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T-cell repertoire profiling by next-generation sequencing reveals tissue migration dynamics of TRBV13-family clonotypes in a common experimental autoimmune encephalomyelitis mouse model

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ABSTRACT

The experimental autoimmune encephalomyelitis (EAE) model is indispensable for autoimmunity research, but model-specific T cell dynamics are sparsely studied. We used next-generation immunosequencing across lymphoid organs, blood and spinal cord in response to immunization with myelin basic protein (MBP) to study T cell repertoires and migration patterns. Surprisingly, most spinal cord T cells were unique to the individual animal despite the existence of shared MBP-specific clones, suggesting a previously underestimated T cell diversity. Almost complete emigration of pathogenic clones from blood to spinal cord indicates that blood is not a suitable compartment to study EAE-mediating T cells.

1. Introduction

Multiple sclerosis (MS) is a T-cell mediated autoimmune disease in which peripheral T-cells are proposed to become activated against central nervous system (CNS) myelin antigens (Sospedra and Martin, 2005). After crossing the blood brain barrier, these cells recognize their cognate antigen and initiate destructive inflammation (Korn and Kallies, 2017). This pathophysiological understanding of the disease is consistent with the experimental autoimmune encephalomyelitis (EAE) mouse model, the most broadly used experimental model of multiple sclerosis (Dendrou et al., 2015). However, a variety of immunologically different EAE models exist, ranging from wild-type mouse models immunized with myelin antigens to models based on T-cell transfer or transgenic overexpression of myelin specific T-cell receptors (TCR) (Constantinescu et al., 2011; Croxford et al., 2011; Glatigny and Bettelli, 2018). The disease mediating T-cell repertoire is influenced by the interaction of the antigen used for immunization and the model specific major histocompatibility complex (MHC), and thus, the repertoire differs substantially between model systems. Myelin basic protein (MBP) remains one of the most studied myelin proteins in EAE, which causes a single acute paralytic episode with subsequent partial recovery (Glatigny and Bettelli, 2018). In the MBP model, there is a strong overlap of the encephalitogenic epitopes between mice and humans (Sospedra and Martin, 2005). Moreover, V-gene restriction of myelin reactive T-cells leading to an oligoclonal T-cell repertoire has been especially found in MBP-induced mouse models (Acha-Orbea et al., 1988; Urban et al., 1988), a phenomenon that is also observed in MS patients (Hafler et al., 1996; Planas et al., 2018). Based on these findings, specific TCR directed therapies targeting the restricted repertoire were developed, although with little success (Killestein et al., 2002; Vandenbark et al., 2008; Wraith, 2009). Despite being extensively used to study T-cell driven autoimmunity, the current understanding of T-cell repertoire metrics in EAE largely relies on Sanger sequencing and CDR3 spectratyping of TCR β-chain variable genes (TRBV) (Kim et al., 1998; Sakuma et al., 2004). Both techniques have significant limitations, because only a small fraction of all T-cells can be analyzed by Sanger sequencing and only a crude repertoire analysis not based on clonal sequences can be performed with CDR3 spectratyping (Lossius et al., 2016). One reason for the failure of therapeutic strategies against specific MS associated TCRs might be a more diverse T-cell repertoire than anticipated. In order to study the T-cell repertoire in response to MBP immunization with high resolution, we used TCR next-generation sequencing, which permits thorough characterization of T-cell repertoire metrics including tracking of individual T-cell clones

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(Akyüz et al., 2017; Schliffke et al., 2016). It is well established that autoreactive T-cells in MS and the EAE model can also be identified in the blood and in lymphoid organs, which are the tissues most frequently studied. With regard to function, MBP specific T-cells isolated from the blood or the CNS might be similar (Hofstetter et al., 2005). Nevertheless, the clonal composition of T-cells in different tissues has not been analyzed in a single comparative study before and the quantitative composition of T-cells in the different compartments is unknown. So far, tissue specific frequencies of EAE-related T-cell receptor beta (*Tcrb*) sequences needed to be extracted from different publications, while a thorough description of sequences was not available. Using next-generation immunosequencing, we comparatively describe the diversity and the clonal composition of T-cell repertoires in response to MBP immunization in the spinal cord, primary and secondary lymphoid tissues and blood. In addition, T-cell receptor sequences are made readily available in a repository.

2. Material and methods

2.1. Mice and induction of EAE

Female 10–20 week old B10.PL-H2uH2-T18a/(73NS)SnJ mice were kept under specific pathogen-free laboratory conditions and experiments were performed in the animal facility of the University Medical Centre Hamburg-Eppendorf, Germany. All experiments were conducted in accordance with the German and European Community laws on protection of experimental animals, and all procedures were approved by the responsible committee of The State of Hamburg, Germany.

