

# Anti-CD20 Depletes Meningeal B Cells but Does Not Halt the Formation of Meningeal Ectopic Lymphoid Tissue

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## Abstract

### Objective

To investigate whether anti-CD20 B-cell-depleting monoclonal antibodies ( $\alpha$ CD20 mAbs) inhibit the formation or retention of meningeal ectopic lymphoid tissue (mELT) in a murine model of multiple sclerosis (MS).

### Methods

We used a spontaneous chronic experimental autoimmune encephalomyelitis (EAE) model of mice with mutant T-cell and B-cell receptors specific for myelin oligodendrocyte glycoprotein (MOG), which develop meningeal inflammatory infiltrates resembling those described in MS.  $\alpha$ CD20 mAbs were administered in either a preventive or a treatment regimen. The extent and cellular composition of mELT was assessed by histology and immunohistochemistry.

### Results

$\alpha$ CD20 mAb, applied in a paradigm to either prevent or treat EAE, did not alter the disease course in either condition. However,  $\alpha$ CD20 mAb depleted virtually all B cells from the meningeal compartment but failed to prevent the formation of mELT altogether. Because of the absence of B cells, mELT was less densely populated with immune cells and the cellular composition was changed, with increased neutrophil granulocytes.

### Conclusions

These results demonstrate that, in CNS autoimmune disease, meningeal inflammatory infiltrates may form and persist in the absence of B cells. Together with the finding that  $\alpha$ CD20 mAb does not ameliorate spontaneous chronic EAE with mELT, our data suggest that mELT may have yet unknown capacities that are independent of B cells and contribute to CNS autoimmunity.

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### Editorial

Can Systemic Anti-CD20 B Cell-Depleting Antibodies Eliminate Meningeal Follicles in Multiple Sclerosis?

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## Glossary

**αCD20 mAb** = anti-CD20 monoclonal antibody; **BCR** = B-cell receptor; **EAE** = experimental autoimmune encephalomyelitis; **mELT** = meningeal ectopic lymphoid tissue; **MOG** = myelin oligodendrocyte glycoprotein; **MPO** = myeloperoxidase; **MS** = multiple sclerosis; **RRMS** = relapsing-remitting multiple sclerosis; **PMS** = progressive MS; **SLO** = secondary lymphoid organ; **LFB-PAS** = Luxol fast blue and periodic acid-Schiff reaction; **TCR** = T-cell receptor.

B-cell-depleting strategies with anti-CD20 monoclonal antibodies (αCD20 mAbs), such as rituximab, ocrelizumab, or ofatumumab, are among the most potent therapies currently available in the treatment of relapsing-remitting multiple sclerosis (RRMS). Modest efficacy has also been shown in progressive MS (PMS), specifically active primary PMS, and early, relapse-independent progression in RRMS.<sup>1-4</sup> Their success highlights the importance of B cells for MS pathogenesis. Among other factors, the therapeutic effect of B-cell depletion may be mediated by interrupting antigen presentation by B cells to T cells in the CNS.<sup>5-9</sup>

Leukocytic infiltrates, forming meningeal ectopic lymphoid tissue (mELT), are found in patients with PMS and to a lesser extent in RRMS. They correlate with an earlier disease onset, a more rapid disease progression, and subpial cortical damage.<sup>10-15</sup> In general, ectopic lymphoid tissue (ELT) is formed by aggregates of B and T cells, which range from tight-cell clusters to highly organized structures and resemble secondary lymphoid organs (SLOs).<sup>16-20</sup> In a model of MS, it was demonstrated that B-cell repertoire expansion occurs in mELT, suggesting germinal center activity in the CNS.<sup>21</sup> Hereby, mELT may facilitate smoldering autoimmunity in the CNS parenchyma sequestered beyond the blood-brain barrier. Yet, it remains unclear whether and how mELT contributes to early or progressive MS.

