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Multiple sclerosis: T-cell receptor expression in distinct brain regions

Andreas Junker,[1,*] Jana Ivanidze,[1,2,*] Joachim Malotka,[2] Ingrid Eiglmeier,[1] Hans Lassmann,[3] Hartmut Wekerle,[2] Edgar Meinl,[1,2] Reinhard Hohlfeld[1,2] and Klaus Dornmair[1,2]

[1]Institute for Clinical Neuroimmunology, Ludwig Maximilians University, D-81377 Munich, [2]Department of Neuroimmunology, Max-Planck-Institute for Neurobiology, D-82152 Martinsried, Germany and [3]Center for Brain Research, Medical University of Vienna, A-1090 Vienna, Austria

*These authors contributed equally to this work.

Correspondence to: Klaus Dornmair or Reinhard Hohlfeld, Institute for Clinical Neuroimmunology, Maximilians University, D-81377 Munich, Germany
E-mail: dornmair@neuro.mpg.de;reinhard.hohlfeld@med.uni-muenchen.de

Multiple sclerosis (MS) is an inflammatory demyelinating disease where T cells attack the brain and the spinal cord. It is known that often particular T-cell clones are expanded in the target tissue, but it is still unknown, whether identical T-cell clones are present at distinct anatomical sites, or whether the T-cell spectrum is locally diverse. Therefore we compared the T-cell receptor (TCR) repertoire in distinct lesions and normal-appearing white matter (NAWM) from post-mortem brains of four MS patients. We analysed 19 lesions (inactive demyelinated, 15; slowly expanding chronic, 3; active lesions, 1) and 5 NAWM regions. The TCR β-chain repertoire was investigated by CDR3 spectratyping. For each anatomical site 325 semi-nested PCR reactions were performed. About 800 Vβ-NDN-Jβ combinations were sequenced. Each of the four patients had distinct T-cell clones that were present in more than two anatomically distinct regions. These clones were not restricted to lesions, but were also present in NAWM. Some clones were present in all investigated lesions, and additionally, in NAWM sites. A single T-cell clone was detected in nine different sites in one patient. None of the clones was shared among different patients. Thus, pervasive T-cell clones exist in distinct regions of MS brain, and these clones are 'private' (unique) to individual patients. Analysis of the hypervariable NDN region revealed 'silent' nucleotide exchanges, i.e. nucleotide exchanges that code for identical amino acids. Such silent nucleotide exchanges suggest that the corresponding T-cell clones were recruited and stimulated by particular antigens. To attribute some of the pervasive clones to particular T-cell subsets, we isolated individual CD8+ T cells from cryosections by laser microdissection and characterized their TCR by single-cell PCR. These experiments revealed that at least some of the pervasive T-cell clones belonged to the CD8+ compartment, supporting the pathogenic relevance of this T-cell subset.

Keywords: multiple sclerosis; T-cell receptor; autoimmune T cells; neuroimmunology; cytotoxic T-cell response

Abbreviations: CDR = complementarity determining region; NAWM = normal-appearing white matter; TCR = T-cell receptor

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Introduction

The pathogenic role of brain-invading autoreactive T-lymphocytes has been firmly established in experimental autoimmune encephalomyelitis, laying the foundation for current concepts of the pathogenesis of multiple sclerosis (MS) [reviewed by (Hohlfeld and Wekerle, 2004)]. There are two major approaches for investigating T-cell responses in MS patients. The first approach relies on specified candidate antigens that have shown encephalitogenic potential in animal models, and are subsequently investigated in MS patients. The second—complementary—approach does not depend on a priori assumptions about the nature of the target antigens, but instead focuses on the properties of the antigen-reactive T-cell receptors (TCR).

With few exceptions, each T-cell clone expresses only one type of TCR. The TCR is a heterodimer, consisting of an α- and a β-chain. Like antibodies, both chains are composed of various genetic elements and contain in addition a hypervariable region. This 'complementarity determining region 3' (CDR3), which plays a major role in antigen

Table 1 Synopsis of clinical features of the four MS patients as extracted from patient' files

Patient	Age (year)	Gender	Duration of MS (year)	Clinical type of MS	Immunosupressive or immunomodulatory therapy for MS	Medical history	Cause of death
MS-1	49	Female	28	Mild relapsing	Steroids as treatment for relapses; intravenous immunoglobulins in 2002 and 2003	Resection of a meningeoma 2 years before death	Unknown
MS-2	77	Female	50	Mild relapsing	No specific therapy for MS	No other diseases known	Left heart failure
MS-3	42	Male	6	Severe relapsing	Approx. one high dose steroid course per year	Asphyxia after aspiration, resuscitation, pulmonary embolism	Brain death after hypoxic brain damage
MS-4	42	Male	4	Primary progressive	No specific therapy for MS	Chronic pain syndrome	Unknown

recognition [reviewed by (Rudolph *et al.*, 2006)], is generated in the thymus by deletion and/or insertion of random nucleotides between germline genetic elements. The theoretical human TCR 'repertoire' comprises approximately 10^{15} different TCR molecules (Davis and Bjorkman, 1988). It was shown experimentally that an individual may have more than 2.5×10^7 different αβ-TCR molecules (Arstila *et al.*, 1999). Previous studies have shown that in MS brains both the TCR α- and β-chain repertoires of tissue-infiltrating T cells are 'skewed', suggesting that a limited number of T-cell clones participate in local immune reactions (Oksenberg *et al.*, 1990, 1993). Subsequently, it has become possible to extend the analysis of TCR repertoires down to the level of single tissue-infiltrating cells (Babbe *et al.*, 2000; Steinman, 2001; Skulina *et al.*, 2004).

By definition, the inflammatory process in MS is multifocal. Despite several previous studies on the TCR repertoire in MS, it is still not known whether or how the TCR repertoire varies between different lesions, and between lesions and NAWM. To address this question we used 'CDR3 spectratyping' (reviewed by Pannetier *et al.*, 1995). This PCR-based technique measures the lengths of the hypervariable TCR CDR3 regions and thus allows identification of deviations from the normal repertoire, which has a Gaussian length distribution. Individual 'expanded' T-cell clones have TCR chains with defined CDR3 lengths and thus may be identified as single peaks in the spectratypes. This method was previously used for TCR repertoire analysis of human blood and tissue samples, including blood and CSF of MS patients (Matsumoto *et al.*, 2003; Muraro *et al.*, 2003; Laplaud *et al.*, 2004). We applied CDR3 spectratyping to conduct a comprehensive survey of the interlesional and interindividual diversity of the TCR repertoire expressed in MS whole brain specimens.