To induce EAE, mice were immunized with 200 µg murine 4YMBP peptide [Ac1–9; Ac-ASQYRPSQR-COOH] (Panatecs, Heidelberg, Germany) emulsified in 100 µl of a 1:1 mixture of PBS and complete Freund's adjuvant (CFA) supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis*, strain H37RA (Difco, USA). Additionally, mice were injected twice intraperitoneally with 200 ng pertussis toxin (Sigma-Aldrich, Germany) at the day of immunization and two days later. The course of EAE was monitored daily for up to 14 days according to the following score: 0, no clinical symptoms; 1, flaccid tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4 fore- and hindlimb paralysis; 5 moribund.

2.2. Genomic DNA isolation, PCR and NGS

Genomic DNA isolation, PCR amplification, NGS and analysis was performed as previously described.(Akyüz et al., 2017; Schliffke et al., 2016) Briefly, mice were euthanized and tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until gDNA isolation. PBMCs were isolated from blood collected into EDTA-tubes (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) using standard lysis buffer (ammonium chloride 8.29 g/l, EDTA 0.372 g/l, potassium hydrogen carbonate 1 g/l). Genomic DNA from tissue and PBMCs was isolated using Gene Elute mammalian genomic DNA miniprep kit (Sigma-Aldrich). *Tcrb* gene amplification was performed by multiplex PCR using forward and reverse primer pools as summarized in supplemental Table S1 (Dash et al., 2011; Pannetier et al., 1993). Note that the Tcrb21-FW primer has not been published previously. All PCRs were performed using Phusion HS II polymerase (Thermo Fisher Scientific Inc., Darmstadt, Germany). Amplicons were tagged with Illumina adapters and indices in 2 consecutive PCR reactions after size separation and purification using the NucleoSpinVR Gel and PCR Clean-up kit (Macherey-Nagel). Concentration and purity of the amplicons were determined by Qubit (QIAGEN, Hilden, Germany) and Agilent 2100 Bioanalyzer (Agilent technologies, Böblingen, Germany). NGS was performed using an Illumina MiSeq sequencer [600 cycle single indexed, paired-end runs (V3 chemistry)]. Demultiplexing and FastQ data output was generated by the MiSeq reporter. FASTQ data are available from the Sequence Read Archive, accession number PRJEB30005.

2.3. Data analysis

Data analysis was performed using the MiXCR analysis tool (Bolotin et al., 2015). A clonotype was defined by the CDR3 amino acid sequence for further analysis and only sequences with a read count ≥2 were included in the analysis. Repertoire metrics were studied using Shannon-Wiener, inverse Simpson and clonality indices (Jost, 2007; Kirsch et al., 2015). Statistical analysis and data visualization were performed using GraphPad Prism and R Foundation's R v3.5.0 using the package “tcR” and the function *heatmap.2* from the package “gplots”, statistical tests as indicated in the figures legends (Nazarov et al., 2015).

3. Results

3.1. T-cell repertoire diversity in wild type mice and in response to immunization with MBP

We analyzed the T-cell repertoire in blood, lymph nodes (axillary, inguinal, popliteal), spleen, thymus and spinal cord tissue from unimmunized B10.PL mice ($N = 2–4$) and from MBP immunized B10.PL mice at day 14 ($N = 6$) using next-generation immunosequencing. The mice showed clinical signs of encephalomyelitis at the time of sampling (mean EAE score = 2). The number of identified T-cell clones was comparable between the same tissues from different mice, but differed substantially between analyzed tissues, being highest in thymus and lowest in spinal cord samples (supplemental Fig. S1). Sequencing depth was consistently high, ranging between 30,000 and 100,000 reads per sample (supplemental Fig. S1). Repertoire diversity was compared using common diversity indices (Shannon-Wiener, Inverse-Simpson). A significantly contracted T-cell repertoire was observed in blood and spleen samples from immunized mice suggesting antigen-driven selection (Fig. 1A and B). In contrast, the thymus repertoire from immunized mice was more diverse in comparison to unimmunized animals, while no difference was observed in lymph node repertoire diversity. T-cells were almost absent in spinal cord samples from unimmunized mice (supplemental Fig. S1), while a diverse repertoire with a large number of T-cells was observed in immunized animals (Fig. 1B). Nevertheless, in immunized mice, the spinal cord repertoire remained significantly less diverse in comparison to all other investigated tissues (Fig. 1 C and D). Highest diversity in response to immunization was observed in the thymus, which was dominated by many small and medium sized clones. In contrast, spinal cord samples showed infiltration of a lower number of more expanded T-cell clones (Fig. 1E).

