups of cells with specific genomic
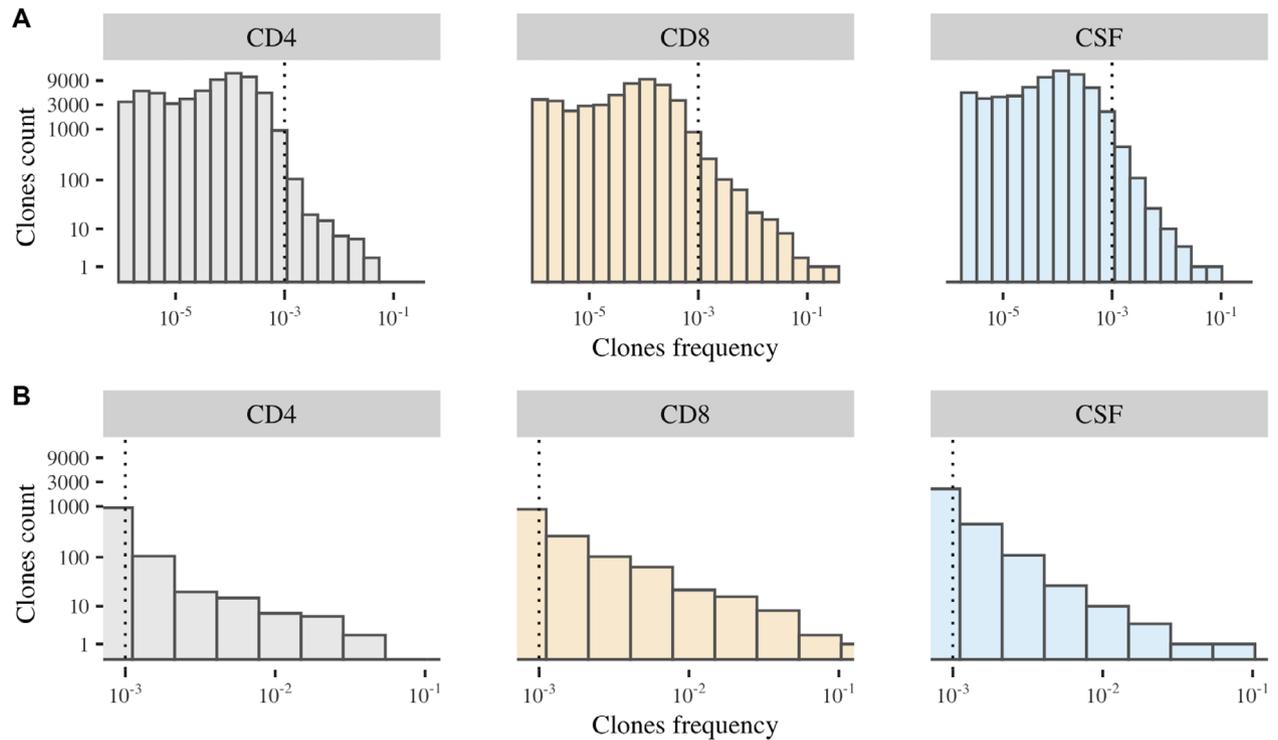
Figure 5. T-cell clone frequency distributions in CD4$^+$, CD8$^+$ T, and CSF cells. (A) Frequency distribution of all identified clones. Vertical dotted line marking the threshold of expansion of 0.1%. (B) Distribution of clones with a frequency ≥0.1%.

characteristics enabling a role in MS pathogenesis. The main results of this analysis revealed CNVs across the TCR regions. Although this is likely a general consequence of an over representation of the proportion of T cells in the CSF as compared to PB, this also enabled us to further describe the TCR rearrangement in T cells from CSF in order to search for potential signs of T-cell clonality in the CNS.