Material and methods

Patients and tissue samples

Brain autopsy specimens from four MS patients were obtained from the Department of Forensic Medicine of the Ludwig-Maximilians University, Munich, Germany. There were two cases of mild relapsing MS (patients MS-1 and MS-2), one case of severe relapsing MS (patient MS-3), and one case of primary progressive MS (patient MS-4). Details are listed in Table 1. All specimens were characterized by genomic HLA typing at the Institute for Immunogenetics, LMU Munich, Germany (Table 2). Tissue blocks that contained either grossly visible lesions or normal appearing CNS tissue were dissected from anatomically distinct areas, snap frozen and stored at –80°C. In addition, we investigated three control brain autopsy specimens from none-MS cases: C-1, male, aged 49 years, cause of death, presumed heart attack; C-2, male, aged 48 years, cause of death, car accident; C-3 male, aged 49 years, cause of death, pneumonia. Furthermore, we used peripheral blood from a healthy volunteer to test the modified CDR3 spectratyping protocol. The study was approved by the Institutional Review Board of the Ludwig-Maximilians University, Munich and conducted according to the Declaration of Helsinki.

Table 2 HLA alleles expressed by the four MS patients as determined by genomic HLA-typing

Patient	HLA-				
	A*	B*	C*	DRBI*	DQBI*
MS-1	0201	4405	0202	04	0304
	2402	5101		15	0602
MS-2	0101	1501	0303	13	0603
	0201	1517	0701		0604
MS-3	0301	1501	0303	0101	0501
	3201	3501	0401	0103	
MS-4	0201	0702	0702	04	0302
	0301	5101	1502	15	0602

Histochemistry, immunohistochemistry

For histochemical analysis 10–30 µm-thick frozen serial sections were mounted onto glass slides. They were stained with luxol fast blue for myelin integrity, Oil Red O for neutral lipids and haematoxylin and eosin according to standard procedures.

For immunohistochemical staining we used 10 µm brain cryostat sections. They were stained with the monoclonal antibodies (mAb) clone PG111 (BD Pharmingen, Erembodegem, Belgium), specific for CD68 on macrophages, RPA-T4 (BD Pharmingen) and

Table 3 Anatomical location and histopathological characterization of the investigated tissue blocks

Patient code	Block designation	Anatomical location	Histopathology of lesion
MS-1	2	Periventricular	Inactive demyelinated
	13	Near cortex in white matter	NAWM with diffuse white matter injury
	14	Capsula interna	Inactive demyelinated
	16	Occipital white matter	Inactive demyelinated
	23	Frontal white matter	Inactive demyelinated
	26	Frontal white matter	Inactive demyelinated
	31	Temporal white matter	NAWM with diffuse white matter injury
	33	Cerebellum	NAWM with diffuse white matter injury
	10	Nucleus caudatus	Inactive demyelinated
MS-2	8	Parietal white matter with cortex	NAWM
	9	Temporal white matter	Slowly expanding chronic
	11	Optic nerve	Inactive demyelinated
	24	Periventricular	Inactive demyelinated
	20	Cerebellum	Inactive demyelinated
MS-3	3	Parietal white matter	Active
	8	Right parietal	Inactive demyelinated
	12	Parietal next to basal ganglia	Inactive demyelinated
	14	Periventricular	Slowly expanding chronic
	23	Periventricular	Inactive demyelinated
MS-4	5	Striatum	Inactive demyelinated
	7	Temporal white matter	Inactive demyelinated
	9	Temporal white matter	Inactive demyelinated
	12	Occipital white matter	NAWM
	6	Periventricular	Slowly expanding chronic

HIT8a specific for T-cell subsets CD4 and CD8. PG111 was diluted as recommended by the manufacturer. RPA-T4 and HIT8a were used at 6 μg/ml. Fab fragments of a rabbit anti-mouse (E413) antibody and standard phosphatase alkaline phosphatase procedures with DAB wre used as secondary reagents. Fab fragments of a rabbit anti-mouse (E413) antibody and standard peroxidase procedures with DAB were used as secondary reagents. All antibodies and reagents other than PG111 and RPA-T4 were from Dako, Hamburg, Germany.

Tissue blocks were classified according to defined criteria (Kutzelnigg *et al*., 2005) (Table 3): *Active lesions* contained abundant macrophages with early (luxol fast blue and Oil Red O positive) myelin degradation products either throughout the whole lesion or in a broad rim at the lesion edge (Brück *et al*., 1995). *Slowly expanding chronic plaques* revealed mild to moderate microglia activation at the lesion margins. Early myelin degradation products, however, were sparse and restricted to single macrophages of microglia cells (Barnett and Prineas, 2004). *Inactive demyelinated lesions* were sharply demarcated from the periplaque white matter and without a rim of microglia activation and no luxol fast blue or Oil Red O reactive myelin degradation products. *Normal appearing white matter* was defined as white matter devoid of demyelinated lesions.

The numbers of CD4+ and CD8+ T cells were determined on serial sections. Cells were counted at 200-fold magnification in 10 representative areas of 1 mm^2 each using 10×10 mm^2 indexing grids. From each lesion or NAWM region the numbers of CD4+ and CD8+ T cells per area were determined to ensure that sufficient cell numbers were prepared for the CDR3 spectratyping experiments. In all patients, control persons and at all sites, the numbers of CD8+ cells ranged from 50 to 80% of all T cells.

CDR3 spectratyping

Total RNA was extracted from every examined lesion using the TRIzol-LS reagent (GIBCO/BRL, Karlsruhe, Germany). To ensure sufficient statistics, we chose the sample size so that each sample from the MS patients contained at least 15 000 T cells. The samples from the healthy control subjects, however, contained only small numbers of parenchymal T cells. TCR β-chain-specific cDNA was synthesized using the primer 'Cβ−RT' (Seitz *et al*., 2006) and Superscript III reverse transcriptase according to the suppliers' recommendations. To enhance the sensitivity of the spectratyping protocol, we performed two rounds of semi-nested PCR reactions with identical forward primers and nested reverse primers. Thus, the cDNA was subjected to a first round of 30 PCR cycles in 25 separate reactions. PCR conditions were as described (Hofbauer *et al*., 2003; Skulina *et al*., 2004). Each reaction comprised one out of 25 Vβ-specific forward primer that hybridize to the V-regions (Monteiro *et al*., 1996) [Vβ10 was omitted because it is a pseudogene (Arden *et al*., 1995; Lefranc and Lefranc, 2001)]. As Cβ-specific reverse primer for the first round of PCR the primer 'SpTy-β-out' (5′-ACACCAGTGTGGCCTTTTGG) was used. For the second 30 cycles we used the same forward primers, but the Cβ-reverse primer described by Monteiro *et al*. (1996) which hybridizes upstream of SpTy-β-out. Each of the 25 PCR products was then subjected to 13 individual 'run-off' reactions (Pannetier *et al*., 1995), with 13 fluorescence-tagged Jβ-specific primers (Puisieux *et al*., 1994) to differentiate individual TCR Jβ-regions. The lengths of the run-off products were analysed with an ABI377 DNA sequencer (Applied Biosystems, Darmstadt, Germany). Apparently monoclonal T-cell expansions yield single peaks of defined lengths, according to the specific characteristics of the hypervariable NDN-regions. However, a single peak is not sufficient for assuming monoclonality, because two or more