3.2. Distribution of Tcrb V-gene usage in response to MBP immunization

Next, we analyzed the V-gene usage of the *Tcrb* repertoires in different tissues. The determined CDR3 nucleotide sequences of *Tcrb* clones were aligned with reference sequences in order to identify the best matching V-genes. In order to verify the analysis with a known T-cell receptor sequence, T-cells from the spleen of a transgenic tg4 mouse were also analyzed. As described before, these showed TRBV13–2 (also known as Vβ8.2) usage by > 96% of the T-cells (Fig. 2A) (Liu et al., 1995). In unimmunized B10.PL mice, similar V-gene distribution was found in blood, thymus, spleen and lymph node tissue. Very few T-cells with a high frequency of TRBV1 and TRBV31 were identified in the spinal cord tissue of unimmunized B10.PL mice (Fig. 2A). Comparison of tissues from thymus, spleen and lymph node from immunized and unimmunized mice showed comparable distributions of V-genes. In the spinal cord tissue and blood samples, however, the distribution differed strikingly, suggesting remodeling of the T-cell repertoire through emerging clones in response to antigen challenge. In blood samples from immunized mice, significantly less T-cell clones with TRBV13-2 and TRBV13-3 usage were observed, while T-cells with TRBV19 and

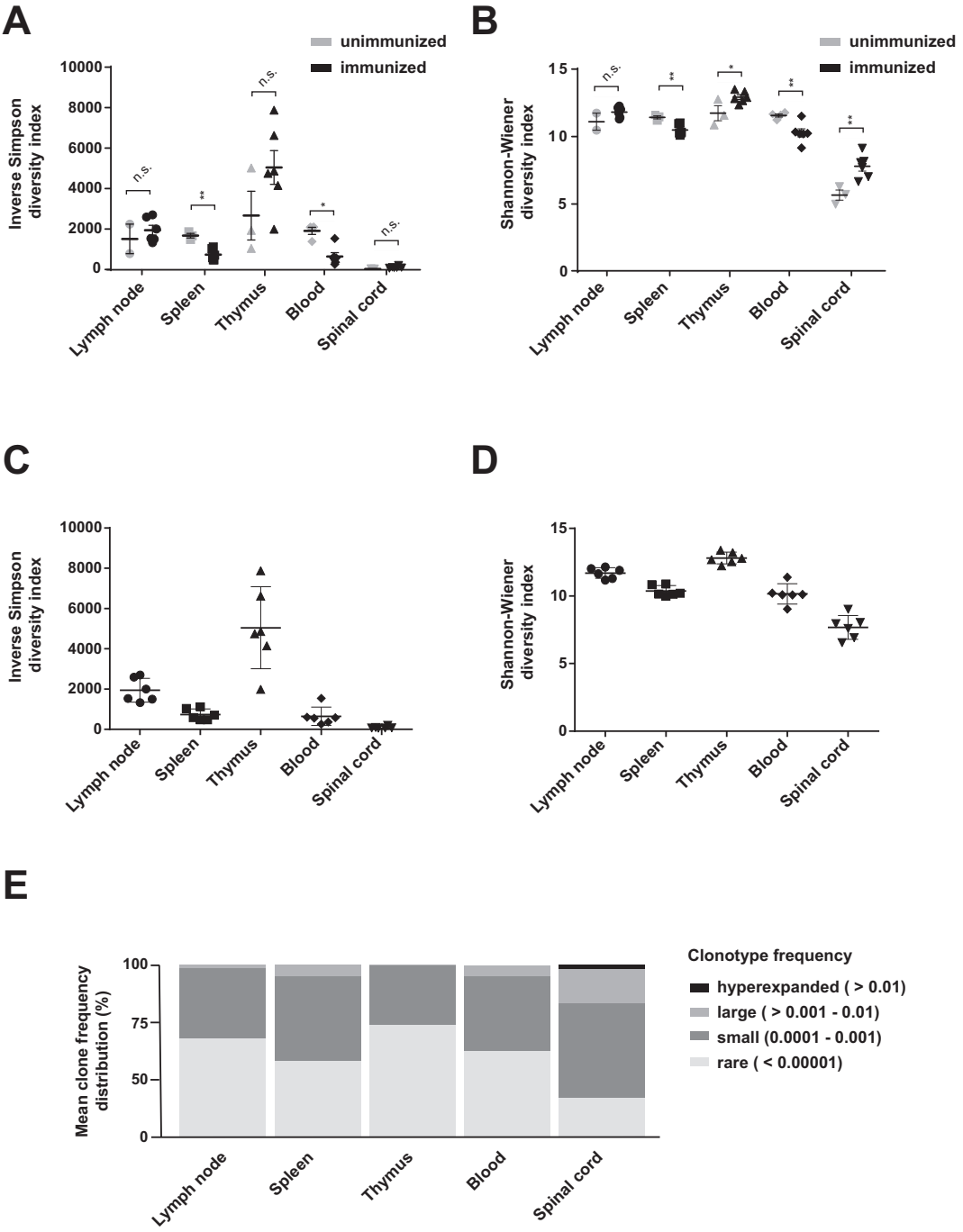

Fig. 1. T-cell receptor repertoire diversity in response to MBP immunization. (A) and (B) Comparison of diversity between unimmunized and immunized mice using the inverse Simpson and Shannon-Wiener diversity indices. (C) and (D) Comparison of diversity between the indicated samples after immunization using the inverse Simpson and Shannon-Wiener diversity indices. (E) Frequency of clones after immunization separated by 4 frequency ranges. One-way ANOVA followed by Tukey's test for multiple tissue comparisons (n = 4–6 per group). P-values are defined as * $<$ 0.05 and ** $<$ 0.01.

TRBV31 usage were overrepresented (Fig. 2B). The opposite occurred in spinal cord tissue, with significantly more TRBV13-2 and TRBV13–3 usage and less clones with TRBV19 and TRBV31 sequences (Fig. 2C). Presumably, upon immunization, migration of TRBV13-2 and TRBV13-3 T-cells into the spinal cord had caused their depletion in the blood.

3.3. Identification of shared clones by T-cell repertoire overlap analysis