Because B cells are an integral part of mELT and αCD20 mAb that targets B cells not only in the periphery but also in the meninges,<sup>22</sup> it seems possible that αCD20 mAb inhibits the formation or retention of mELT. Assuming that mELT plays a role in MS pathogenesis, preventing or suppressing mELT may contribute to the efficacy of B-cell depletion in MS. In this study, we investigated the effects of αCD20 mAb on mELT in a B-cell-dependent and T-cell-dependent experimental autoimmune encephalomyelitis (EAE) model of MS. Approximately, 50% of 2D2 (TCR<sup>MOG</sup>) × Th (IgH<sup>MOG</sup>) mice develop spontaneous chronic EAE and exhibit meningeal clusters of T and B cells in the spinal cord, reminiscent of those found in MS.<sup>23-25</sup>

In this model, we depleted B cells to investigate whether αCD20 mAb prevents mELT formation. Surprisingly, our results showed that, despite depleting virtually all B cells from the meninges, mELT still formed or persisted.

## Materials and Methods

### Mice

2D2xTh mice, developing spontaneous chronic EAE with mELT, were generated as previously described by crossing myelin oligodendrocyte glycoprotein (MOG)-specific T-cell receptor (TCR) transgenic 2D2 (TCR<sup>MOG</sup>) mice with MOG-B-cell receptor (BCR) knockin Th mice (IgH<sup>MOG</sup>).<sup>23,24</sup> Mice were bred and maintained in-house at the Center of Preclinical Research (CPR) at Klinikum rechts der Isar (Technical University of Munich) under specific pathogen-free conditions, according to FELASA recommendations with free access to water and standard rodent chow.

### EAE and Clinical Assessment

2D2xTh mice develop spontaneous chronic EAE with an incidence of approximately 30–50% in our facility. Clinical scores of mice were evaluated on a daily basis and assessed as follows: 0 = no clinical signs, 1 = tail weakness, 1.5 = additional impaired tail erection, 2 = ataxia and hind limb weakness, 2.5 = hind limb paresis, 3 = high-grade hind limb paresis, 3.5 = additional minor weakness of the fore limbs, 4 = additional fore limb paresis, 4.5 = tetraplegia, and 5 = moribund.