different NDN regions might accidentally have identical lengths. To prove the monoclonality of candidate clones, the semi-nested Vβ-Cβ PCR products were amplified using non-tagged Jβ-specific primers and the amplification products were sequenced. Only if a readable sequence was obtained, the clone was considered truly monoclonal.

Laser microdissection and single-cell PCR analysis

For laser microdissection, 10 µm cryostat sections from tissue specimens of patient MS-1 were double-stained by Cy3-labelled anti-CD8 antibodies and Alexa-488 labelled anti Vβ-antibodies. All aqueous solutions were previously inactivated by DEPC and contained 1 U/µl Protector RNase inhibitor (Roche, Mannheim, Germany) and purified BSA (B4287, Sigma, Deisenhofen, Germany). Sections were mounted on PET-films (P.A.L.M Microlaser, Bernried, Germany) that had been previously baked at 180°C for 4 h, UV-irradiated and coated with poly-L-lysine. Mounted sections were stored at –80°C. After thawing, rehydration in phosphate-buffered saline (PBS) for 10 s, and blocking by PBS with 2% BSA for 3 min, the sections were co-incubated for 5 min with the anti-CD8 antibody LT8 (Serotec, Düsseldorf, Germany), which had been previously labelled with the Cy3-mAb Labeling Kit (GE/Amersham, Freiburg, Germany) and with the FITC-labelled anti-TCR β-chain antibodies anti-Vβ5.1: clone IMMU157 and anti-Vβ5.2: clone 36213 (both Beckman/Coulter, Krefeld, Germany). All antibodies were diluted 1:25. After the sections were rinsed with 2 ml of PBS, they were incubated for 3 min with the 1:100 diluted Alexa-488-labelled polyclonal goat anti-FITC antibody preparation A11096 (MolecularProbes/Invitrogen, Karlsruhe, Germany) to increase the fluorescence signal. The tissue was rinsed with PBS, covered with 1-propanol to prevent drying and to inhibit RNAse activity, and immediately transferred to a P.A.L.M Microbeam-Z microscope. The specimens were examined within 10 min for CD8- and Vβ-double positive cells. The double positive cells were marked electronically. After evaporation of the 1-propanol each marked cell was microdissected and laser pressure catapulted into single reaction tubes, which were immediately stored on dry ice.

Single cells were analysed for expression of the expanded TCR β-chains by RT-PCR. The RT-reaction and two cycles of double-nested RT PCR were performed essentially as described (Seitz *et al.*, 2006), but all TCR α-chain-specific primers were omitted. For patient MS-1 the following outer primers were used: BV5.1u.2-for-out: 5′-GATCAAAACGAGAGGACAGC; together with a combination of 403-5.1-2.7-rev-out: 5′-CGAAGTACTGCTCGTATACC and 403-5.2-1.1-rev-out:5′-GTGCCTTGTCCAAAGAAAGC. The combinations of BV5.1.1-for-in: 5′-TTCCCTGGTCGATTCTCAGG, BV5.1.2-for-in: 5′-TTCCTTGGTCGATTCTCAGG and BV5.2-for-in: 5′-TTCCCTGATCGATTCTCAGG, with 403-5.1-2.7-rev-in: 5′-TACCCTGTCCCATTCCAAGC, and 403-5.2-1.1-rev-in: 5′-AAGCTTCAGTGTACCTGTCG were used as inner primers. The obtained PCR products were sequenced to confirm the correct sequences.

Results

Screening of the TCR β-chain expression by CDR3 spectratyping

A total of 19 distinct lesional sites and 5 NAWM sites from whole-brain specimens of four MS patients were investigated. For comprehensive analysis of the TCR repertoire, cDNA from each site was prepared and the CDR3 spectratyping technique applied to determine the length distribution of the hypervariable CDR3 regions of the TCR β-chains (Pannetier *et al.*, 1995). For each anatomical site, 25 Vβ-forward primers were combined in individual reactions with each of 13 Jβ-reverse primers, which resulted in 325 PCR products. Thus, a total of $24 \times 325 = 7800$ individual PCR products were analysed. Each of the products was run on high resolution polyacrylamide gels to separate the nucleotide fragments according to their lengths. Polyclonal populations yield Gaussian length distributions, whereas oligo- or monoclonal populations yield distinct single peaks characteristic for a highly skewed TCR repertoire (Pannetier *et al.*, 1995). To demonstrate that our modified, more sensitive spectratyping protocol does not introduce any skewings, we analysed peripheral blood cells from a healthy subject. As expected, we found polyclonal (Gauss-distributed) populations in all Vβ–Jβ combinations in this sample (data not shown).

Our analysis of the MS patients focused on detection of defined clones, which are characterized by clearly identifiable, distinct spectratyping peaks, that yielded a readable nucleotide sequence and occurred in at least two distinct anatomical sites. A total of 790 candidate clonal expansions were sequenced from the four patients. This approach allows detection of TCR repertoire skewings and identification of individual T-cell clones at different sites of each individual brain.