In order to identify common T-cell clones possibly mediating autoimmune encephalomyelitis, shared clones were identified between T-cell repertoires of spinal cord tissues from different immunized mice (supplemental table S2). T-cell clones identified in spinal cord samples from at least two different mice were searched in all other analyzed tissues from immunized and unimmunized animals. As expected, the identified clones were specifically enriched in the spinal cords of immunized animals. At lower frequencies, some of the identified clones could also be found in the blood, spleen and especially lymph nodes of immunized mice, while only very few could be found in the thymus. Rarely, these shared clones could also be identified in blood, lymph nodes, spleen and thymus of unimmunized mice, suggesting that the baseline T-cell repertoires in these tissues harbor some MBP-reactive clones that expand after additional antigen challenge (Fig. 3A). In

A

	Blood		Thymus		Spleen		Lymph node		Spinal cord		TG4
	unimm.	imm.	unimm.	imm.	unimm.	imm.	unimm.	imm.	unimm.	imm.	unimm.
TRBV1	17,42	25,59	15,20	13,90	13,24	17,72	12,40	18,86	31,92	7,7	0,00
TRBV2	8,98	6,13	5,62	5,67	6,50	6,42	6,01	6,21	7,62	4,9	0,00
TRBV3	0,59	0,01	0,34	0,21	0,24	0,16	0,29	0,18	0,06	0,5	0,00
TRBV4	2,44	0,59	0,82	0,85	2,07	1,86	1,52	1,98	0,61	2,0	0,00
TRBV5	5,05	10,04	4,41	4,56	4,39	6,55	2,96	5,29	7,68	2,4	0,00
TRBV12-1	0,32	0,02	2,59	2,06	0,21	0,12	0,22	0,15	0,10	0,2	0,08
TRBV12-2	0,11	0,00	0,65	0,41	0,10	0,03	0,08	0,05	0,34	0,1	0,01
TRBV13-1	15,03	4,28	16,21	15,53	18,52	14,67	20,58	16,22	2,26	19,9	0,00
TRBV13-2	26,67	6,83	25,89	27,79	32,04	28,75	31,84	26,41	8,23	40,1	96,28
TRBV13-3	8,90	0,88	7,10	7,90	12,36	9,38	14,97	10,48	1,73	16,1	0,00
TRBV14	0,05	0,06	0,04	0,02	0,06	0,06	0,03	0,08	0,06	0,1	0,00
TRBV15	1,18	0,24	7,64	5,96	0,51	0,68	0,72	0,64	0,88	1,0	0,00
TRBV16	0,17	0,08	1,01	0,77	0,11	0,18	0,13	0,21	0,29	0,4	0,01
TRBV17	1,17	0,66	1,28	0,73	1,17	0,84	0,87	0,82	1,07	1,0	0,00
TRBV19	0,44	4,27	0,65	1,16	0,24	1,39	0,16	0,56	1,60	0,1	0,00
TRBV20	0,33	0,14	0,17	0,06	0,40	0,35	0,34	0,34	0,37	0,6	0,00
TRBV23	0,03	0,01	0,11	0,05	0,05	0,07	0,02	0,04	0,00	0,0	0,00
TRBV24	0,77	0,28	0,51	0,41	0,55	0,57	0,46	0,54	2,59	0,5	0,00
TRBV26	0,65	0,33	0,48	0,33	0,88	0,63	0,48	1,26	0,85	0,4	0,00
TRBV29	0,51	1,65	0,94	0,90	0,32	0,62	0,15	0,82	0,61	0,1	0,03
TRBV30	0,06	0,02	0,02	0,01	0,06	0,05	0,05	0,07	0,20	0,1	0,00
TRBV31	8,77	37,76	7,82	9,93	5,73	8,70	5,32	8,58	30,71	1,7	3,59

