Siponimod Inhibits the Formation of Meningeal Ectopic Lymphoid Tissue in Experimental Autoimmune Encephalomyelitis

Rosa Margareta Brand, PhD, Jolien Diddens, MSc, Verena Friedrich, PhD, Monika Pfaller, BSc, Helena Radbruch, MD, Bernhard Hemmer, MD, Katja Steiger, VMD, and Klaus Lehmann-Horn, MD

Correspondence
Dr. Lehmann-Horn
klaus.lehmann-horn@tum.de

Abstract

Background and Objectives
To investigate whether the formation or retention of meningeal ectopic lymphoid tissue (mELT) can be inhibited by the sphingosine 1-phosphate receptor 1,5 modulator siponimod (BAF312) in a murine model of multiple sclerosis (MS).

Methods
A murine spontaneous chronic experimental autoimmune encephalomyelitis (EAE) model, featuring meningeal inflammatory infiltrates resembling those in MS, was used. To prevent or treat EAE, siponimod was administered daily starting either before EAE onset or at peak of disease. The extent and cellular composition of mELT, the spinal cord parenchyma, and the spleen was assessed by histology and immunohistochemistry.

Results
Siponimod, when applied before disease onset, ameliorated EAE. This effect was also present, although less prominent, when treatment started at peak of disease. Treatment with siponimod resulted in a strong reduction of the extent of mELT in both treatment paradigms. Both B and T cells were diminished in the meningeal compartment.

Discussion
Beneficial effects on the disease course correlated with a reduction in mELT, suggesting that inhibition of mELT may be an additional mechanism of action of siponimod in the treatment of EAE. Further studies are needed to establish causality and confirm this observation in MS.

From the Department of Neurology (R.M.B., J.D., V.F., M.P., H.R., B.H., K.L.H.), School of Medicine, Technical University of Munich; Department of Neuropathology (H.R.), Charité-Universitätsmedizin Berlin; Munich Cluster of Systems Neurology (SyNergy) (B.H.); Comparative Experimental Pathology (CEP), Department of Pathology (K.S.), School of Medicine, Technical University of Munich, Germany.

Glossary

BBB = blood-brain barrier; **BW** = body weight; **CMC** = carboxymethylcellulose; **EAE** = experimental autoimmune encephalomyelitis; **GMW** = gray matter width; **HE** = hematoxylin and eosin; **IHC** = immunohistochemistry; **LFB-PAS** = Luxol fast blue–periodic acid–Schiff reaction; **mELT** = meningeal ectopic lymphoid tissue; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **RMS** = relapsing MS; **S1PR** = sphingosine 1-phosphate receptor; **MPO** = myeloperoxidase; **SLO** = secondary lymphoid organ; **SPMS** = secondary progressive MS; **TCR** = T-cell receptor.

Although the options to treat relapsing multiple sclerosis (RMS) have dramatically increased and improved over the last 2 decades, the options to treat secondary progressive MS (SPMS) are still very limited. To date, the next-generation sphingosine 1-phosphate receptor (S1PR) 1,5 modulator siponimod (BAF312) is, among the agents approved for the treatment of SPMS, that with the most convincing clinical trial results. The EXPAND trial demonstrated the efficacy of siponimod in reducing 3-month confirmed disability progression and brain atrophy compared with placebo in SPMS.[1]

An important limitation in the advancement of SPMS therapies is our insufficient understanding of what drives progression in MS. Impaired remyelination, mitochondrial failure, and membrane channel dysfunction may result in increasingly vulnerable axons and subsequent neuronal degeneration. This process may be driven by compartmentalized chronic inflammation, occurring behind an, compared with RMS, increasingly impermeable blood-brain barrier (BBB), supported by B and T cells and activated microglia.[2,3]

Leukocytic infiltrates, forming meningeal ectopic lymphoid tissue (mELT) in MS, have first been described in the brain and spinal cord in patients with SPMS.[4,5] They are associated with an early disease onset, subpial cortical demyelination, cortical atrophy, and a rapid disease progression.[6-9] Resembling secondary lymphoid organs (SLOs), ectopic lymphoid tissue (ELT) in general and mELT in particular consists of B and T cells, other immune cells, and stromal cells, with varying degrees of organization, ranging from dense cell clusters to highly organized structures.[10-12] According to its putative function, which includes affinity maturation of B cells,[13] mELT may be regarded as tertiary lymphoid tissue. Thus, mELT represents a potential therapeutic target in MS.

Lymphocyte egress from lymphoid organs is dependent on the S1PR 1.[14-16] S1PR modulators, such as siponimod, interfere in this process and hereby, potentially, in the formation of ELT. The lipophilic small molecule siponimod crosses the BBB,[17] easily reaching the meningeal space and CNS parenchyma, even in later stages of MS, when the BBB is restored.

In this study, we investigated whether siponimod was able to prevent or deplete mELT in a B- and T-cell–dependent spontaneous chronic experimental autoimmune encephalomyelitis (EAE) model of MS.[18,19] We demonstrate that siponimod improved the clinical course of EAE and reduced mELT, suggesting that targeting meningeal infiltrates may be an additional mechanism of action of S1PR modulators in the treatment of SPMS.

Methods

Mice, EAE, and Clinical Assessment

2D2xTh mice were generated, held, and assessed as previously described.[20] From a score of 3, mice were additionally provided with watered oats and glucose per os. A score of 4 for 72 hours or a score of 4.5 served as termination criterion.

Siponimod Treatment

Siponimod (BAF312; ADV638392161, Sigma-Aldrich) was suspended in 0.5% carboxymethylcellulose (CMC) medium viscosity (C4888, Sigma-Aldrich) and dosed adjusted to body weight (BW) with 3 mg/kg per day. This dosing was chosen because it has been shown to ameliorate EAE in rats, whereas a dose similar to that used in patients with MS (0.03 mg/kg BW) did not.[21] The vehicle alone, 0.5% CMC, was administered to a control group. In an attempt to prevent EAE (prevention paradigm), mice received the first dose at the age of 26 ± 2 days (n = 17 siponimod treated; n = 14 vehicle treated). For the evaluation of the therapeutic potential (treatment paradigm), mice were treated when reaching a clinical score of ≥3 (n = 7 siponimod treated; n = 8 vehicle treated). Considering the sex and the age when EAE started, animals were alternately assigned to treatment groups and received the respective agent daily via oral gavage. To minimize potential confounders, only mice of the same treatment group were held together in 1 cage. Mice were killed 30 days after the first application. For histologic reference at peak of disease, 5 mice were dissected when they reached a score of 3 without any further treatment.