The main reason for choosing CNVs to study genomic variants is that CNVs are known to occur frequently in connection with mitosis and can hence provide the basis for somatic genomic rearrangements and the establishment of mosaicism. Our genomic comparison of CSF cells to PBMCs identified deletions in the TRA/D, TRG, and TRB loci in CSF cells, which were confirmed by qPCR. Comparison of sorted peripheral CD4$^+$ and CD8$^+$ T cells to CSF cells showed similar deletions in the TRA/D and TRG loci, while the TRB locus displayed a more complex structure with inter-individual and intra-variations between CD4$^+$ and CD8$^+$ T cells and CSF cells. The deletions in CSF cells were more specific to CD4$^+$ and CD8$^+$ than to γδ T cells, indicating that CSF cells seem to be predominantly T cells of the αβ and not the γδ type. Surprisingly though, we observed deletions in the TRB locus in peripheral γδ T cells. This would not be expected based on the sequential TCR rearrangement[18]

and we do not believe this is caused by contamination of the samples with αβ T cells as we would then have expected deletions in the TRA locus too, which we did not find. However, deletions in the TRB locus in γδ T cells have been observed before and attributed to the concurrent rearrangement of β, γ, and δ loci.[19]

The TCR loci undergo rearrangement during the maturation of the T cells in the thymus and unselected genes are spliced out.[20] Hence the deletions in the TCR loci, when comparing CSF cells to PBMCs, suggest that these T cells display the required diversity of adaptive immunity.

The presence of deletions in the TRG loci of CD4$^+$ and CD8$^+$ T cells could be explained by the successive model of TCR rearrangement,[21] where TRG and TRD are first rearranged and if there is a γδ TCR product the cell commits to being a γδ T cell, if not, the next step is to rearrange the TRB and TRA loci and commit to being an αβ T cell. TCR rearrangement is a classic form of somatic variation and the detection of TCR regions in the initial CNV screening, as previously pointed out, is likely the result of the proportion of T cells in the CSF being higher than in the PBMCs, that is, T lymphocyte count bias. This allowed us to further characterize the TRB locus rearrangements using next generation sequencing and profile the αβ T-cell repertoire in paired CSF, CD4$^+$, and
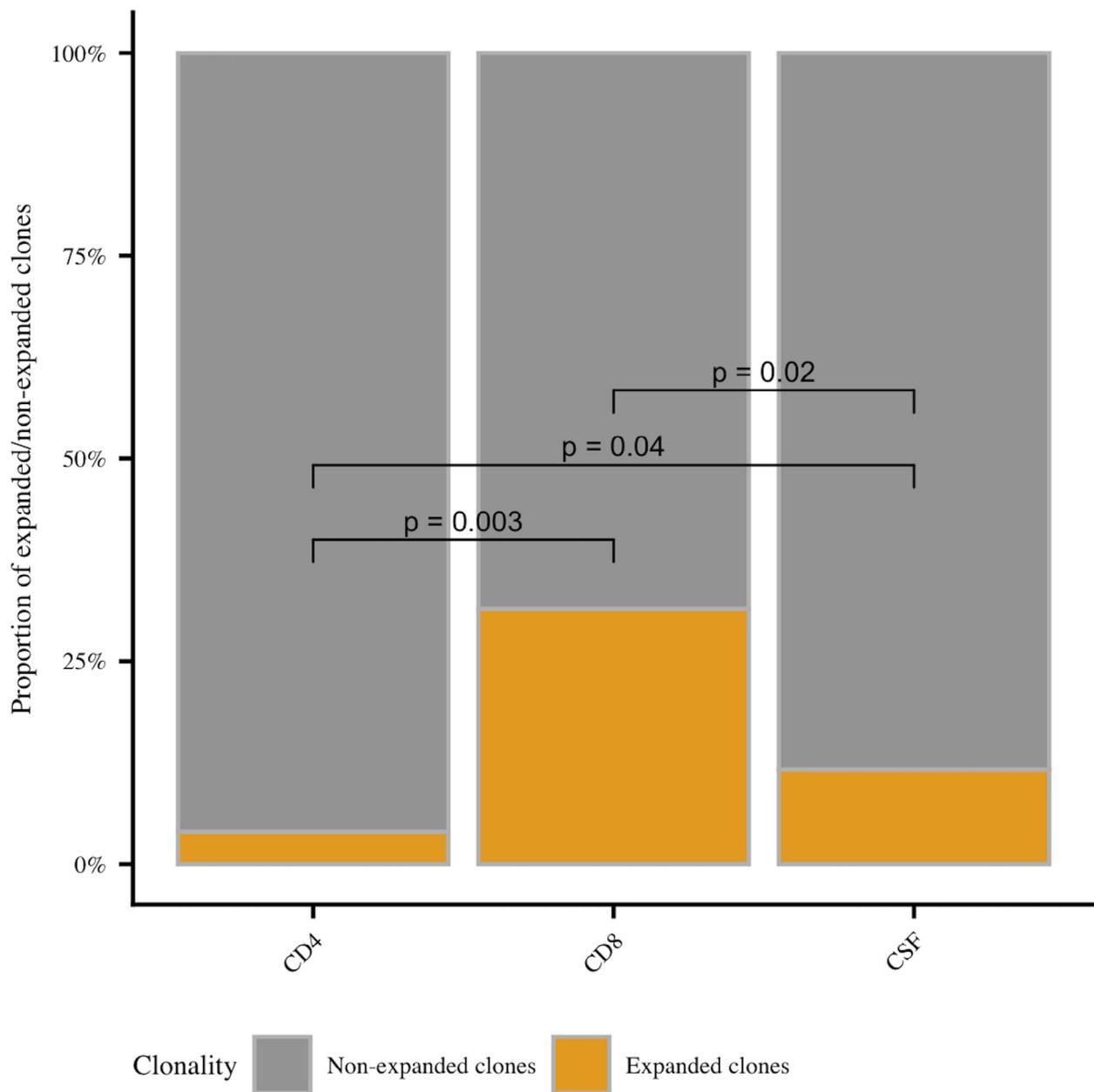
Figure 6. Proportion of expanded and non-expanded clones in CD4^{+} T cells, CD8^{+} T cells, and CSF cells. Clones with a frequency of either ≥0.1 or <0.1% of all clones are considered expanded and non-expanded respectively, calculated as the number of clones of the respective category compared to the total sum of clones per sample. The bars represent the median of the expanded and non-expanded clones per cell compartment. Differences in the proportion of expanded clones between the three cell compartments were tested using Wilcoxon rank-sum test. CSF, cerebrospinal fluid.