V gene frequency (%)

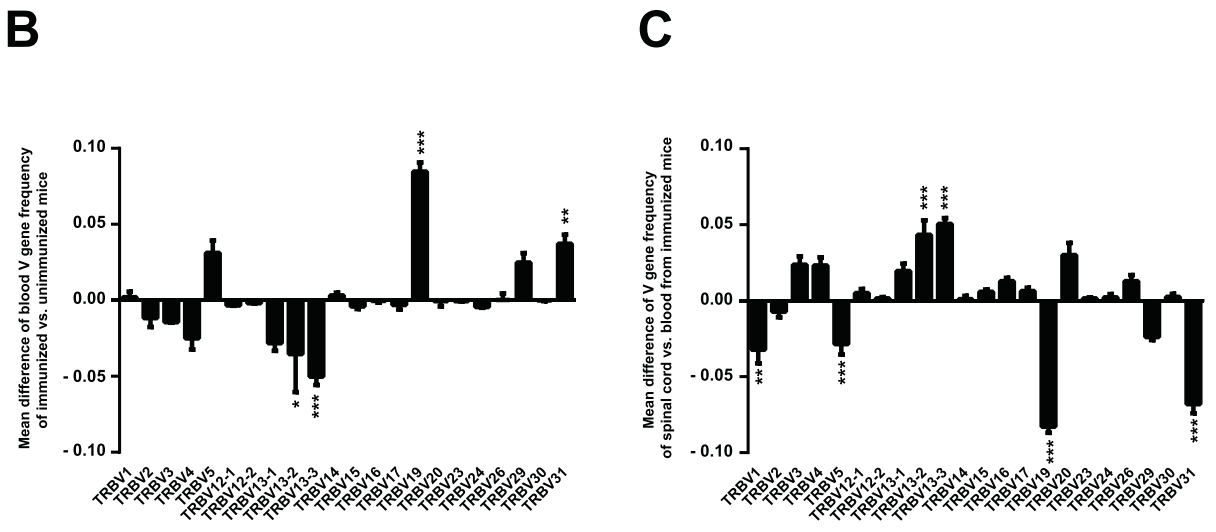

Fig. 2. V gene usage in the T-cell receptor beta repertoire of unimmunized and MBP immunized mice. (A) Heatmap of mean V-gene frequencies in the indicated samples from unimmunized and immunized mice. Numbers indicate the average V gene frequency in percent (n = 2–6 per group). (B) Mean differences of blood V gene frequency (± SEM) between immunized and unimmunized mice. ANOVA with Šídák's multiple comparisons test (n = 6 per group). (C) Mean differences of V gene frequency (± SEM) between blood and spinal cord samples from immunized mice. One-way ANOVA with Šídák's multiple comparisons test (n = 5–6 per group). P-values are defined as * < 0.05, ** < 0.01 and *** < 0.001.

addition, the fraction of shared clones in different tissues was analyzed, which was highest in lymph nodes and thymus and rather low in blood, spleen and spinal cord samples (Fig. 3B). Highly shared clones, however, occurring at significant frequencies in all six animals, could especially be identified in spinal cord samples and only at rare frequencies in other tissues (Fig. 3C). On the clonal level, two highly expanded T-cell CDR3 amino acid sequences occurring in all animals (CASGDAGSQNTLYF and CASGDAGGGYEQY) could be identified significantly more often in spinal cord samples in comparison to blood, lymph node, spleen and thymus (Fig. 4A). One of these highly expanded shared sequences (CASGDAGSQNTLYF) also occurred significantly more often in the spleen and lymph nodes in comparison to the thymus (Fig. 4B), suggesting enrichment in secondary lymphoid organs of this clone. Nevertheless, highly shared clones represented only a minority of the expanded T-cells in spinal cord tissues, as most clones were unique in each animal (Fig. 4C). Thus, even the restricted T-cell repertoire in the spinal cord tissue of each mouse remained highly diverse in spite of inflammation induced by the same antigen.