Blinding

Siponimod or 0.5% CMC was applied in a blinded manner, with only M. Pfaller knowing the group allocation. Also, all histologic evaluations were performed blinded.

Organ Preparation, Cell Isolation, and Flow Cytometric Analysis

Spleen cells were processed and analyzed as previously published.[20] The following anti-mouse antibodies were used: CD16/CD32 (BD, 2.4G2), CD45.2 (BioLegend, 104, FITC), CD19 (BioLegend, 6D5, PerCP), CD45R/B220 (BioLegend, RA3-6B2, AF647), CD3e (BD, 145-2C11, PE), and CD4 (BD, RM4-5, PB).

Histology, Immunohistochemistry, and Imaging

Histologic sections of the spinal cord and spleen were prepared, stained with hematoxylin and eosin (HE), Luxol fast blue–periodic acid–Schiff (LFB-PAS) reaction as well as immunohistochemistry (IHC) and evaluated as recently described.[20] Additional IHC staining with an anti-mouse F4/80 antibody (BioRad, MCA497G, 1:50) was performed. F4/80⁺-positive cells were counted using a membrane algorithm (Membrane v9, Image Scope; Leica). Determined by the degree of demyelination, LFB-PAS–stained sections were classified with a score from 0 to 4 categorized as follows: 0 = no demyelination, 1 = minor demyelination, 2 = 1–2 quadrants showing demyelination with less than half of gray matter width (GMW) affected or 3–4 quadrants with less than a quarter of GMW affected, 3 = 1–2 quadrants showing demyelination with over half of GMW affected or 3–4 quadrants with less than half of GMW affected, and 4 = 3–4 quadrants with more than half of GMW affected to complete demyelination.

Statistical Analysis

Data are shown as mean ± SEM or 95% CI. Statistical significance between groups was analyzed using the unpaired Student t test when normally distributed or otherwise Mann-Whitney U test (GraphPad Prism, version 8). A value of $p \leq 0.05$ was considered significant, with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Standard Protocol Approvals, Registrations, and Patient Consents

All animal experiments were approved by the competent authority, Regierung von Oberbayern, Munich, Germany (ROB-55.2-2532.Vet_02-16-100).

Data Availability

All data supporting our findings can be found within the article and will be made available by request to any qualified investigator.

Results

Preventive Siponimod Administration Attenuates the Clinical Course of Spontaneous Chronic EAE and Decreases Splenic T Cells

To test the selective S1PR modulator siponimod in the context of spontaneous chronic EAE and determine its effects on mELT, we crossed myelin oligodendrocyte glycoprotein (MOG) Ig-VH knock-in (IgH^MOG, Th) mice, in which B cells express an MOG-specific B-cell receptor heavy chain, with MOG-specific T-cell receptor (TCR) transgenic mice (TCR^MOG, 2D2). 2D2xTh mice spontaneously develop EAE and form lymphocytic aggregates in the meninges, resembling those found in MS.[18,19] In a preventive setting, mice received the first dose shortly after weaning, at an age of 26 ± 2 days. Siponimod or vehicle was administered daily by oral gavage in a weight adapted manner (3 mg/kg BW) over a period of 30 days (Figure 1A). Although we did not observe a decline in incidence with 64.3% developing EAE in the vehicle and 58.8% in the siponimod-treated group (Figure 1B), siponimod-treated mice recovered soon after the initial peak (Figure 1C). Regarding its effects on the peripheral immune compartment, the selective S1PR modulator massively reduced the CD45.2⁺ population in splenic tissue in our model after 30 days of application. This may be explained by a decrease in CD3⁺CD4⁺ as well as in CD3⁺CD4⁻ T cells in the spleen, whereas the CD19⁺B220⁺ B cells seemed to remain unaffected in comparison with vehicle-treated controls (eFigures 1A and 1B, links.lww.com/NXI/A664). Morphologically, the periarteriolar lymphocyte sheet—which mainly consists of T lymphocytes—is clearly narrower after siponimod treatment (eFigure 1B). Also, spleens of siponimod-treated mice weighed less than spleens of control-treated animals (eFigure 1C).

Siponimod Administered Before EAE Onset Prevents the Formation of mELT

When screening the meningeal compartment for immune cells along the spine, 11.6% of vehicle and 17.1% of siponimod-treated animals had minor cell infiltrates, not

**Figure 1** Preventively Administered Siponimod Ameliorates EAE
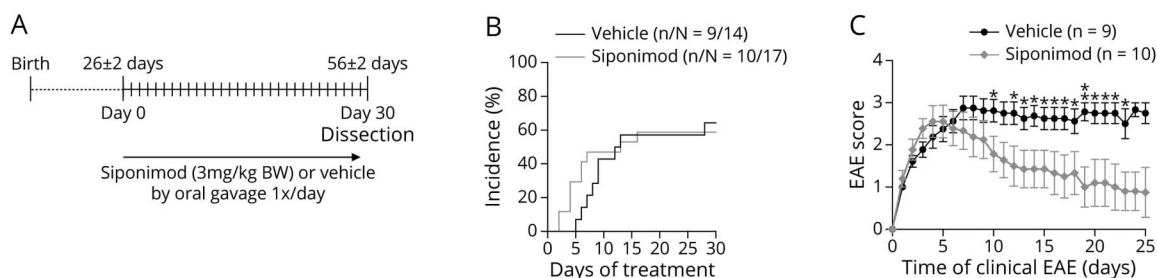
(A) Experimental setup for the prevention paradigm. After weaning, at age 26 ± 2 days, 2D2xTh mice were treated with 3 mg per kg bodyweight siponimod (n = 17) or vehicle (n = 14) by daily oral gavage for 30 days. (B) EAE incidence over the treatment period of 30 days. (C) EAE score evaluated daily, aligned to EAE onset. Data shown as mean ± SEM. Statistical significance between groups was analyzed using the Mann-Whitney U test. $*p \leq 0.05$ and $**p \leq 0.01$. BW = body weight; EAE = experimental autoimmune encephalomyelitis; n/N = mice with EAE/all mice observed.