Regional distribution of TCR repertoires in MS whole brain specimens

Figures 1 to 4 give an overview of the regional variation of the TCR repertoire. All spectratyping peaks occurring at two or more anatomical sites are presented for each patient. In all figures, black areas indicate confirmed monoclonal expansions. Light grey areas indicate oligoclonal Vβ-Jβ combinations. Figure 1 illustrates the distribution of distinct T-cell clones observed at different sites in whole brain of patient MS-1, who had a large number of lesions. Six distinct lesions and three regions of NAWM were investigated. Eleven T-cell clones were detected at 2 sites; 8 at 3 sites; 3 at 4 sites; 1 at 5 sites; 2 at 6 sites; 2 at 7 sites; 2 at 8 sites; and one at all 9 investigated sites. It is important to note that the TCR Vβ–Jβ combinations shown in black represent clonal expansions, the TCR β-chain sequences of which were identified by direct sequencing. It is possible, however, that the same clones are also present in other lesions where oligo- or polyclonal populations were observed. In such areas (indicated in grey) the relevant β-chains might be obscured by other chains with identical Vβ- and Jβ-regions but different NDN sequences. Therefore, the numbers of expansions given here are low estimates. There was no obvious bias towards any particular

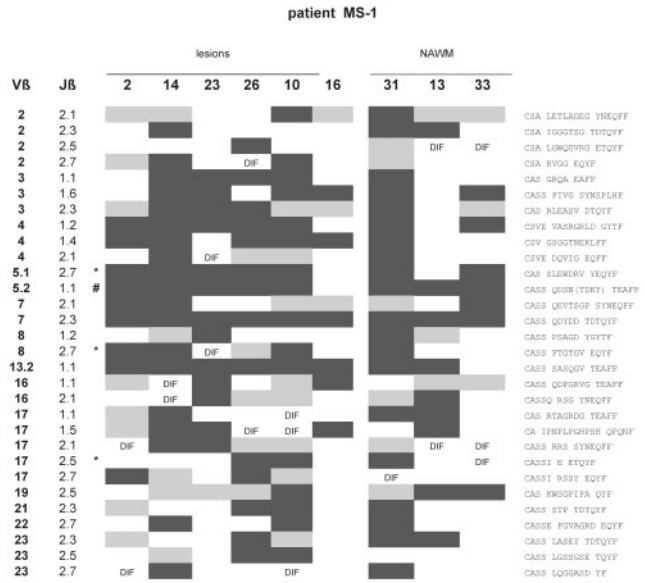

Fig. 1 Distinct T-cell clones in lesions and NAWM of patient MS-1 as detected by CDR3 spectratyping of the TCR β-chains. The Vβ–Jβ combinations that revealed monoclonal expansions for the lesions and NAWM regions are listed (see Table 2 for anatomical and pathophysiological details). For each individual lesion or NAWM region 325 Vβ–Jβ combinations were investigated. A particular Vβ–Jβ combination was considered monoclonal only if CDR3 spectratyping revealed a single peak that yielded a readable nucleotide sequence. Monoclonal expansions that were found at two or more anatomical sites are shown in black. Monoclonal Vβ–Jβ combinations found only in one site are not shown. Light-grey fields indicate that in the particular Vβ–Jβ combination an oligo- or polyclonal population was detected. Oligoclonality was inferred either because several peaks appeared in the spectratype, or because the spectratypes revealed single peaks, which by sequencing turned out to be composed of different clones with identical CDR3 region lengths. Here a clone of interest may be present, but hidden by other clones. A white field indicated that a PCR product was not obtained in the spectratyping reaction. In some Vβ–Jβ combinations we identified readable NDN-sequences that were different from the sequences of the pervasive clone. This is indicated by the term 'DIF' at the respective site. An asterisk (*) at the Vβ–Jβ combinations Vβ5.1–Jβ2.7, Vβ8–Jβ2.7 and Vβ17–Jβ2.5 denotes that silent nucleotide exchanges were detected in the *N*-nucleotides of the CDR3-regions. The symbol # denotes that two independent NDN sequences with identical CDR3 lengths were detected together with identical Vβ- and Jβ-elements. One of the two sequences of the Vβ5.2–Jβ1.1-combination is listed in brackets. In the last column, the amino acid sequences of the V–NDN–J transition are listed in single letter code. All sequences start at the conserved cysteine of the V-region and end with the conserved phenylalanine of the J-element. V-, NDN- and J-regions are separated by a blank.

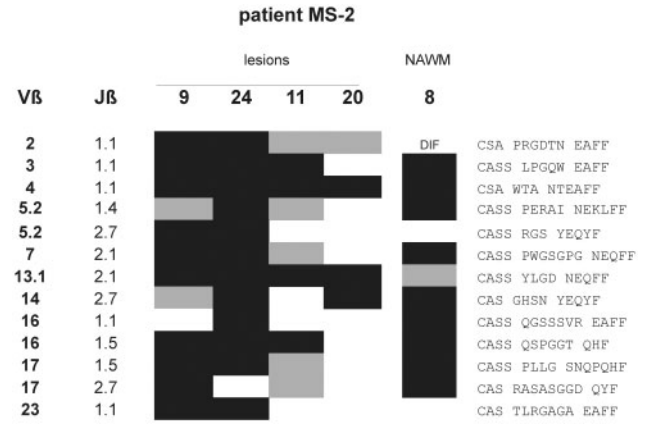

Fig. 2 Distinct T-cell clones in lesions and NAWM of patient MS-2. See Fig. 1 for details.

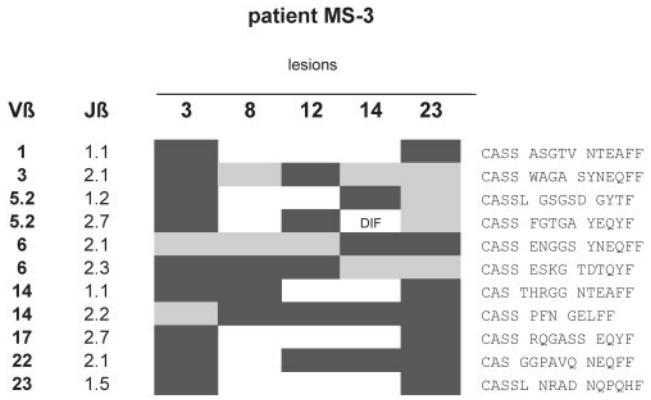

Fig. 3 Distinct T-cell clones in active and inactive lesions of patient MS-3. The active lesion is designated 'block 3'. See Fig. 1 for details.