CD8^{+} T cells. The results revealed intra- as well as interindividual diversity across the TRB locus.

We profiled the TCR repertoire in the periphery and the CSF using a combination of multiplex PCR and next generation sequencing which provided the frequencies and CDR3 sequences of the T-cell clones. Clones with a frequency ≥0.1% were considered clonally expanded. We examined the TCR repertoire in the CSF cells and compared to the peripheral CD4^{+} and CD8^{+} T cells and found no significant overlap between the TCR repertoire in the CSF and the periphery, which indicates a divergence in the TCR profile between the two compartments. Within the peripheral compartment we observed that the CD8^{+} T cells were less diverse than the CD4^{+} T cells,

Table 4. For each patient, the top five frequent clones in the CSF and their corresponding frequencies in the CD4^{+} and CD8^{+} T cells peripheral compartments when available.

id	CDR3aa	V gene	D gene	J gene	CSF	CD4^{+}	CD8^{+}
17-465	CASSLTQGGGETQYF	TRBV12-4	TRBD2	TRBJ2-5	**0.0031**	2.93E-06	**0.0032**
17-465	CASRSQ_G*QYF	TRBV4-3	TRBD1	TRBJ2-7	**0.0029**	0.0001	**0.0015**
17-465	CASSRQNSPLHF	TRBV25-1	.	TRBJ1-6	**0.0026**	0	0.0007
17-465	CASSQGSSGRLAGSYEQYF	TRBV3-1	TRBD2	TRBJ2-7	**0.0026**	2.93E-06	0.0007
17-465	CASSSQSGVNNEKLFF	TRBV12-4	TRBD1	TRBJ1-4	**0.0023**	0	**0.0059**
17-8801	CASSPAMNTEAFF	TRBV14	.	TRBJ1-1	**0.0327**	3.77E-05	**0.0085**
17-8801	CASSQVLLGQAFF	TRBV14	.	TRBJ1-1	**0.0127**	**0.0013**	**0.0300**
17-8801	CASSGTEAFF	TRBV4-1	.	TRBJ1-1	**0.0054**	4.19E-06	**0.0046**
17-8801	CASSLGQGNAYGYTF	TRBV12-4	TRBD1	TRBJ1-2	**0.0043**	1.04E-06	**0.0010**
17-8801	CASSQRSGSTPYEQYF	TRBV5-1	TRBD2	TRBJ2-7	**0.0036**	4.19E-06	**0.0021**
17-8823	CASSRTGRVDEQFF	TRBV18	TRBD1	TRBJ2-1	**0.0155**	**0.0058**	0.0008
17-8823	CATSRGLGQ_GFGANVLTF	TRBV15	TRBD1	TRBJ2-6	**0.0105**	0	**0.0028**
17-8823	CASSQVDRTHDGNEQFF	TRBV3-1	TRBD1	TRBJ2-1	**0.0092**	0	0.0007
17-8823	CASSPDGMNTEAFF	TRBV10-2	.	TRBJ1-1	**0.0091**	0.0002	0.0004
17-8823	CASSV*LTTNTGELFF	TRBV10-1	TRBD2	TRBJ2-2	**0.0065**	0.0004	**0.0019**
16-223	CSASQGYGATEAFF	TRBV29-1	TRBD1	TRBJ1-1	**0.0064**	3.84E-06	**0.0049**
16-223	CAWSVLGPAPGGGYTF	TRBV30	TRBD1	TRBJ1-2	**0.0052**	0.0001	**0.0067**
16-223	CAHERTAGELFF	TRBV29-1	.	TRBJ2-2	**0.0037**	0	0
16-223	CSVEDLLWADYGYTF	TRBV29-1	.	TRBJ1-2	**0.0031**	7.68E-06	**0.0011**
16-223	CASSLYRGTEAFF	TRBV12-4	TRBD1	TRBJ1-1	**0.0031**	0	**0.0106**