fulfilling our criteria for mELT ($<1 \times 10^4$ μm² per section). mELT formed in 68.0% of all sections analyzed in the control group in 9/9 EAE mice, whereas siponimod-treated animals formed mELT in only 29.5% of all sections, with 2/7 mice forming no mELT at all (Figure 2A, bar charts). We grouped mELT into 4 categories according to the area of mELT per section. Under siponimod therapy, mELT tended to be small: three-quarters of all mELT sections fell into the smallest category ($\geq 1 \times 10^4$ μm², $<3 \times 10^4$ μm²; category +), whereas in controls, only one-quarter fell into that category. One-third of sections in control mice fell into category +++

($\geq 1 \times 10^5$ μm², $<3 \times 10^5$ μm²) or ++++ ($\geq 3 \times 10^5$ μm²), whereas hardly any siponimod-treated mice showed mELT of that size (Figure 2A, pie charts). mELT in vehicle-treated mice was predominantly observed along the thoracic part of the spinal cord, whereas in the siponimod-treated group, no such peak was notable (Figure 2B). Overall, siponimod drastically reduced the mean size of mELT per section to 13,403 μm² compared with 100,261 μm² in controls (Figure 2C). Reflecting the strongly reduced size of mELT, the numbers of immune cells, especially B220$^+$ B cells and CD3$^+$ T cells, but also F4/80$^+$ macrophages, were

Figure 2 Siponimod Halts the Formation of mELT and Reduces Immune Cells in the Meningeal Compartment
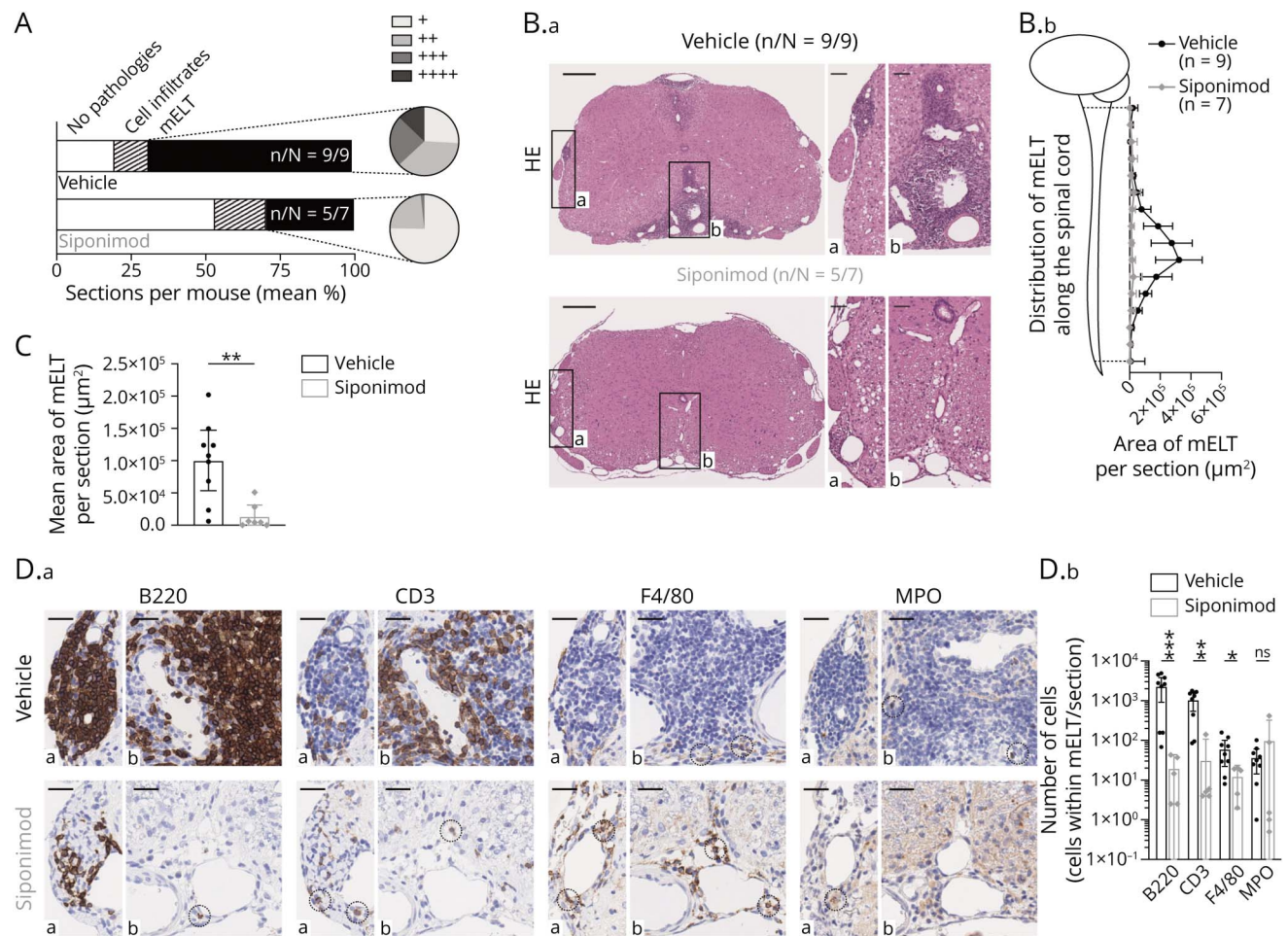
After weaning, 2D2xTh received a daily dose of siponimod (n = 17) or vehicle (n = 14) and were observed for 30 days. Of those mice, n = 10 in the siponimod group and n = 9 in the vehicle group developed EAE. In the siponimod-treated group, 7/10 mice were subsequently assessed histologically as 3 mice died prematurely and had to be excluded from histological analysis. (A) Proportion of spinal cord sections of EAE mice after 30 days of treatment showing no meningeal pathologies, loose meningeal cell infiltrates, or full mELT (defined as >1 × 10⁴ μm²), displayed as the mean percentage of sections within each group (left panel, horizontal bars). Semiquantitative analysis of the size of mELT ranging from + (≥1 × 10⁴ μm², <3 × 10⁴ μm²), ++ (≥3 × 10⁴ μm², <1 × 10⁵ μm²), +++ (≥1 × 10⁵ μm², <3 × 10⁵ μm²) to ++++ (≥3 × 10⁵ μm²) per section, demonstrated as the mean percentage of sections within each category per group (right panel, pie chart). (B.a) Representative HE-stained cross-sections of the thoracic part of the spine with corresponding lateral (a) and ventral (b) close-ups. Mean area of mELT per segment showing the distribution of mELT along the spinal cord with 13–16 cross-sections analyzed for each animal (B.b). (C) Quantitative analysis of mELT calculated as the mean value of all sections taken into analysis per mouse. *p ≤ 0.05 and **p ≤ 0.01; statistical significance between groups was analyzed using the Mann-Whitney U test. (D.a) Representative cross-section through the spinal cord showing lateral (a) and ventral (b) close-ups of mELT. Quantification of B cells (B220), T cells (CD3), macrophages (F4/80), and neutrophil granulocytes (MPO) in the meningeal compartment with 1–2 randomly chosen cross-sections analyzed per animal. (D.b) Values are plotted on a logarithmic scale. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001; Mann-Whitney U test. Scale bars: 250 μm (HE overview), 50 μm (HE close-ups), and 25 μm (IHC-stained sections). When not stated differently, data shown as mean ± 95% CI. EAE = experimental autoimmune encephalomyelitis; HE = hematoxylin eosin; IHC = immunohistochemistry; mELT = meningeal ectopic lymphoid tissue; MPO = myeloperoxidase; n/N = mice with mELT/all mice analyzed; ns = not significant.