4. Discussion

Previous studies with MBP immunization in B10.PL mice identified MBP specific T-cell clones and described a restricted T-cell repertoire with a bias towards TRBV13–2 usage (Acha-Orbea et al., 1988; Zamvil et al., 1986; Zamvil and Steinman, 1990). However, these studies relied on MBP driven in-vitro selection, and methods to confirm clonality of isolated T-cells differed between studies, with *Tcrb* sequences rarely being available for comparison. In addition, only a very small fraction of the T-cell repertoire was studied. In order to comprehensively analyze the T-cell repertoire in MBP immunized mice with introduction of minimal bias, we used high-throughput next-generation sequencing and made sequences accessible for comparison in a repository.

On the clonal level, two dominant shared clones (CDR3 amino acid sequences CASGDAGSQNTLYF and CASGDAGGGYEQY) were identified

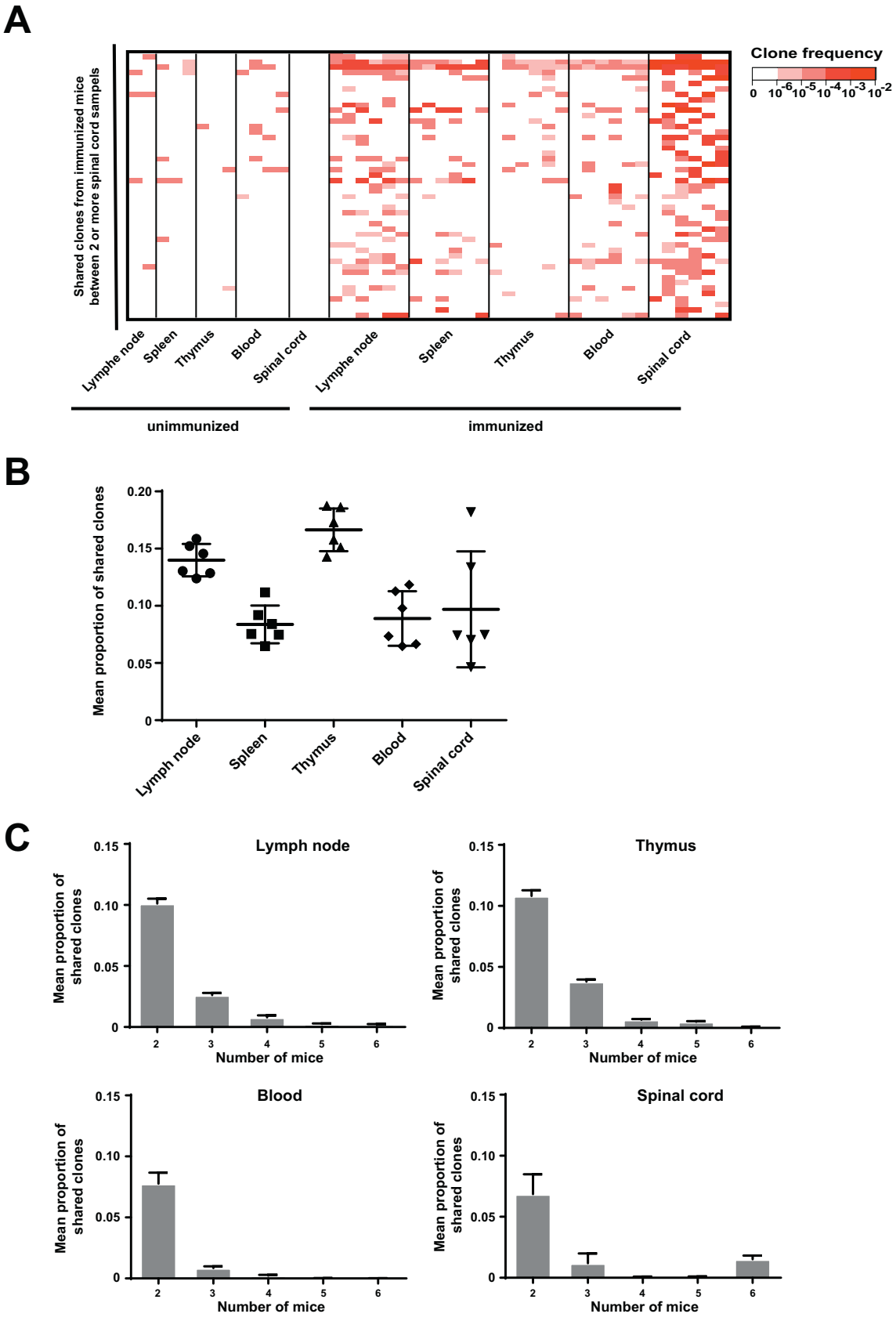

Fig. 3. Identification of shared T-cell clones in response to MBP immunization. (A) Heatmap of shared clones. After MBP immunization, clones found in two or more spinal cord samples were identified in the other indicated samples from unimmunized and immunized mice. Clonotype frequency is shown in a logarithmic scale. (B) Proportion of clones shared between two or more immunized mice in the indicated tissues. (C) Proportion of shared clones according to the number of immunized mice a clone could be identified in.