17-8838	CSASLAGR_NTGELFF	TRBV20-1	TRBD2	TRBJ2-2	**0.0052**	8.91E-06	**0.0376**
17-8838	CASSERGQGETQYF	TRBV25-1	TRBD2	TRBJ2-5	**0.0040**	0	**0.0111**
17-8838	CASSLELASYGYTF	TRBV5-1	.	TRBJ1-2	**0.0026**	0	**0.0170**
17-8838	CASSLEDR_INQPQHF	TRBV7-4	.	TRBJ1-5	**0.0025**	0.0004	**0.0254**
17-8838	CATSRDSGLRANGYTF	TRBV15	.	TRBJ1-2	**0.0025**	0	0
17-8838	CASSLGQAYEQYF	TRBV7-8	TRBD1	TRBJ2-7	**0.0023**	**0.0010**	**0.0402**
18-8856	CASSYLPGQQNTEAFF	TRBV6-5	TRBD1	TRBJ1-1	**0.0082**	0.0001	0.0009
18-8856	CATSWDNQPQHF	TRBV15	.	TRBJ1-5	**0.0063**	4.49E-06	0
18-8856	CASSLRGSNQPQHF	TRBV12-4	.	TRBJ1-5	**0.0057**	0.0005	0.0004
18-8856	CSVEPDRVENGYTF	TRBV29-1	TRBD1	TRBJ1-2	**0.0054**	**0.0024**	**0.0063**
18-8856	CATSREKGQNTEAFF	TRBV15	TRBD1	TRBJ1-1	**0.0044**	0.0000	0.0007
16-098	CAISEQQGEGYTF	TRBV10-3	TRBD1	TRBJ1-2	**0.0091**	**0.0042**	0
16-098	CASSLWTFNTGELFF	TRBV7-8	.	TRBJ2-2	**0.0050**	0	**0.0012**
16-098	CASSRGR_DTEAFF	TRBV14	TRBD2	TRBJ1-1	**0.0049**	0	0.0003
16-098	CASSFGSPGSTEAFF	TRBV27	TRBD2	TRBJ1-1	**0.0049**	0	0.0006
16-098	CASSESTEFTEAFF	TRBV10-1	.	TRBJ1-1	**0.0049**	0.0008	0
14-137	CASSQESGPFYEQYF	TRBV4-1	TRBD2	TRBJ2-7	**0.0133**	**0.0015**	**0.0153**
14-137	CASSESISNQPQHF	TRBV10-2	.	TRBJ1-5	**0.0081**	**0.0029**	**0.0168**
14-137	CSASNRGTSNQPQHF	TRBV20-1	TRBD1	TRBJ1-5	**0.0065**	**0.0018**	**0.0147**
14-137	CASSERGNSDYGYTF	TRBV2	TRBD2	TRBJ1-2	**0.0059**	**0.0145**	**0.0687**
14-137	CSASLQLTTYGYTF	TRBV20-1	.	TRBJ1-2	**0.0047**	**0.0030**	0.0008
14-87	CASSWGSGSNYGYTF	TRBV11-2	.	TRBJ1-2	**0.0574**	NA	NA
14-87	CASSQDRLTGGYTF	TRBV4-1	.	TRBJ1-2	**0.0229**	NA	NA
14-87	CASSPLPPSNTGELFF	TRBV18	TRBD1	TRBJ2-2	**0.0226**	NA	NA
14-87	CASSPSRGEGYTF	TRBV18	.	TRBJ1-2	**0.0100**	NA	NA
14-87	CASSLYSATGEAFF	TRBV28	.	TRBJ1-1	**0.0051**	NA	NA
14-155	CASSGGVGSYEQYF	TRBV18	TRBD2	TRBJ2-7	**0.0122**	NA	NA
14-155	CASSE*RP_GVRGGYTF	TRBV25-1	TRBD1	TRBJ1-2	**0.0060**	NA	NA
14-155	CASSQRGPGVAVKNEKLFF	TRBV4-1	TRBD1	TRBJ1-4	**0.0058**	NA	NA
14-155	CASSLSRGGELFF	TRBV7-8	TRBD1	TRBJ2-2	**0.0055**	NA	NA
14-155	CASRLGGLGYGYTF	TRBV13	TRBD2	TRBJ1-2	**0.0041**	NA	NA
14-250	CASSHPRENTYEQYF	TRBV14	TRBD1	TRBJ2-7	**0.0150**	NA	NA
14-250	CARRVG_NTEAFF	TRBV12-3	.	TRBJ1-1	**0.0059**	NA	NA
14-250	CASSYVGDRTEAFF	TRBV6-3	.	TRBJ1-1	**0.0038**	NA	NA
14-250	CASSQAGRSYEQYF	TRBV14	TRBD2	TRBJ2-7	**0.0038**	NA	NA
14-250	CASSQDRLTGGYTF	TRBV4-1	.	TRBJ1-2	**0.0030**	NA	NA