Figure 3 Siponimod Prevents Demyelination and the Migration of Lymphocytes Into the Parenchyma
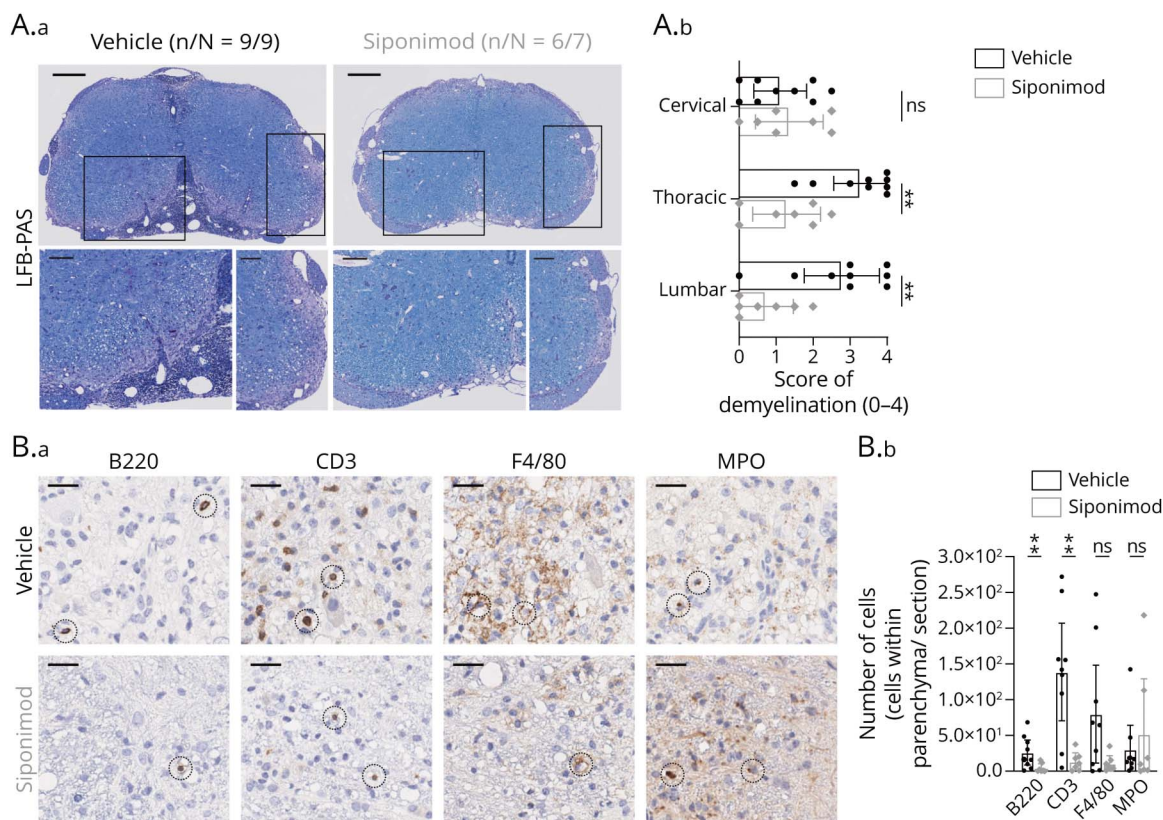
After weaning, 2D2xTh received a daily dose of siponimod (n = 17) or vehicle (n = 14) and were observed for 30 days, of which mice developing EAE (n = 10 siponimod, n = 9 vehicle treated) were assessed histologically (n = 7 siponimod, n = 9 vehicle treated). Three siponimod-treated mice died prematurely and had to be excluded from histological analysis. (A.a) LFB-PAS–stained cross-sections of the spinal cord of the thoracic part and corresponding close-ups below. (A.b) 1–2 randomly chosen cross-sections of the cervical, thoracic, and lumbar part of the spinal cord were analyzed. (B.a–B.b) Quantification of B cells (B220), T cells (CD3), macrophages (F4/80), and neutrophil granulocytes (MPO) infiltrating the parenchyma of the spinal cord. Close-ups of the parenchyma (B.a) with corresponding cell counts per section (B.b). Scale bar: 250 μm (LFB-PAS overview), 100 μm (LFB-PAS close-ups), and 25 μm (IHC-stained sections). Data presented as individual data points and as mean ± 95% CI. *$p \leq 0.05$ and **$p \leq 0.01$; Mann-Whitney U test. EAE = experimental autoimmune encephalomyelitis; IHC = immunohistochemistry; MPO = myeloperoxidase; n/N = mice with demyelination/all mice analyzed; ns = not significant; LFB-PAS = Luxol fast blue–periodic acid–Schiff reaction.

significantly decreased within the meningeal compartment (Figure 2D).

Preventively Administered Siponimod Reduces Demyelination and Lymphocytic Infiltration to the Spinal Cord Parenchyma

As mELT is often correlated with a pronounced underlying cortical demyelination,[5,6] we performed LFB-PAS staining on cross-sections of the cervical, the thoracic, and the lumbar part of the spinal cord. Consistent with the improved clinical outcome, siponimod-treated animals showed less demyelination (Figure 3A). In contrast, controls were heavily affected in the thoracic and lumbar part of the spine (Figure 3A), the area where most mELT was found (Figure 2B). Correspondingly, less B220^{+} and CD3[+-] lymphocytes infiltrated the spinal cord parenchyma under siponimod treatment, whereas CD3^{+} T cells represented the largest group of infiltrating immune cells under control conditions (Figure 3B). In contrast to all other investigated cell types, myeloperoxidase (MPO[+]) neutrophil granulocytes were not decreased in the siponimod group.