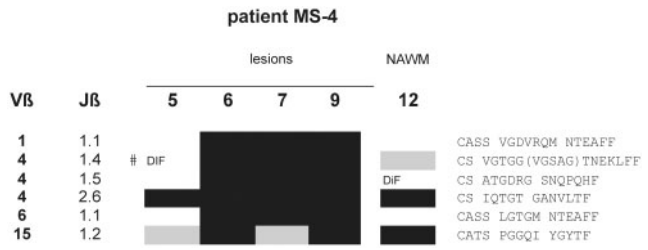

Fig. 4 Distinct T-cell clones in lesions and NAWM of patient MS-4. See Fig. 1 for details.

anatomical or functional brain region. Thus, a Vβ7–Jβ2.3-clone was detected in all sites, and the clones Vβ5.1–Jβ2.7 and Vβ13.2–Jβ1.1 were detected in all sites but one. A number of other clones were detected at lower frequencies in lesions or in NAWM. Interestingly, there seemed to be no striking difference in the TCR repertoires of lesions as compared to NAWM. In lesions and NAWM, there were sites that contained many pervasive T cells, e.g. lesion 14 and NAWM 31, and sites that contained a few different T cells, e.g. lesion 2 or 16 and NAWM 13 or 33.

The other patients exhibited very similar patterns (Figs 2–4). Notably, the TCR repertoires expressed in different types of lesions—inactive demyelinated, slowly expanding chronic and active—and NAWM sites were remarkably similar in that several identical T-cell clones pervaded distinct regions.

In control brains, we observed by immunohistological analysis scattered CD4+ and CD8+ T cells at perivascular

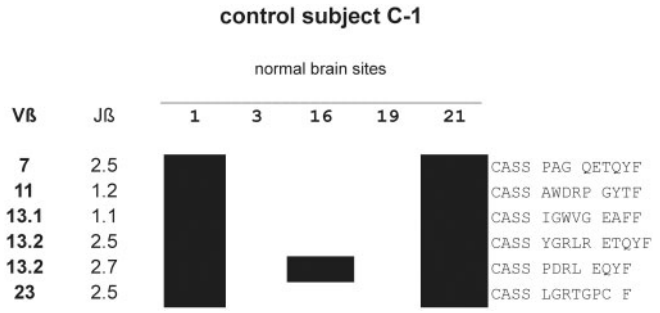

Fig. 5 Distinct T-cell clones in lesions and NAWM of control subject C-1. See Fig. 1 for details.

and deep parenchymal sites. CDR3 spectratype analysis revealed several distinct peaks, some of which occurred at several sites (e.g. control subject C-1, Fig. 5).

Inter-individual comparison of TCR repertoires in whole brain specimens of different patients

Comparison of the patterns of expanded clones and the CDR3 sequences obtained from all four MS brains revealed that not a single TCR sequence was shared among different patients (Figs. 1–4). Thus, the TCR repertoires were completely 'private' to each patient, although several HLA class I and class II alleles were shared among the patients (Table 2). From a technical perspective, the complete absence of inter-individually shared sequences rules out that PCR contaminations gave rise to the occurrence of identical clones at different sites.

A search of the non-redundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), which contains many published TCR sequences, including TCR chains from many anti-myelin T cells, and a collection of sequences from TCRs recognizing viral epitopes (Daniel C. Douek, personal communication) revealed that none of the TCR sequences identified in the MS brains were present in the databases.

Silent mutations in the TCR CDR3 regions

Some of the Vβ-Jβ-PCR products contained silent nucleotide exchanges coding for identical protein sequences. An example from patient MS-1 is shown in Fig. 6. Single peaks were detected by CDR3-spectratyping of lesions 2, 23 and 10. The electropherograms obtained by sequencing of the Vβ5.1-Jβ2.7 PCR products are shown. The positions of interest are indicated by red and green arrows. In addition, the relevant nucleotide triplets and the corresponding amino acids are shown. Two independent clones were found in lesions 2 and 23. In lesion 10 both sequences were superimposed. Strikingly, all sequences from the different lesions coded for identical amino acids. These clones also occurred in lesions 14 and 26, and in NAWM regions

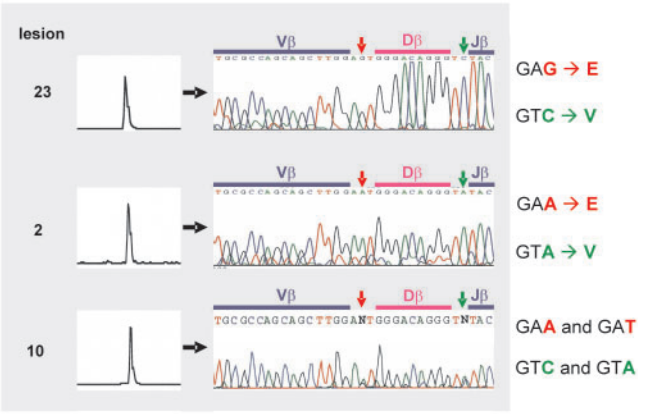

Fig. 6 Silent nucleotide exchanges in different Vβ5.1–Dβ1–Jβ2.7 T-cell clones of patient MS-1. CDR3 spectratyping of lesions 23, 2 and 10 revealed single peaks suggesting strong clonal expansions. The PCR products were sequenced to confirm that they were monoclonal and to determine the sequences of the NDN regions. The electropherograms are shown and the Vβ-, Dβ- and Jβ elements are indicated by bars. The nucleotide sequences are identical except for two nucleotides indicated by red and green arrows. Both exchanges are located in the N regions. The respective nucleotide-triplets code for the same amino acids: in lesion 23 the triplets read GAG and GTC, whereas in lesion 2 they read GAA and GTA, respectively. GAG and GAA both code for glutamic acid (E), and GAA and GTA both code for valine (V). In lesion 10 we observed two superimposed sequences at these positions, coding for GAA or GAG, and for GTC or GTA, respectively.

31 and 33 (Fig. 1). Comparable silent nucleotide substitutions were also detected with the combinations Vβ8–Jβ2.7 and Vβ17–Jβ2.5 in patient MS-1. The presence of silent mutations suggests antigen-driven stimulation of brain-infiltrating T cells.