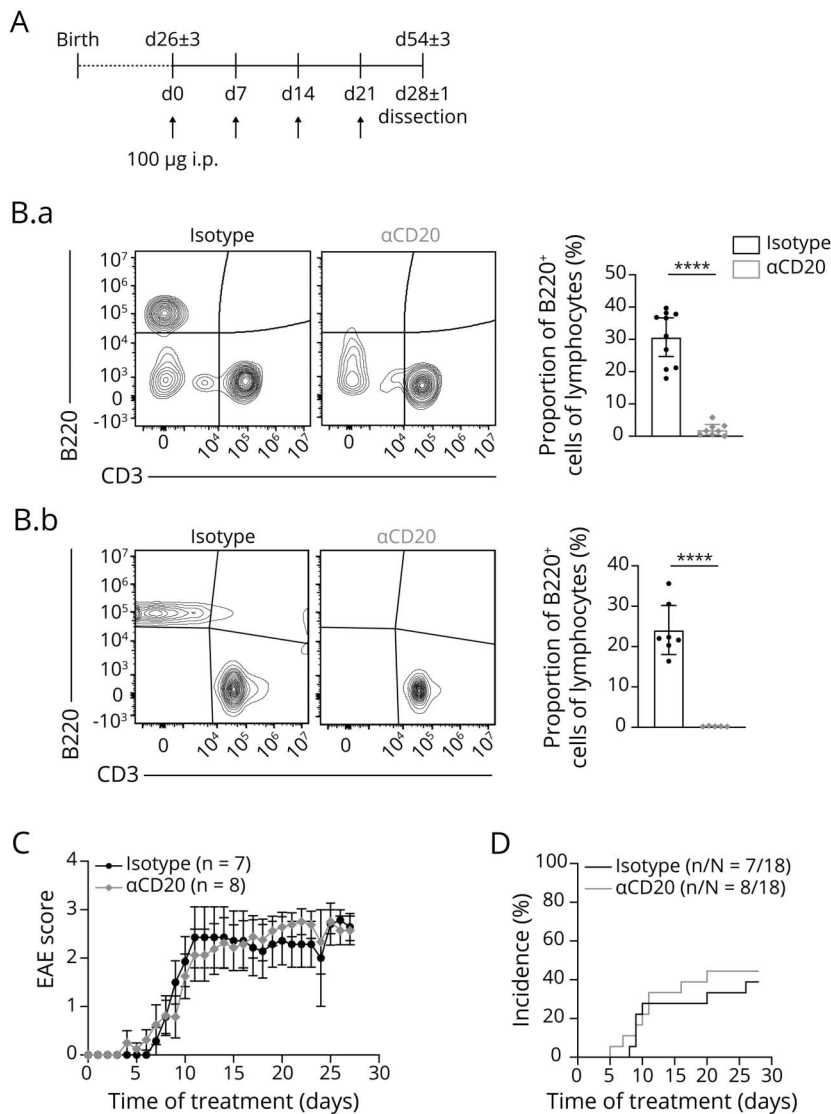
### αCD20 mAb Treatment

100 μg of antimouse CD20 mAb (clone 18B12, provided by F. Hoffmann-La Roche, Switzerland) in a murine IgG2a Fc-format or antihen egg lysozyme isotype control (Crown bio, Cat: C0006) in 100 μL PBS was injected intraperitoneally (i.p.) at day 0, 7, 14, and 21 for a total of 4 doses. Mice were grouped for prevention or treatment of EAE. In an attempt to prevent EAE, mice received the first injection at the age of 26 ± 3 days (n = 18 in both groups). For the evaluation of the therapeutic potential, mice were treated when reaching a clinical score of ≥3 (n = 11 αCD20 mAb-treated, n = 14 isotype-treated). Mice were killed 28 days after the first injection. Reference mice at the onset of disease (figure 3C) were dissected immediately after reaching an EAE score of ≥3 without treatment.

### Cell Isolation and Flow Cytometric Analysis

Blood was freshly obtained by terminal cardiac puncture and mixed with 500 μL 2 mM EDTA. Cervical lymph nodes were carefully dissected and passed through a 30-μm cell strainer. Samples were washed with RPMI medium, and erythrocytes were lysed using BD Pharm Lyse Buffer (BD Biosciences). Cells were washed again, and the number of living cells was determined using a hemocytometer (Cellometer Auto 2000, Nexcelom Bioscience). Single-cell suspensions were stained with antibodies in PBS containing 2% fetal calf serum for 20

**Figure 1** Spontaneous EAE Occurs in the Absence of Peripheral B Cells in Mice Preventatively Treated With  $\alpha$ CD20 mAb



(A) Experimental setup. Mice were treated with a weekly dose of 100 µg of  $\alpha$ CD20 mAb (n = 18) or isotype control (n = 18) after weaning. (B) Flow cytometric analysis of B220<sup>+</sup> and CD3<sup>+</sup> cells in blood (B.a) and lymph nodes (B.b) after 4 weeks of treatment. The representative flow cytometry contour plot (left) and quantification (right). (C) Daily evaluated EAE scores over the experimental period. Data are shown as mean  $\pm$  SEM. (D) Incidence of mice developing clinical signs of disease. When not stated differently, data shown as mean  $\pm$  95% CI. \**p* < 0.05; \*\*\*\**p* < 0.0001; statistical significance between groups was analyzed using the Student *t* test.  $\alpha$ CD20 mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; i.p. = intraperitoneally; n/N = mice with EAE/all mice observed.