EAE-Induced Autoimmune and Demyelinating Damage to the Spinal Cord Is Only Partially Restored by Siponimod Treatment Initiated at Peak of Disease

In a second experimental setup complementing the prevention paradigm, we investigated a scenario more resembling a clinical MS setting. Here, we commenced the application of siponimod or vehicle at the peak of disease, when mice had developed an EAE score of ≥3 (Figure 4A). Again, animals were treated per oral gavage daily over a period of 30 days. Serving as a histologic reference for the time point when treatment commenced, we killed 5 mice at the peak of EAE. At that stage, mice already displayed massive demyelination, especially along the thoracic and lumbar part of the spinal cord (Figure 4B). We observed a strong influx of immune cells to the parenchyma with F4/80[+] macrophages presenting the largest fraction with a mean of 1,644 cells per section (Figure 4C). In contrast, in mELT, which had already formed at that stage, B220^{+} B cells and CD3^{+} T cells predominated, whereas F4/80^{+} macrophages and MPO^{+} neutrophil granulocytes were less frequent (Figure 4D). Over 30 days of

Figure 4 Massive Demyelination and Influx of Immune Cells Into the CNS at the Peak of EAE
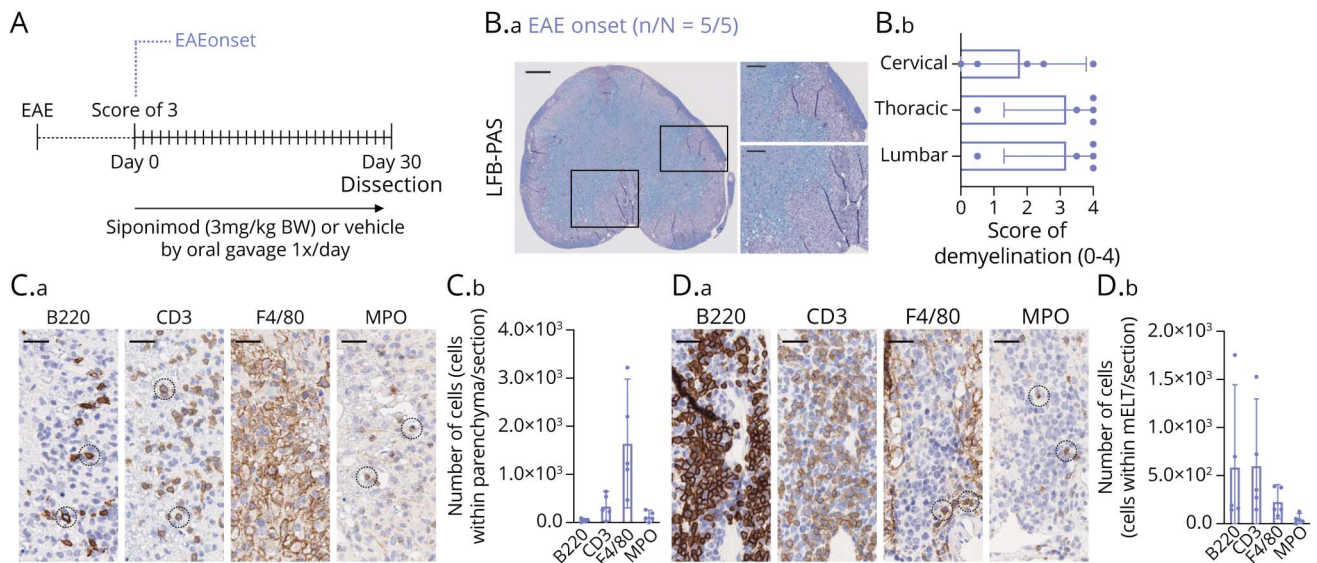
(A) Experimental setup for the treatment paradigm. 2D2xTh mice were treated with either 3 mg oral siponimod per kg bodyweight (n = 7) or vehicle (n = 8) daily from the time they developed an EAE score of 3 for 30 days. Five additional mice were dissected at that same time point, when otherwise treatment started and served as a histological reference for that stage of disease. (B.a) 1–2 LFB-PAS stained cross-sections of the cervical, thoracic, and lumbar part of the spinal cord were analyzed. A thoracic cross-section of the spinal cord of 1 representative mouse at EAE onset and corresponding close-ups are shown. Representative close-ups of parenchyma (C.a) and mELT (D.a) stained for B cells (B220), T cells (CD3), macrophages (F4/80), and neutrophil granulocytes (MPO) (left panel). Corresponding cell counts showing the number of those immune cells in the respective compartments (B.b, C.b, D.b) Scale bar: 250 μm (LFB-PAS overview), 100 μm (LFB-PAS close-ups), and 25 μm (IHC-stained close-ups). Data presented individual data points and as mean ± 95% CI. BW = body weight; EAE = experimental autoimmune encephalomyelitis; IHC = immunohistochemistry; MPO = myeloperoxidase, n/N = mice with demyelination/all mice analyzed; LFB-PAS = Luxol fast blue–periodic acid–Schiff reaction.

treatment with siponimod, the EAE course was modestly ameliorated (Figure 5A). Accordingly, there was a minor reduction of demyelination at the thoracic level of the spinal cord (Figure 5B). Noting that generally few immune cells were detectable in the parenchyma at that chronic phase of EAE, we found a trend toward a decrease for all leukocytes, but no statistically significant changes except for the B220$^+$ B-cell fraction. F4/80$^+$ macrophages remained the most prevalent population in the parenchyma in both groups (Figure 5C).

Siponimod Treatment Initiated at Peak of Disease Effectively Diminishes mELT and Reduces Not Only T Cells But Also B Cells in the Meningeal Compartment

Of note, 7/7 mice, which received vehicle, had mELT in 51.0% of all spinal cord sections analyzed. In contrast, treated with siponimod, only 5/7 animals exhibited mELT in 13.5% of all analyzed cross-sections (Figure 6A, bar chart). Regarding its size, mELT in siponimod-treated mice did never exceed $3 \times 10^4 \ \mu m^2$ per section (category +), whereas controls also developed large areas of mELT with a size $\geq 1 \times 10^5 \ \mu m^2$ in 20.7% (categories +++ and ++++) (Figure 6A, pie chart), peaking at the thoracic part of the spine (Figure 6B). Siponimod lead to a 94.8% reduction of the mean area of mELT per section (Figure 6C). Consequently, B220$^+$ B cells, CD3$^+$ T cells, and MPO$^+$ neutrophil granulocytes were notably less frequent in the meningeal compartment compared with controls (Figure 6D). Of interest, unlike in the spleen, not only T cells but also B cells were significantly reduced.