Laser microdissection and single-cell PCR analysis of tissue-infiltrating T cells

For morphological identification of *in situ* expanded, tissue-infiltrating T cells, lesions were double-stained with Cy3-labelled anti-CD8 antibody and Alexa-488-labelled anti-TCR Vβ-antibodies. Single double-positive T cells were then isolated by laser microdissection, and single-cell PCR was used to assess whether the microdissected T cells expressed a CDR3 region sequence that had been identified previously by CDR3 spectratyping. Fig. 7A shows a cluster of CD8+ Vβ5.2+ T cells identified in and microdissected from lesion 14 of patient MS-1. From such stained cryosections single cells were isolated by laser-microdissection and their CDR3 nucleotide sequences were determined by single-cell PCR. In Fig. 7B, the CDR3 nucleotide sequences obtained by spectratyping are compared to sequences identified in single cells. By CDR3 spectratyping two distinct Vβ5.2–Dβ1–Jβ1.1 clones using different N-nucleotides were identified: one clone used an NDN region that coded for the amino acid sequence QDSN

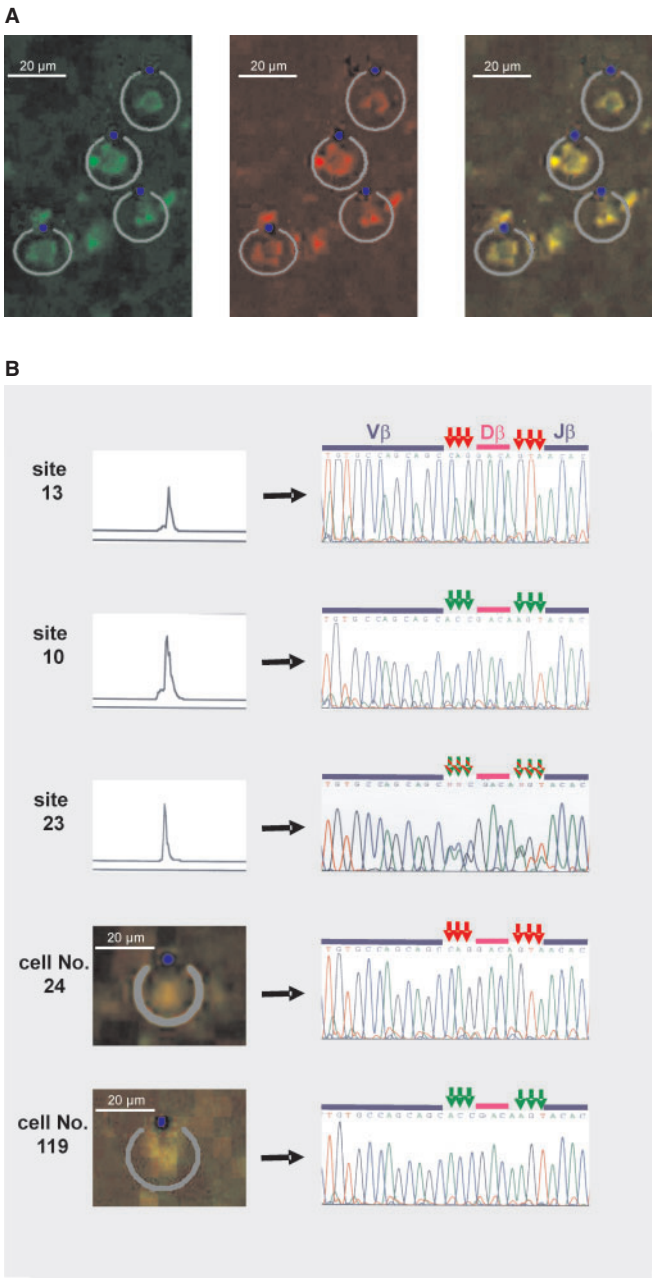

Fig. 7 Immunolocalization and single-cell analysis of T cells in the brain parenchyma of patient MS-1. (**A**) Immunolocalization of CD8 and Vβ5 double-positive T cells. Cryosections were stained with a Cy-3-labelled anti-CD8 antibody (red) and with Alexa-488-labelled anti-Vβ-5.1 and -5.2 antibodies (green). On the right side is an overlay of both pictures that provides evidence that the cells are double-positive. The circles around the cells indicate that these cells were isolated by laser microdissection and later analysed by single-cell PCR. (**B**) Comparison of results from CDR3 spectratyping and from single-cell analysis. We show the Vβ5.2–Jβ1.1 spectratype analysis from three different sites (NAWM 13, lesions 10 and 23), and two of the cells isolated by laser microdissection together with the corresponding electropherograms and nucleotide sequences of the TCR β-chains. The germline coded sequences of the Vβ5.2-, Dβ1- and Jβ1.1-elements are indicated by bars. In the NAWM region 13 we found an NDN-region sequence that coded for the amino acids QDSN, and in lesion 10 we identified an NDN-region that coded for TDKY. The *N*-nucleotides responsible for the differences are indicated by red (site 13) and green arrows (site 10). In lesion 23 we found a superimposition of both sequences, indicating that both clones were present (hatched arrows). In the lower two panels we show the single cells no. 24 and 119, which were double-stained with an Cy3-labelled anti-CD8 antibody and Alexa-488 anti-Vβ5 antibodies, laser-microdissected and subjected to single-cell PCR using Vβ5 forward and Jβ1.1 reverse primers. Cell no. 24 revealed a nucleotide sequence coding for the amino acid sequence QDSN and cell no. 119 revealed a sequence coding for TDKY. These sequences are identical to the sequences identified by CDR3 spectratyping in sites 13 and 10, respectively.

(lesion 13), whereas the other coded for TDKY (NAWM region 10) (Fig. 1). In lesions 2, 14, 23 and 26, and in NAWM regions 31 and 33, spectratype analysis revealed superimposed sequences, indicating that both clones co-existed in these lesions. One such example from lesion 23 is shown in Fig. 7B. Sixty-four individual CD8+ Vβ5+ cells were then isolated from lesion 14. In two microdissected cells, cells no. 24 and 29, the sequence coding for QDSN was identified, and in cell no. 119 the sequence coding for TDKY. This experiment shows that the results of CDR3 spectratyping and single-cell analysis are consistent, and that the investigated Vβ5.2 clones belong to the CD8+ population.

Discussion

The main findings of our comprehensive survey of the TCR β-chain repertoire in four MS whole brain specimens are as follows: (a) identical T-cell clones were detected in separate brain regions, including NAWM, although the TCR repertoire in each brain was oligoclonally diverse; (b) some spectratyping-defined TCR sequences identified in individual tissue-infiltrating T cells were CD8+ by immunohistochemical staining; (c) several TCR sequences contained silent mutations in the CDR3 region, suggesting antigen-driven recruitment and (d) the TCR repertoire was strictly private to each patient, although some frequently occurring HLA class I and class II alleles were shared among patients.