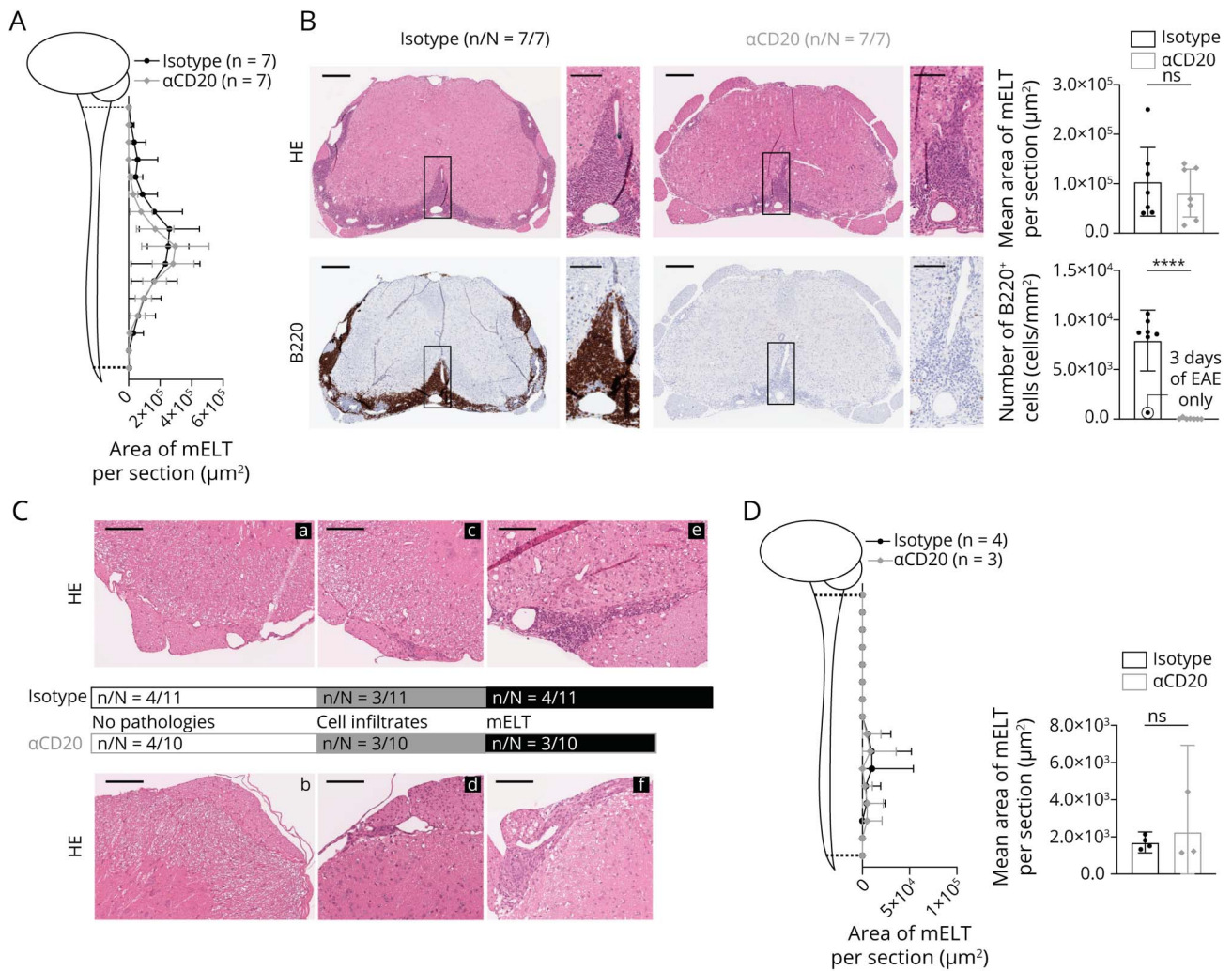
minutes at 4°C in the dark. Fc-receptors were blocked to prevent nonspecific antibody binding. Dead cells were excluded using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). Cells were washed twice and acquired on a CytoFLEX flow cytometer (Beckman Coulter). The following antimouse antibodies were used: CD16/CD32 (2.4G2), CD45R/B220 (RA3-6B2, FITC), and CD3e (145-2C11, PE) (BD Bioscience). Data were analyzed using FlowJo version 10 (TreeStar).