In summary, the selective S1PR modulator siponimod ameliorated spontaneous chronic EAE and strongly reduced the formation and retention of mELT along the spinal cord, bringing up the possibility that there may be a causative correlation.

Discussion

As our understanding of the forces that drive progression in MS is still limited, therapeutic options to treat SPMS remain scarce. Sequestered meningeal inflammation may be the link between early, highly inflammatory RMS, and chronic progression in later SPMS. We set out to study the impact of a moderately effective SPMS therapeutic, siponimod, on meningeal inflammation in a murine model of MS.

In line with the clinical observation that siponimod reduces disability progression in SPMS, it ameliorated spontaneous chronic EAE both when applied in a preventive fashion before onset of the disease and, although less pronounced, in treatment of fully established paralysis. The damage to the spinal cord present at the peak of disease, few days after onset, was so severe that mice only partially recovered when treated with siponimod at that stage. This clinical benefit of siponimod correlated with a marked reduction in the presence of mELT in siponimod-treated EAE mice compared with controls. Formally, we could not determine whether preventive

Figure 5 Siponimod Administered From the Peak of Disease Slightly Improved EAE and the Degree of Thoracic Demyelination
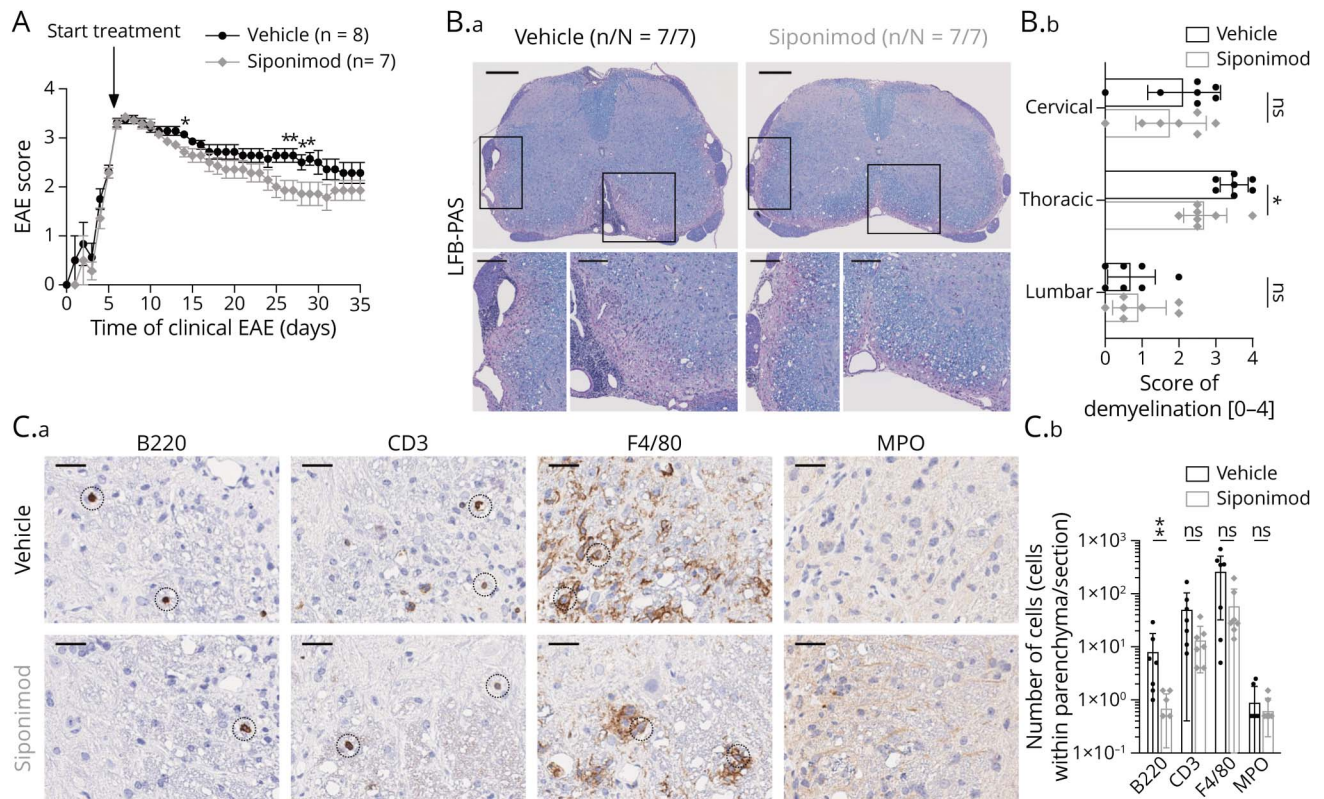
2D2xTh mice were treated with siponimod (n = 7) or vehicle (n = 8) daily for 30 days as soon as they reached an EAE score of 3. One vehicle-treated mouse died prematurely and had to be excluded from histological analysis. (A) Clinical course of EAE aligned to the start of treatment with a score of 3. Data shown as mean ± SEM. (B.a) Representative LFB-PAS stained thoracic cross-sections with corresponding close-ups are shown. (B.b) Various levels of spinal cord sections were semi-quantitatively analyzed. (C.a) Representative close-ups of the spinal cord parenchyma, stained for B cells (B220), T cells (CD3), macrophages (F4/80) and neutrophil graulocytes (MPO) after 30 days of treatment with siponimod or vehicle and (C.b) corresponding counts of infiltrating cells per section. Two sections of the cervical, thoracic, and lumbar part were analyzed from each animal. Values are plotted on a logarithmic scale. Scale bar, 250 μm (LFB-PAS), 100 μm (LFB-PAS, close-ups), and 25 μm (IHC-stained close-ups). Data are presented as mean ±95% CI. *$p \leq 0.05$ and **$p \leq 0.01$; statistical significance between groups was analyzed using the Mann-Whitney U test. EAE = experimental autoimmune encephalomyelitis; IHC = immunohistochemistry; LFB-PAS = Luxol fast blue–periodic acid–Schiff reaction; MPO = myeloperoxidase; n/N = mice with demyelination/all mice analyzed; ns = not significant.