Similarity of TCR repertoires in lesions and NAWM

We found a very similar pattern of regional distribution and variation of the expanded T-cell clones in all the four investigated brains. All cases had clones that (a) pervaded the entire brain, (b) were detectable in some but not all of the investigated sites and (c) occurred in only one site. This heterogeneity in clonal spatial distribution might be related to technical reasons. For example, the sensitivity of the PCR protocol might depend on a certain threshold of cell numbers reached at one site, but not at others. Alternatively, it might be an intrinsic feature of the TCR repertoire of the infiltrating T cells. For example,

there might be a hierarchy of disease-inducing clones including driver, regulator and bystander clones (Cohen, 1992; Sercarz, 2000). Notably, there was no obvious difference between the repertoire patterns observed in the 15 inactive demyelinated, 3 slowly expanding chronic, 1 active lesion, and the 5 NAWM sites. Several dominant clones were observed at many different locations and types of sites, suggesting that part of the T-cell response was clearly common to the entire brain and not restricted to particular lesions or lesion types.

Diffuse infiltration of the so-called 'NAWM' has been previously reported to be a characteristic feature of chronic MS (Kutzelnigg *et al.*, 2005). Injury of the NAWM, reflected by diffuse axonal injury with profound microglia activation, occurred on the background of a global inflammatory response in the whole brain and meninges, suggesting that with chronicity diffuse inflammation accumulates throughout the whole brain (Kutzelnigg *et al.*, 2005). Our present results suggest that in chronic stages of MS, similar antigens are recognized by tissue-infiltrating T cells in lesions and so-called NAWM. By comparison, our control specimens contained small numbers of T cells that were detectable by CDR3 spectratyping peaks, some of which were present at different sites. This is consistent with the present concepts of immune surveillance of the healthy CNS (Wekerle *et al.*, 1986; Hickey, 2001; Kivisäkk *et al.*, 2003). According to these concepts, the CNS is constantly patrolled by T cells activated outside the CNS, for example during infections.

Several previous studies have examined the TCR repertoires in MS lesions. Oksenberg and colleagues (Oksenberg *et al.*, 1990, 1993) were the first to investigate the α- and β-chain repertoires expressed in MS brain. Wucherpfennig and colleagues (Wucherpfennig *et al.*, 1992) analysed the TCR repertoire on the level of TCR α- and β-chain V-regions, but did not identify particular clones. At this level of analysis they noted differences between different plaques. This does not contradict our findings, because we also noted variability between sites. However, Wucherpfennig and colleagues did not track individual clones, because they did not resolve explicitly the nucleotide sequences of the hypervariable CDR3 regions.

CNS versus blood and CSF

We could not compare the TCR repertoires expressed in the target organ with those in peripheral immune compartments, because no concomitant blood or CSF samples were available from any of the patients whose brains we studied. However, we know from a previous study that some brain-infiltrating, expanded T-cell clones, which were initially identified in a biopsy of an MS brain lesion, could also be detected in the blood and CSF of the same patient, in some instances more than 5 years after the biopsy (Skulina *et al.*, 2004). Thus, the blood TCR repertoire partly reflects the repertoire in the CNS. This is indirectly supported by a series of studies which revealed highly skewed TCR repertoires in blood or CSF (Matsumoto *et al.*, 2003; Laplaud *et al.*, 2004). They indicated that changes in the TCR repertoires of CSF and blood may be linked to disease activity (Muraro *et al.*, 2006; Laplaud *et al.*, 2006). All in all, these observations support the concept that there is continuous recruitment of immune cells from the periphery into the target organ during the course of disease. This provides the conceptual basis for therapeutic inhibition of the migration of immune cells, notably T cells, across the blood–brain barrier (Steinman, 2005).

Inter-individual comparison; public clonotypes

None of the TCR sequences were shared between patients. T cells recognize peptide antigens only when bound to autologous HLA class I (for CD8+ T cells) or HLA class II molecules (for CD4+ T cells). However, HLA typing of each MS brain revealed that several HLA class I and class II molecules were shared among patients. For example, patients MS-1 and MS-4 were typed positive for the alleles HLA-A*0201, -B*5101, -DRB1*04, and the MS linked alleles -DRB1*15, and -DQB1*0602 (Olerup and Hillert, 1991) (Table 2). Hence, in principle, different patients could use the same TCR β-chains to recognize the same target antigen/HLA combination. Indeed, such 'public' clonotypes have been described for various human T-cell responses, mostly directed against viral epitopes [reviewed in (Turner *et al.*, 2006)]. Our analysis, however, revealed that the TCR repertoire identified in each MS brain was strictly private. Despite several matching HLA alleles among several patients, they did not share a single TCR β-chain.

We checked all identified TCR β-chain sequences against the non-redundant sequence databases, and against a sequence database containing sequences of TCRs specific for different viral antigens. There were no hits, suggesting that the dominant T-cell responses in the investigated MS brains did not include any known public clonotypes. In particular, we could not identify any matches to known anti-myelin-basic-protein clones (Wucherpfennig *et al.*, 1990; Giegerich *et al.*, 1992; Meinl *et al.*, 1993; Wucherpfennig *et al.*, 1994; Muraro *et al.*, 1997). Interestingly, although we could not identify any published hypervariable CDR3 sequences, we found expanded Vβ5.2 sequences in three out of four patients: in each patient (MS-1, MS-2 and MS-3) two Vβ5.2-positive T-cell clones were observed (Figs. 1–4). The clones from patient MS-1 were CD8+, as shown by microdissection and single-cell PCR (Fig. 7). Expansions of Vβ5.2+ T-cell clones were described earlier in MS brain, CSF and blood (Oksenberg *et al.*, 1993; Matsumoto *et al.*, 2003), although Vβ5.2 is not particularly prominent in healthy individuals (Grunewald *et al.*, 1991). Based on these observations, Vβ5.2 T cells were targeted in pilot clinical trials (reviewed by Hohlfeld and Wekerle, 2004).

Silent mutations in the CDR3 regions

Interestingly, the CDR3 regions of several TCR β-chains contained 'silent' mutations, i.e. changes of the nucleotide sequences, which do not change the encoded amino acid sequences. Such silent mutations were previously observed in the CDR3 regions of TCR β-chains from encephalitogenic T cells in experimental autoimmune encephalomyelitis, the rodent model of MS (Acha-Orbea *et al.*, 1988; Chluba *et al.*, 1989; Zhang and Heber-Katz, 1992), and more recently in the repertoire of brain-infiltrating T cells in MS (Babbe *et al.*, 2000). If silent mutations occur in the CDR3 region, they can provide tale-tell evidence that the corresponding T cells were recruited and stimulated by the same antigen. The reason for this conclusion is that the T-cell repertoire, unlike the B cell receptor repertoire, is shaped in the thymus, and does not mutate during peripheral immune reactions. Therefore, the presence of silent mutations in the TCR CDR3 region proves that these T cells are not merely bystanders or random travellers, but that they selectively accumulate in MS brain, because they recognize a locally presented target antigen.