### Histology, Immunohistochemistry, and Imaging

Spinal cord, spleen, and inguinal lymph nodes were perfused before dissection, fixed with 4% paraformaldehyde, and paraffin embedded. The spinal cord was additionally decalcified with Osteosoft (Sigma-Aldrich) 72 hours before embedding. To determine the degree of meningeal infiltrates, the spinal

cord was sectioned horizontally into 13–16 tissue blocks (thickness: 2  $\times$  5 mm cervical, subsequently 2.5 mm) per mouse and stained with hematoxylin and eosin according to standard protocols. The stained tissues were digitized (Leica AT2 scanner) and examined using Image Scope (Pathology Slide Viewing Software, version 12.4.3, Leica Biosystems). Infiltrates with a size of  $\geq 1 \times 10^4 \mu\text{m}^2$  per section were considered as mELT. The cell density was analyzed using a nuclear algorithm (Nuclear v9, Image Scope; Leica). To characterize the cellular composition of mELT, immunohistochemistry was performed on representative sections with antimouse CD45R/B220 (BD, 550286, 1:50), CD3 (DCS, CI597R0, 1:50), and myeloperoxidase (MPO; Thermo Fisher, RB-373-A, 1:50). Mouse spleens served as positive controls. Numbers of B220<sup>+</sup> and CD3<sup>+</sup> positive cells were determined using a membrane algorithm (Membrane v9, Image Scope; Leica). In the CNS, MPO<sup>+</sup> cells were counted

**Figure 2** mELT Formation Occurs in the Absence of B Cells in Mice Preventatively Treated With  $\alpha$ CD20 mAb



After weaning, 2D2xTh mice received a weekly dose of 100  $\mu\text{g}$   $\alpha$ CD20 mAb (n = 18) or isotype control mAb (n = 18) and were observed for 28 days (A + B) 8 of 18  $\alpha$ CD20 mAb-treated mice and 7 of 18 isotype mAb-treated mice developed EAE. One  $\alpha$ CD20 mAb-treated mouse died prematurely and had to be excluded from the histologic analysis. (A) Quantitative distribution of mELT along the spinal cord with 12–16 cross-sections per animal analyzed. (B) Representative HE-stained (upper left panel) and B220-stained (lower left panel) sections of the thoracolumbar part of the spinal cord and corresponding close-up views of mELT. Quantification of the mean area of mELT including all sections (upper right panel) and the number of B220<sup>+</sup> cells in mELT, analyzed in 2 randomly selected sections per animal, for all mice (lower right panel). (C) Analysis of histologic signs of disease in mice without clinically manifest EAE (11 of 18 isotype mAb-treated mice and 10 of 18  $\alpha$ CD20 mAb-treated mice). Representative HE sections given in (a) and (b) for mice with no pathologies, in (c) and (d) for mice with minor cellular infiltrates in the meninges, and in (e) and (f) for mice with mELT. (D) Quantitative analysis of the size and distribution of mELT along the spine in mice without clinically manifest EAE. In total, 12–16 sections analyzed per animal. Scale bars: 250  $\mu\text{m}$  (overviews) and 100  $\mu\text{m}$  (close-ups). When not stated differently, data shown as mean  $\pm$  95% CI. \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ ; statistical significance between groups was analyzed using the Student *t* test.  $\alpha$ CD20 mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; HE = hematoxylin & eosin; mELT = meningeal ectopic lymphoid tissue; n/N = mice showing mELT or noted feature/total number of mice analyzed; ns = not significant.

manually. In the periphery, MPO<sup>+</sup> cells were quantified by applying a nuclear algorithm (Membrane v9, Image Scope; Leica). Two cross-sections of the cervicothoracic, the thoracolumbar, and the lower lumbar part were stained with Luxol fast blue and periodic acid-Schiff reaction (LFB-PAS) according to standard protocols. LFB-PAS staining was analyzed semiquantitatively by dividing spinal cord sections into quadrants and counting quadrants with demyelination. All histologic evaluations were performed in a blinded manner.