treatment prohibited the initiation of the formation of mELT in the first place or rather inhibited its growth and proliferation. Furthermore, we were unable to prove that inhibiting the formation of mELT was the cause for the clinical benefit. To demonstrate that would require a model, which is dependent on mELT, which is not available to date. It is, however, noteworthy that this correlation could also be found using another drug: In a similar experimental setup, laquinimod, like siponimod, inhibited EAE and the formation of meningeal B-cell aggregates.[22] Recently, we demonstrated that anti-CD20 monoclonal antibodies, despite its superior efficacy in treating RMS and moderate efficacy in treating primary progressive MS, neither ameliorated EAE nor reduced the extent of mELT formation in the 2D2xTh mouse model.[20] Apparently, anti-CD20 monoclonal antibodies only depleted B cells from mELT without changing any other obvious features. Of interest, a very recent study demonstrated that evobrutinib, a Bruton tyrosine kinase inhibitor, reduced the areas of meningeal contrast enhancement in an EAE model in Swiss Jim Lambert J mice immunized with proteolipid protein peptide (PLP139-151) using serial ultra-high-field MRI.[23]

Taken together, our present findings suggest that this correlation may be relevant and potentially causal. Consequently, the role of mELT in CNS autoimmunity in general and in the treatment with immunomodulatory agents, specifically, should be studied in more detail, ideally in patients with MS.

How does siponimod inhibit the formation of mELT? Immunomodulatory effects related to lymphocyte trafficking regulation are likely to be at the center of its effects on mELT. Fingolimod (FTY720), the first S1PR modulator approved for the use against MS, has been shown to accelerate lymphocyte homing and hereby decreases the number of lymphocytes in peripheral blood and spleen and increases the number in peripheral and mesenteric lymph nodes.[24] Its immunomodulatory effects are expected to be similar to the closely related but more selective S1PR modulator siponimod (class effects). One study showed that the

Figure 6 Siponimod Treatment Effectively Resolves mELT
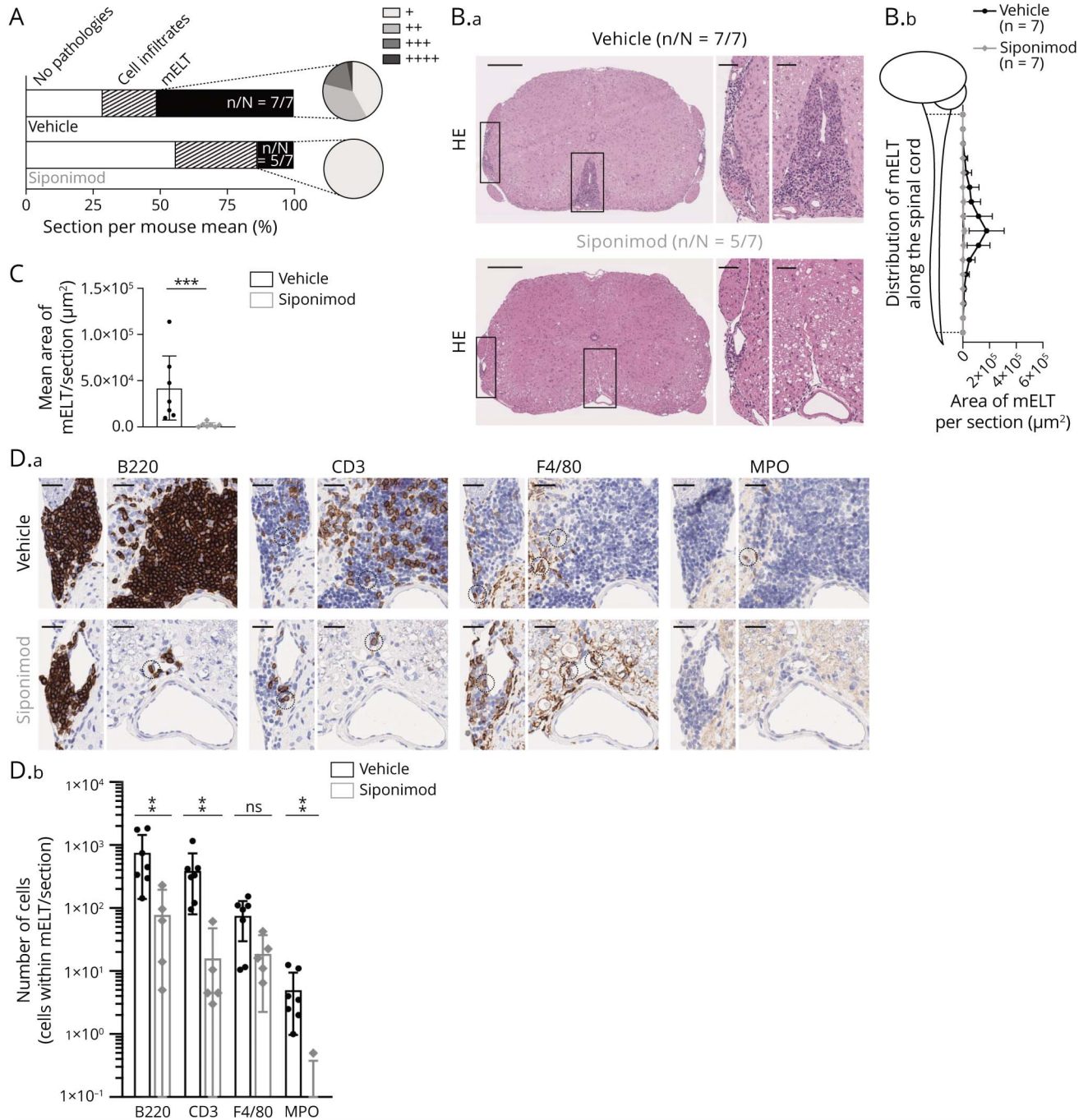
2D2xTh mice treated with oral siponimod (n = 7) or vehicle (n = 8) daily for 30 days, starting as they reached an EAE score of 3. One vehicle-treated mouse died prematurely and had to be excluded from histological analysis. (A) Proportion of spinal cord sections of EAE mice showing no meningeal pathologies, loose meningeal cell infiltrates, or full mELT (defined as >1 × 10^4 μm²), displayed as the mean percentage of sections within each group (left panel, horizontal bars). Semiquantitative analysis of the size of mELT, ranging from + (≥1 × 10^4 μm², <3 × 10^4 μm²), ++ (≥3 × 10^4 μm², <1 × 10^5 μm²), +++ (≥1 × 10^5 μm², <3 × 10^5 μm²) to ++++ (≥3 × 10^5 μm²) per section, demonstrated as the mean percentage of sections within each category per group (right panel, pie chart). (B.a) Representative HE stained cross-sections of the thoracic part of the spine with corresponding lateral (a) and ventral (b) close-ups of mELT. (B.b) Mean area of mELT per segment showing the distribution of mELT along the spinal cord with 13–16 cross-sections analyzed for each animal. (C) Comparable statistics applied to the mean area of mELT of all cross-sections per animal. *$p ≤ 0.05$ and ***$p ≤ 0.001$; statistical significance between groups was analyzed using the Mann-Whitney U test. (D.a) Representative cross-section through the spinal cord showing lateral (a) and ventral (b) close-ups of mELT. (D.b) Quantification of B cells (B220), T cells (CD3), macrophages (F4/80), and neutrophil granulocytes (MPO) in the meningeal compartment with 1–2 randomly chosen cross-sections analyzed per animal. Values are plotted on a logarithmic scale. *$p ≤ 0.05$ and **$p ≤ 0.01$; Mann-Whitney U test. Scale bar: 250 μm (HE overview), 50 μm (HE close-ups), and 25 μm (IHC-stained close-ups). Data are presented as mean ± 95% CI. EAE = experimental autoimmune encephalomyelitis; HE = hematoxylin eosin; IHC = immunohistochemistry; MPO = myeloperoxidase; n/N = mice with mELT/all mice analyzed; ns = not significant.