Expanded clone frequencies are in bold. CSF, cerebrospinal fluid; NA, not available; Dot (·), sequencing reads not aligned to a D gene.

Table 5. Interindividual overlap of expanded T-cell clones.

id	Cell type	CDR3nt	CDR3aa	V gene	D gene	J gene	Frequency
16-223	CD8+	TGTGCCAGCAGTGAAGGTTATGGCTACACCTTC	CASSEGYGYTF	TRBV25-1	.	TRBJ1-2	0.0037
17-465	CD4+	TGTGCCAGCAGTGAAGGCTATGGCTACACCTTC	CASSEGYGYTF	TRBV25-1	.	TRBJ1-2	0.0014
14-137	CSF	TGTGCCAGCAGTATACAGGGGGCGAACTATGGCTACACCTTC	CASSIQGANYGYTF	TRBV27	TRBD1	TRBJ1-2	0.0031
14-250	CSF	TGTGCCAGCAGTATCCAGGGGGCGAACTATGGCTACACCTTC	CASSIQGANYGYTF	TRBV27	TRBD1	TRBJ1-2	0.0012
14-87	CSF	TGCGCCAGCAGCTTGGCACTGAACACTGAAGCTTTCTTT	CASSLALNTEAFF	TRBV5-1	.	TRBJ1-1	0.0012
17-8801	CD8+	TGTGCCAGCAGTTTAGCGCTGAACACTGAAGCTTTCTTT	CASSLALNTEAFF	TRBV12-4	.	TRBJ1-1	0.0015
14-250	CSF	TGCGCCAGCAGCCAAGACAGGCTGACGGGCGGCTACACCTTC	CASSQDRLTGGYTF	TRBV4-1	.	TRBJ1-2	0.0030
14-87	CSF	TGCGCCAGCAGCCAAGATCGCTTAACGGGGGGCTACACCTTC	CASSQDRLTGGYTF	TRBV4-1	.	TRBJ1-2	0.0229
14-137	CD4+	TGCAGCGTTGTCGCGGGGTACTATGGCTACACCTTC	CSVVAGYYGYTF	TRBV29-1	TRBD2	TRBJ1-2	0.0011
17-8801	CSF	TGCAGCGTTGTGGCAGGGTACTATGGCTACACCTTC	CSVVAGYYGYTF	TRBV29-1	TRBD1	TRBJ1-2	0.0016