### Detection of Anti-MOG Antibodies

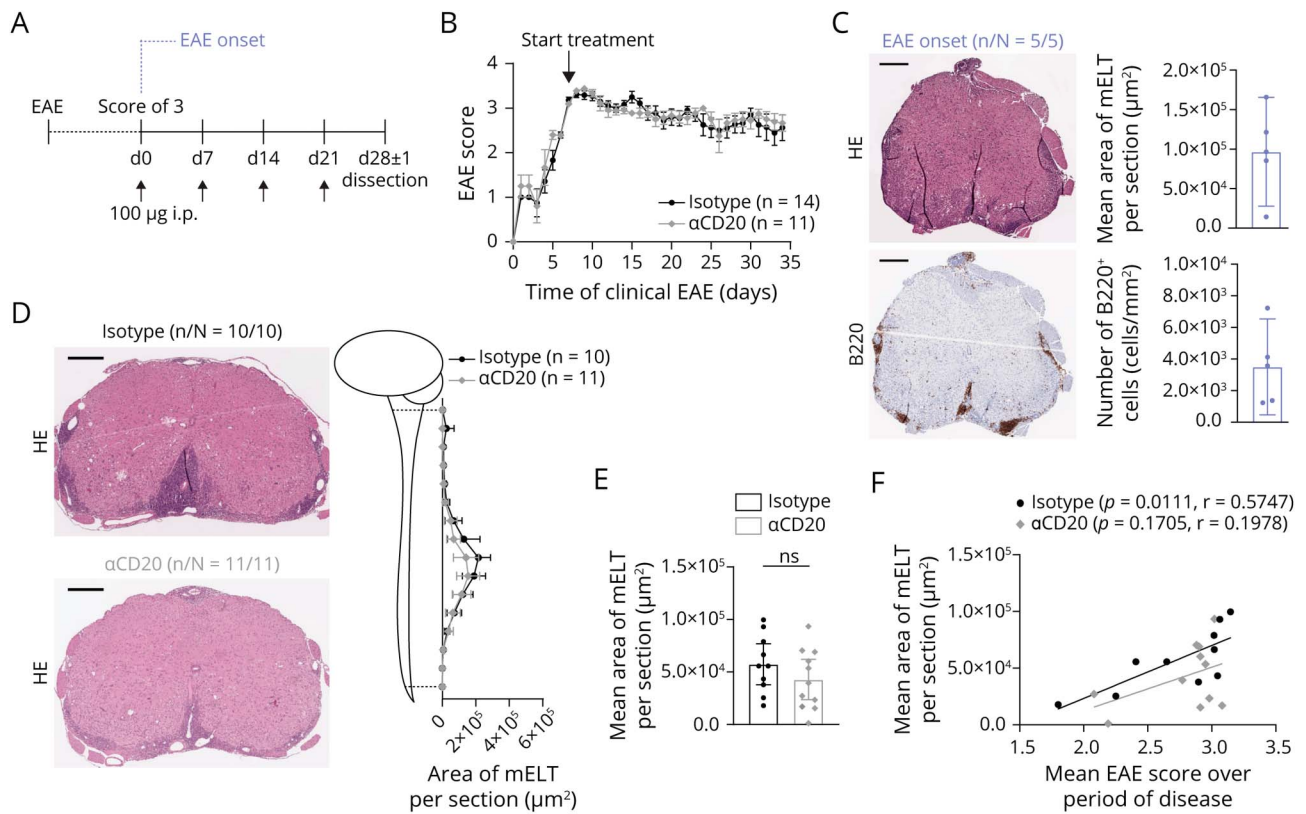
Antirecombinant mouse MOG (1–125) (Eurogentec, Cat: AS-55150-100) IgG was measured with a noncommercial

ELISA in duplicates as described,<sup>26</sup> using MOG at a final concentration of 10  $\mu\text{g}/\text{mL}$  and horseradish peroxidase-labeled goat antimouse IgG (Thermo Fisher Scientific). The optical density was measured at a wavelength of 450 nm with 540 nm as a reference wavelength using the ELISA reader (infinite M200 PRO, TECAN).