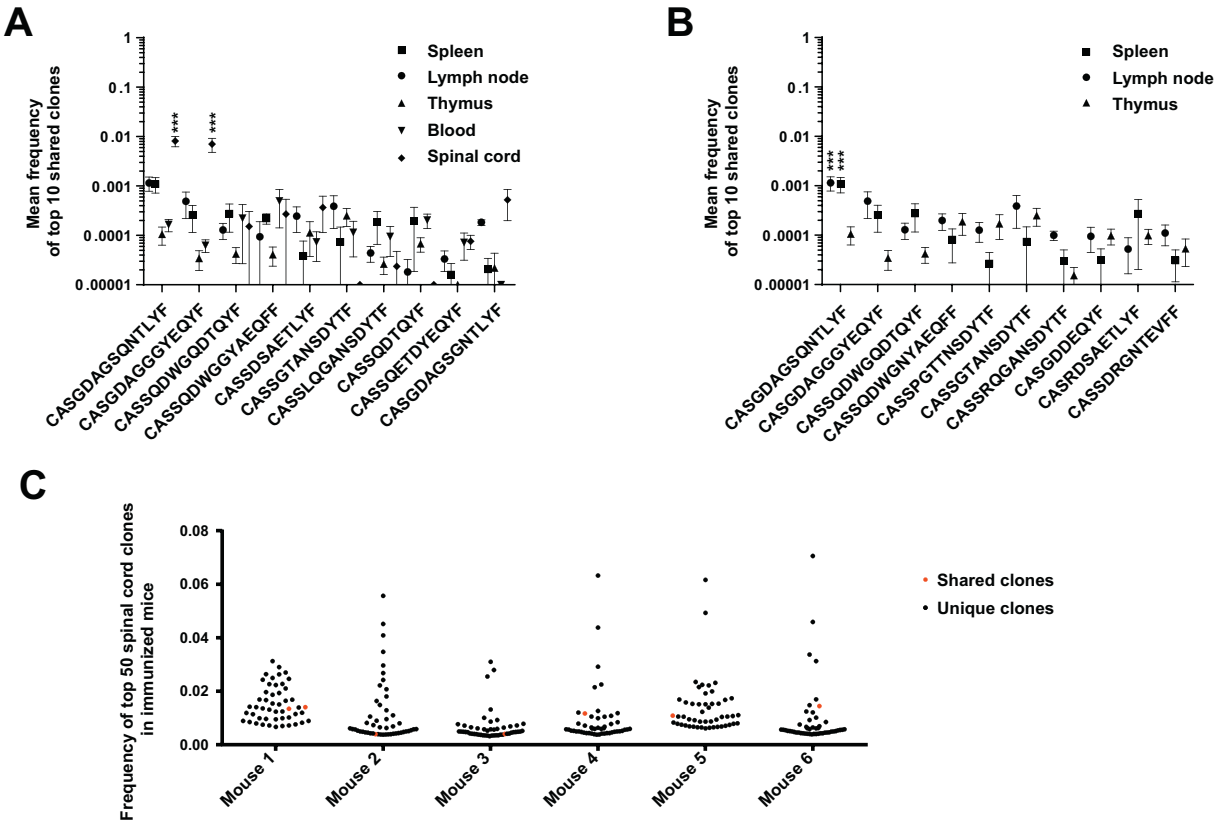

Fig. 4. Highly shared CDR3 amino acid sequences. (A) and (B) Comparison of the mean clone frequency (± SEM) of the top 10 shared CDR3 amino acid sequences after MBP immunization. (C) Frequencies of the top 50 clones of 6 different spinal cord samples from MBP immunized mice. Unique clones are shown in black, shared are indicated in red. ANOVA with Tukey's test for multiple comparisons (n = 6 per group). *P*-values are defined as * < 0.05, ** < 0.01 and *** < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the spinal cords of immunized animals, which could also be found in the lymph nodes and spleens, while being almost absent in the blood and in all other tissues. Most likely, these clones are MBP-specific and drive EAE, since one of the clones (CASGDAGGGYEQY) has previously been identified as a public sequence mediating EAE (Maynard et al., 2005). Clonal T-cells with the DAGGGY motif were isolated from immunized mice before and could transfer EAE (Menezes et al., 2007). TCR carrying this motif show a strong affinity to the MHC bound MBP peptide and TCR transgenic mice expressing that motif develop spontaneous EAE (Goverman et al., 1993; Huang et al., 2005). Nevertheless, we observed that most expanded spinal cord T-cells in immunized mice were unique to the individual animal and unique T-cells could not transfer EAE in previous experiments (Menezes et al., 2007). However, attempts to develop TCR antagonist peptides based on isolated public encephalitogenic T-cell clones proved to be difficult (Anderton, 2015; Anderton et al., 1998). In fact, other EAE mouse models using PLP or MOG for immunization show a more diverse T-cell repertoire after immunization, and in the MOG model diversity is a prerequisite for recurrence of EAE (Ben-Nun et al., 2006; Fazilleau et al., 2007; Kuchroo et al., 1994). Thus, it is conceivable that the failure of previous strategies directed against specific disease associated T-cell clones can be explained by a higher T-cell repertoire diversity than has been anticipated. This notion is in agreement with the low overlap we observed between spinal cord T-cell repertoires from different mice and also with recent studies showing large T-cell diversity in multiple sclerosis despite existence of public clones (Lossius et al., 2014; Planas et al., 2018).

In MBP-immunized mice, comparative analysis of V gene usage revealed a contracted blood T-cell repertoire due to depletion of TRBV13–2 rearranged T-cell clones and a relative increase of TRBV31 clones, while a significant overrepresentation of clones using TRBV13–2 was observed in spinal cords. Functional assays previously suggested that MBP-reactive cells are equally found in blood, secondary lymphatic tissues and the central nervous system (Hofstetter et al., 2005). However, the significant enrichment of TRBV13-2 usage in spinal cord T-cells and TRBV31 usage in blood T-cells suggests profound repertoire differences. T-cell clones with TRBV13-2 usage