reduction of circulating lymphocytes by fingolimod in mice was due to the inhibition of lymphocyte egress, particularly of mature T cells, from the bone marrow.[25] Circulating monocytes, like lymphocytes, were reduced by the S1PR receptor modulators fingolimod and siponimod, whereas monocytes were increased in the bone marrow and spleen, suggesting that monocyte egress from hematopoietic organs is restricted by S1PR receptor modulation.[26] Consistent with the literature,[24] spleens of siponimod-treated mice in our experiments were lighter and contained less T cells. In our model, which is genetically determined, mELT formed at some stage of the autoimmune process directed against the CNS and is fully developed shortly after disease onset. To form mELT, lymphocytes have to migrate to the meningeal compartment, either from primary or SLOs or both. It is plausible that this process may be impaired under the influence of siponimod. Once mELT is formed, it is possible that there is persistent influx of peripheral lymphocytes, or even bidirectional exchange, as it has been demonstrated for the CSF and B cells.[27] Again, siponimod may interfere with that process, resulting in shrinkage of mELT. Of interest, in its response to S1PR modulation, mELT does not seem to behave like a lymph node, as it would be expected to increase in size. Its decreasing size rather resembles the spleen, but unlike in that SLO, B cells in mELT are also decreased. Further research is required to clarify whether mELT shares certain properties with SLOs (and which) or whether it needs to be considered independent thereof.

Finally, microarray and flow cytometric analysis in patients with SPMS treated with S1PR modulator showed that both T- and B-cell populations were shifted toward a more anti-inflammatory and regulatory phenotype.[28] They were characterized by decreased expression of genes involved in T- and B-cell activation and receptor signaling and an enrichment of T effector memory cells, anti-inflammatory Th2, and T regulatory cells as well as transitional regulatory B cells ($CD24^{hi}CD38^{hi}$) and B1 cell subsets ($CD43^{+}CD27^{+}$).[29] Thus, the inhibitory effect of siponimod on mELT may be independent of leukocyte trafficking but due to its anti-inflammatory properties. Given the differential effects of various immunomodulatory therapies in the treatment of MS, some of which we have discussed above, combining therapies, for example, such that act mainly in the periphery with such that act centrally and inhibit mELT formation, may be helpful.[30]

In conclusion, the S1PR modulator siponimod ameliorated spontaneous chronic EAE and reduced the formation of mELT. Together, our results suggest that inhibiting sequestered inflammation in the meningeal compartment, by the mechanisms discussed above or by other, yet unknown means, may be an additional mechanism of action of siponimod in the treatment of MS. More studies are required to prove causality. We propose to directly target mELT formation in future experimental approaches addressing SPMS.

Disclosure
R.M. Brand, J. Diddens, V. Friedrich, M. Pfaller, and H. Radbruch report no disclosures. B. Hemmer has served on scientific advisory boards for Novartis; he has served as DMSC member for AllergyCare, Polpharma, and TG Therapeutics; he or his institution has received speaker honoraria from Desitin; his institution received research grants from Regeneron for MS research. He holds part of 2 patents: 1 for the detection of antibodies against KIR4.1 in a subpopulation of patients with MS and 1 for genetic determinants of neutralizing antibodies to interferon. All conflicts are not relevant to the topic of the study. K. Steiger has received research support (to TUM) from FHLR and holds a patent for a radiopharmaceutical. K. Lehmann-Horn has received research support (to TUM) from Novartis and honoraria and compensation for travel expenses from Novartis, FHLR, Biogen, Teva, and Merck Serono. Go to Neurology.org/NN for full disclosures.

Appendix Authors

Name	Location	Contribution
Rosa Margareta Brand, PhD	Department of Neurology, School of Medicine, Technical University of Munich, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data

Continued

Appendix *(continued)*

Name	Location	Contribution
Jolien Diddens, MSc	Department of Neurology, School of Medicine, Technical University of Munich, Germany	Drafting/revision of the manuscript for content, including medical writing for content
Verena Friedrich, PhD	Department of Neurology, School of Medicine, Technical University of Munich, Germany	Drafting/revision of the manuscript for content, including medical writing for content
Monika Pfaller, BSc	Department of Neurology, School of Medicine, Technical University of Munich, Germany	Major role in the acquisition of data
Helena Radbruch, MD	Department of Neurology, School of Medicine, Technical University of Munich, Germany, Department of Neuropathology, Charité-Universitätsmedizin Berlin, Berlin, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; and analysis or interpretation of data
Bernhard Hemmer, MD	Department of Neurology, School of Medicine, Technical University of Munich, Germany; Munich Cluster of Systems Neurology (SyNergy), Germany	Drafting/revision of the manuscript for content, including medical writing for content
Katja Steiger, VMD	Department of Pathology, School of Medicine, Technical University of Munich, Germany	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; and analysis or interpretation of data
Klaus Lehmann-Horn, MD	Department of Neurology, School of Medicine, Technical University of Munich, Germany	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; and analysis or interpretation of data

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