### Statistical Analysis

Data are shown as mean  $\pm$  SEM or 95% CI. Statistical significance between groups was analyzed using the unpaired Student *t* test. For correlation analysis, simple linear regression was calculated for each group. A value of  $p \leq 0.05$  was considered significant with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

**Figure 3** αCD20 mAb Applied After EAE Onset Does Not Change the Clinical Course of Spontaneous EAE or the Size of mELT



(A) Experimental setup. As soon as 2D2xTh mice developed a score of  $\geq 3$ , they received a weekly dose of 100  $\mu\text{g}$  of  $\alpha\text{CD20}$  ( $n = 11$ ) or isotype control mAb ( $n = 14$ ) over a period of 28 days. Another 5 mice were euthanized and dissected directly after developing a clinical score of  $\geq 3$ , without receiving treatment. They served as histologic reference at disease onset for the remainder of the mice for which treatment commenced at that stage. (B) Clinical course of EAE during application of  $\alpha\text{CD20}$  or isotype mAb. Data expressed as mean  $\pm$  SEM. (C) Representative HE (upper left panel) and B220 (lower left panel) staining of the spinal cord mELT of mice at EAE onset and respective quantification in all mice (right panels). (D) Representative HE-stained cross-sections of the thoracolumbar part of the spinal cord of  $\alpha\text{CD20}$  mAb-treated and isotype-treated mice (left) and representation of the distribution of mELT along the spine with 14–16 sections analyzed per animal (right). (E) Quantification of the mean area of mELT per section. (F) Correlation of the mean area of mELT per section with the mean EAE score over the observation period. Simple linear regression was calculated for each group. Scale bars, 250  $\mu\text{m}$ . When not indicated differently, data shown as mean  $\pm$  95% CI. Statistical significance between groups was analyzed using the Student  $t$  test.  $\alpha\text{CD20}$  mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; HE = hematoxylin & eosin; i.p. = intraperitoneally; mELT = meningeal ectopic lymphoid tissue; n/N = mice showing mELT/total number of mice analyzed; ns = not significant.

## 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

Data not published within this article will be made available by request from any qualified investigator.

## Results

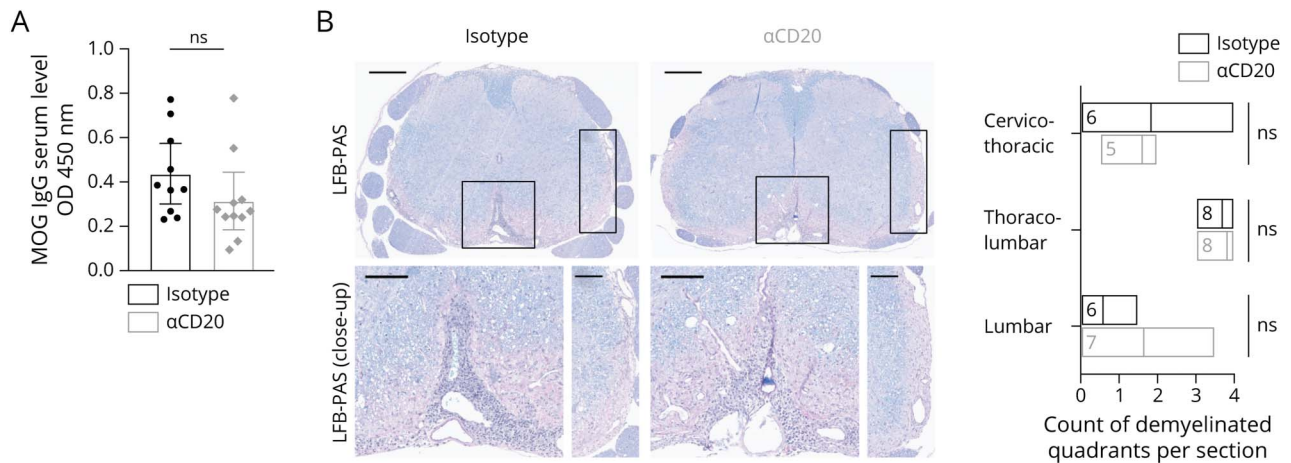
### αCD20 mAb Efficiently Depletes B Cells in the Periphery but Does Not Affect the Clinical Course of Spontaneous Chronic EAE

To analyze the effects of  $\alpha\text{CD20}$  mAb on mELT in the context of EAE, we crossed MOG Ig-VH knock-in ( $\text{IgH}^{\text{MOG}}$ , Th) mice, in which B cells express a MOG-specific BCR heavy chain, with MOG-specific TCR transgenic mice ( $\text{TCR}^{\text{MOG}}$ ,