were previously described as pathogenic and anti-TRBV13-2 treatment can prevent MBP-induced EAE in B10.PL mice (Acha-Orbea et al., 1988; Fernández-Malavé and Stark-Aroeira, 2011; Urban et al., 1988). Structural studies suggest that TRBV13–2 usage is essential for MBP (p1-11) specificity of T-cells in B10.PL mice due to a high complementarity between TRBV13-2 and the peptide-MHC-I-A^u complex (Maynard et al., 2005). Moreover, the TRBV13-2 is unique, because it uses glycine instead of serine at position 107, which results in decreased affinity while preserving the TCR structure and specificity (Alli et al., 2011). Low but sufficient antigen affinity is an important feature of autoreactive T-cells, as it allows to escape negative selection (Koehli et al., 2014). In contrast to the spinal cord, a significant bias towards TRBV31 was observed in the blood of immunized mice. TRBV31 is known to be overrepresented among regulatory T-cells, mediating recovery from EAE (Kumar et al., 1996; Kumar et al., 1995; Madakamutil et al., 2008).

As we used MBP peptide emulsified in CFA for immunization, we cannot differentiate between T-cell clones recognizing MBP or a CFA component with certainty. However, we observed a T-cell spinal cord infiltration that is generally not observed in mice immunized with CFA only (Traugott, 1989). In addition, the observed bias of spinal cord T-cells towards TRBV13–2 usage is highly suggestive of cells recognizing the MBP derived peptide used for immunization (p1–9), as this is not

observed in mice immunized with CFA and alternative MBP derived peptides (Acha-Orbea et al., 1988).

In summary, we present a sequencing repository encompassing *Tcrb* sequences observed in MBP immunized B10.PL mice. Next-generation immunosequencing revealed a remarkable diversity in the repertoire despite also confirming the existence of public, disease-mediating T-cell clones shared between affected animals. Of note, one of the identified public clones (CASGDAGSQNTLYF) has not been described before. Comparative repertoire analysis revealed that besides the spinal cord, the blood repertoire is especially altered in immunized mice, while the T-cell repertoire in primary and secondary lymphoid tissues is relatively stable. Overall, this clearly indicates that in the MBP-induced EAE mouse model blood is no suitable material to study autoreactive T-cells.

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Disclosure of conflict of interest

The authors declare no potential conflicting interests.

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Authorship contributions

M.B. designed the study, supervised, analyzed and interpreted the experiments and wrote the manuscript. S.S. analyzed and interpreted the experiments and wrote the manuscript. A.C. and N.A designed and performed experiments. B.T. assisted with visualization of the NGS data. J.H. critically revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.03.014.

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