CSF, cerebrospinal fluid; Dot (·), sequencing reads not aligned to a D gene.
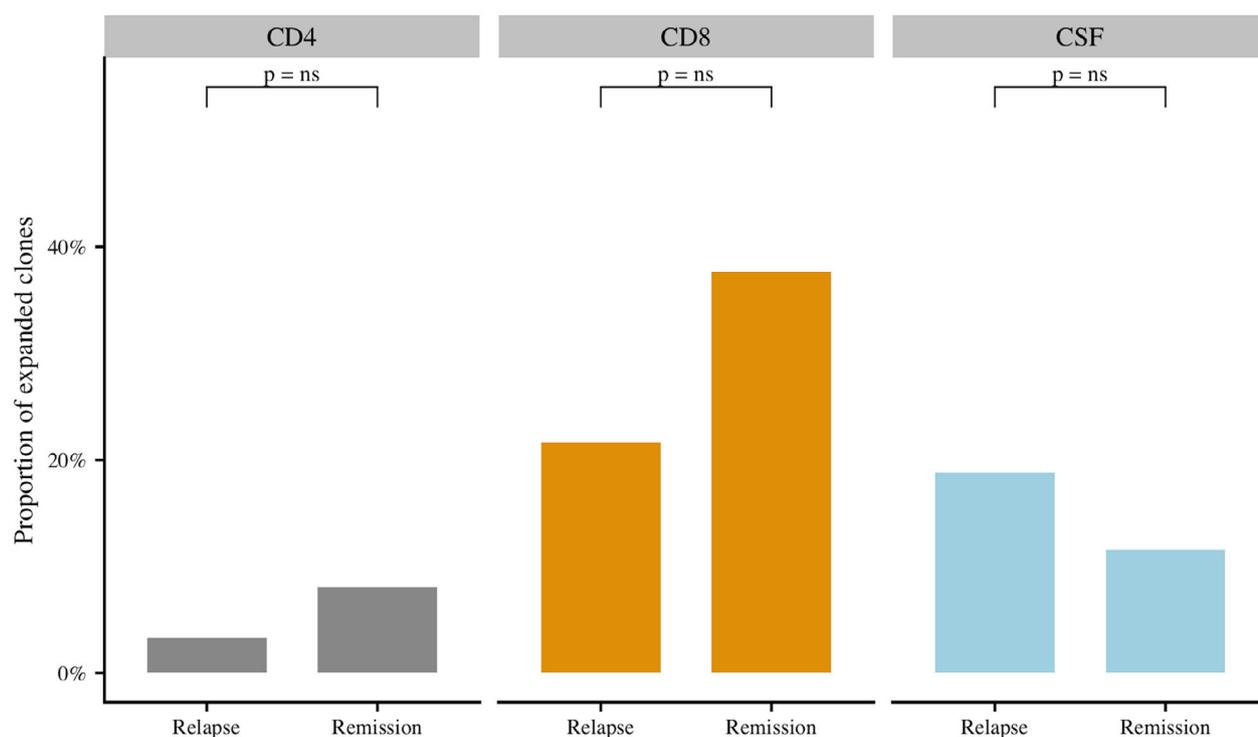

Figure 7. Difference in the proportion of expanded clones between MS patients under relapse or remission in CD4+, CD8+ T, and CSF cell compartments. Expanded clones are clones with a frequency of ≥0.1% and the proportion of expanded clones is calculated as the number of expanded clones compared to the total sum of clones per sample. The bars represent the median of the expanded clones per cell compartment. Differences of the proportion of expanded clones between relapse and remission were tested using Wilcoxon rank-sum test. ns, not significant; CSF, cerebrospinal fluid.

which was also reported in MS patients before undergoing autologous stem cell transplantation.[22] The TCR repertoire analysis was performed in samples from RRMS patients before the initiation of treatment, and it would be interesting to follow up the TCR repertoire in these same patients after initiating treatment to track the frequencies of the clones that were abundant pretreatment.

Our results, indicate an increased proportion of expanded clones in samples from patients during relapse as compared to remission in CSF cells, while the proportion of expanded clones is higher in PB samples from patients during remission compared to relapse in both CD4+ and CD8+ T cells. Although the results were not statistically significant, this observation may propose a

role for clonal expansion in MS disease as well as a possible shift of expanded clones from the periphery into the CNS during relapse.