CD4+ versus CD8+ T cells

MS tissue lesions are known to contain both CD8+ and CD4+ T lymphocytes. It is also known that there is a preponderance of CD8+ cells [reviewed by (Friese and Fugger, 2005)]. Furthermore, CD8+ T cells were shown to be encephalitogenic in EAE [reviewed by (Steinman, 2001; Gold *et al.*, 2006; Ji and Goverman, 2007)]. Consistent with these earlier observations, the CD8+ T cells contributed to more than 50% to the total number of T cells in our tissue samples. It should be noted that the CDR3 spectratyping profiles do not allow distinction between the repertoires of CD4+ and CD8+ αβ-T-cells. In order to relate spectratyping-defined TCR sequences to individual morphologically identifiable cells, one needs to apply single-cell PCR technology (Babbe *et al.*, 2000; Dornmair *et al.*, 2003; Seitz *et al.*, 2006). For technical reasons, this demanding method could only be applied to a small 'spot sample' of cells. First, we selected candidate CDR3 spectratype peaks that predominated in a given tissue sample and belonged to a Vβ-family that could be stained with one of the available anti-TCR Vβ-mAb. Next, we double-immunostained the tissue with the relevant anti-Vβ mAb and with an anti-CD8+ mAb. We then excised the double-stained T cells by laser-microdissection, and analysed each cell by single-cell PCR. In this way we could unequivocally relate three cells of the expanded Vβ5-positive populations to the CD8+ tissue-infiltrating T-cell population. These findings provide proof of principle that the spectratyping-defined TCR sequences belong to tissue-infiltrating T cells, and that at least some of the expanded sequences belong to CD8+ T cells. However, this does not rule out the possibility that some of the reported sequences belong to CD4+ instead of CD8+ cells.

Functional relevance and perspectives for identification of target antigens

The question arises as to the functional role of tissue-infiltrating T cells in MS brain. First of all, it is important to note that each whole brain specimen can provide only a 'snapshot' of the TCR repertoire in the CNS. Although we know from independent earlier studies that CNS infiltrating T cells can persist for many years in blood and CNS in individual patients (Skulina *et al.*, 2004), we have no means to identify the chronicity of the T cell infiltrates of the autopsy cases studied here. Second, we cannot exclude the possibility that some of the infiltrating T cells have nothing to do with the MS inflammatory process, but are part of the ongoing physiological surveillance of the CNS (Wekerle *et al.*, 1986; Hickey, 2001; Kivisäkk *et al.*, 2003). Third, some T cells in lesions might represent bystander cells which were non-specifically attracted by the inflammatory process. In contrast to other diseases, e.g. polymyositis and inclusion body myositis, where it is possible to identify autoaggressive CD8+ T cells by morphological criteria (Goebels *et al.*, 1996), this is not possible in MS tissue owing to the dense packing of many different cell types in brain parenchyma. It appears likely, however, that the relevant, pathogenic T-cell clones are the ones that are expanded *in situ* and carry silent nucleotide exchanges in their TCR, whereas irrelevant bystander cells should neither be expanded nor carry such TCR mutations.

In principle, both CD4+ and CD8+ T cells could act as (pathogenic) effector cells, or (potentially beneficial) regulatory or 'suppressor' cells. At this point we can only speculate about their function. However, two arguments favour the possibility that at least some of these cells act as effectors rather than regulators. First, cytotoxic mediators like granzyme B have been found to be upregulated in MS lesions (Neumann *et al.*, 2002). Second, Foxp3, a tentative marker of CD4+ regulatory T cells, seems to be virtually absent from MS tissue (H. Lassmann, unpublished). However, these indirect arguments by no means exclude the possibility that some of the infiltrating T cells have as yet undefined regulatory functions. To address this question, it will be necessary to co-localize the expanded TCR together with cytokines, cytotoxic effector molecules, and relevant transcription factors, such as Foxp3, in individual T cells. This will be a major technical challenge for the future, because it requires a combination of microdissection, single-cell PCR, multi-colour immunocytochemistry and possibly also *in situ* hybridization techniques.

Clearly, the most challenging open question relates to the nature of the target antigens recognized by the oligoclonal, pervasive T cells identified by our study. To approach this problem, several consecutive steps are required, as reviewed by Dornmair *et al.* (2003). First, as was done in the present study, the TCR repertoire needs to be carefully analysed to identify expanded clones. Second, additional information must be gathered, to distinguish between pathogenic,

regulatory and irrelevant cells (see previous section). Third, matching pairs of TCR α- and β-chains need to be identified in and isolated from individual T cells, and fourth, the TCR α- and β-chains need to be expressed in a recombinant system, e.g. an immortalized T hybridoma cell line. The 'revived' T cells can then be used to investigate the antigens, e.g. by screening peptide or cDNA libraries (Van den Eynde *et al.*, 1995; Hemmer *et al.*, 1997, 1999; Wong *et al.*, 1999). We have recently for the first time succeeded in identifying several αβ-TCR pairs from muscle biopsy samples of patients with myositis (Seitz *et al.*, 2006). However, particularly the identification of the TCR α-chains from microdissected single T cells and the subsequent search for target antigens still remain significant technical challenges. Nevertheless, the repertoire study conducted here provides a platform for further experiments that may reveal the matching β-chains, and eventually will help move towards identification of the antigenic specificity of the T cell infiltrates in MS lesions.

In conclusion, our results show that the TCR expression in MS brain is diverse, but dominated by a limited number of clones, that is, the T-cell response is 'oligoclonal'. Notably, expanded T-cell clones can be found in the entire brain. They are present in different types of lesions, and remarkably, also in the NAWM. Our findings have two implications. First, as regards the immunopathogenesis of MS, our results suggest (a) that at least part of the pervasive T-cell clones belong to the CD8+ compartment, and (b) that some of these T cells respond to a common antigen or to common antigens. Second, as regards the therapeutic implications, it is especially noteworthy that the T-cell response seems to be strictly 'private' to each patient. This re-emphasizes the need for 'individualized' approaches as far as selective, TCR-directed therapies, such as T-cell and TCR vaccination, are concerned (Wraith *et al.*, 1989; Waisman *et al.*, 1996; Hohlfeld and Wekerle, 2004; Bourdette *et al.*, 2005). Furthermore, intra-individual diversity implies that it would be necessary to simultaneously target multiple TCRs.

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