The obvious question is if the observed TCR rearrangements in the CSF cells are unique to MS. We only had access to a very limited number of non-MS samples but we did see less deletions in the TRB locus in these as compared to the MS samples. Although this may suggest differences in αβ T cells ratio as well as levels of clonality between MS and non-MS samples, the number of available samples in the latter group was too small to determine such an effect. However, the sheer number of cells in CSF from the non-MS samples is surprising, although the samples were selected to have high numbers of cells.

Determining the antigen specificity of the expanded T-cell clones is a major step in deciphering the autoimmune response. Due to the environmental association of EBV infection with MS risk,[23] there is an interest in studying T-cell clones reactivity or specificity to EBV antigens.[24,25] In addition to other viral antigens, we have also identified clones that are targeting EBV antigens, with expanded clones specific for EBNA3A and EBNA3B antigens observed in one of the remission patients. This same clone, CASSLGQAYEQYF, targeting EBNA3A antigen was also observed in another study in MS patients who developed a new EBV infection or reactivation of EBV following autologous stem cell transplantation.[22] We detected an expanded clone targeting the same EBNA3A antigen but at another epitope, RPPIFIRRL, in the CSF samples of two patients, one in relapse and the other in remission. This clone did not share the same nucleotide sequence but translated into the same amino acid sequence. The sharing of clones that are identical in amino acid but different in the nucleotide sequences has been attributed to the process known as convergent recombination of TCR[26,27] and this process can be explained by the redundancy of the genetic code. Cytomegalovirus (CMV) infection is also another environmental risk factor for MS, however in the contrary to EBV infection a previous infection with CMV protects against MS.[28,29] Here, we have also identified clones that are targeting CMV antigens, mainly phosphoprotein 65 (pp65). Interestingly, clones targeting one of the pp65 epitopes, RPHERNGFTVL, were recently reported being only present in Japanese MS patients compared to healthy controls and mainly in patients carrying HLA-DRB1*04:05 and associated with a favorable disability progression.[30] However, there are other expanded or non-expanded clones for which we did not identify antigen specificity. This does not minimize their importance or significance as the investigation of the specificity of the T-cell clones was in silico and is dependent on previously reported findings, of which a majority is viral antigen-specific TCRs. Another limitation of this in silico investigation is that we searched for matches in the VDJdb using only TRB CDR3 sequences and not also including the CDR3 sequences of the paired TRA. Although therefore not conclusive, our findings still provide indications of potential target antigens.

In a GWAS, Sato et al. using DNA from PBMCs, reported CNVs that were also deletions in the TRG and TRA/D loci to be associated with MS and neuromyelitis.[31] Highlighting the importance of sample selection and DNA source when studying somatic variations,[32] they also attempted to correct for the DNA source and concentration when they sorted for different subsets of white bloods cells and the deletions were validated only in T cells. Interestingly they speculated that due to the large size of the deletions they are unlikely to be a result of rearrangement.

The original hypothesis of this study was the presence of sub-populations of cells within the CNS that are established as a result of genomic rearrangements and which may be involved in MS pathogenesis. By genomic comparison of cells from the CSF and PB we could detect an over representation of T cells in the CSF based on rearrangements of TCR. We further explored this finding by characterizing the genomic rearrangements of the TRA, TRB, and TRG regions and used the diversity of the TRB locus rearrangements to explore the character and clonal expansion of the T cells in the CNS. Although we did not detect any evidence of CNVs around the IGH, IGK, and IGL loci on chromosomes 14q32, 2p12, and 22q11 which may be an indication of an over representation of B cells in the CSF we can not guarantee that the available genetic markers from the CytoScan HD Array across the relevant genomic regions would allow such an observation. Nor did we see any other chromosomal regions indicating CNVs between CSF and PBMCs in multiple samples although the number of samples was limited.

In conclusion, in search of sub-populations of immune cells in the CNS, we have identified deletion type CNVs in the TCR loci of cells in the CSF consistent with specific TCR rearrangements of the T cells. We have further characterized these T cells and identified clonality which may advance the understanding of the neuro-inflammation process and eventually contribute to the development of therapeutics.

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Conflict of Interest

No author has any conflict of interest related to this study to report.

Author Contributions

Conception and study design: A. G. and S.K.B. Data acquisition: all authors, Data analysis: S.K.B. and A.G. Data interpretation: S.K.B., A.G., and F.A.N. Drafting of the manuscript: S.K.B. and A.G. Critical revision of the manuscript: all authors.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. The specificity of the identified T-cell clones when matching their CDR3 sequences with previously known targets in the VDJdb database.