2D2). As previously described, 2D2xTh mice develop spontaneous chronic EAE with B-cell-rich and T-cell-rich mELT along the spinal cord.<sup>23,24</sup>

2D2xTh mice were treated weekly for a total of 4 intraperitoneal (i.p.) doses with antimurine CD20 or isotype control mAb. Two treatment paradigms were pursued in parallel: (1) to prevent EAE and mELT formation, mice received the first dose as soon as they were weaned about 26 days after birth (figure 1A) and (2) to treat fully established EAE,  $\alpha\text{CD20}$  mAb was first administered after mice developed an EAE score of  $\geq 3$  (figure 3A).  $\alpha\text{CD20}$  mAb efficiently depleted B cells in the peripheral blood (figure 1B.a, upper panel) and lymph nodes (figure 1B.b, lower panel). However, its application neither attenuated the rather severe and chronic course of EAE compared with the control groups in either condition (figures 1C and 3B) nor changed the incidence of EAE. In total, 44% of preventatively treated mice developed spontaneous chronic EAE, which was similar to 39% of control treated mice (figure 1D).

**Figure 4** B-Cell-Depleted Spontaneous Chronic EAE Mice Exhibit High Titers of Anti-MOG Antibodies and Extensive Demyelination, Reflecting Their Severe Disease Course



2D2xTh mice received 4 doses of αCD20 mAb (n = 11) or isotype control (n = 10) as soon as they reached an EAE score of ≥3. (A) Antimurine MOG IgG serum levels determined by ELISA (dilution 1:300). Data are shown as individual data points and mean ± 95% CI. (B) LFB-PAS staining of the spinal cord, showing representative thoracolumbar cross-sections and corresponding close-ups. For each mouse, 2 randomly chosen cross-sections per spinal cord segment—cervicothoracic (n = 5 for αCD20 mAb and n = 6 for isotype), thoracolumbar (n = 8 per group), and lumbar (n = 7 for αCD20 mAb and n = 6 for isotype)—were examined. Data are presented as minimum and maximum values and mean. Scale bars: 250 μm and 100 μm (close-ups). Statistical significance between groups was analyzed using the Student *t* test. αCD20 mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; LFB-PAS = Luxol fast blue and periodic acid-Schiff reaction; MOG = myelin oligodendrocyte glycoprotein; ns = not significant; OD = optical density.

### mELT Formation and Retention Occurs in the Absence of B Cells in Preventatively Treated Mice

To investigate whether mELT forms and persists under B-cell-depleting conditions, we analyzed cross-sections along the spinal cord of 2D2xTh mice, which developed clinical signs of EAE during the observation/treatment period. With a mean EAE duration of 19 days (range 13–24 days) in the αCD20 mAb-treated group and 16 days (range 3–21 days) in the isotype-treated group, all mice showed mELT in at least 5 of 12–16 segments with a maximum along the thoracolumbar part of the spinal cord (figure 2A). Infiltrates were considered as mELT when their size was  $\geq 1 \times 10^4 \mu\text{m}^2$  per section. Surprisingly, the mean area of mELT per section, including sections in which no mELT was detected, was similar in both groups with a mean of  $81,152 \mu\text{m}^2$  per section in αCD20 mAb and  $103,968 \mu\text{m}^2$  in isotype-treated controls (figure 2B, upper panel). Hence, despite the absence of B cells in circulation and cervical lymph nodes, structures of mELT formed. With B cells being a main component of mELT,<sup>21,23,24</sup> we performed B220 immunohistochemistry on sections of the thoracolumbar part of the spinal cord. We found that, although mELT was present to a similar extent, mELT of αCD20 mAb-treated mice only exhibited 0.59% of B220<sup>+</sup> B cells observed in controls (figure 2B, lower panel).

Interestingly, in both groups, mice without clinically apparent EAE over the treatment period of 28 days also showed histologic signs of disease in approximately 60% of cases, ranging from scattered cellular infiltrates in the meninges in some cases to mELT in 30% of the αCD20 mAb-treated mice and 36% of the isotype controls (figure 2C). In these clinically unremarkable animals, mELT was also mainly found in the

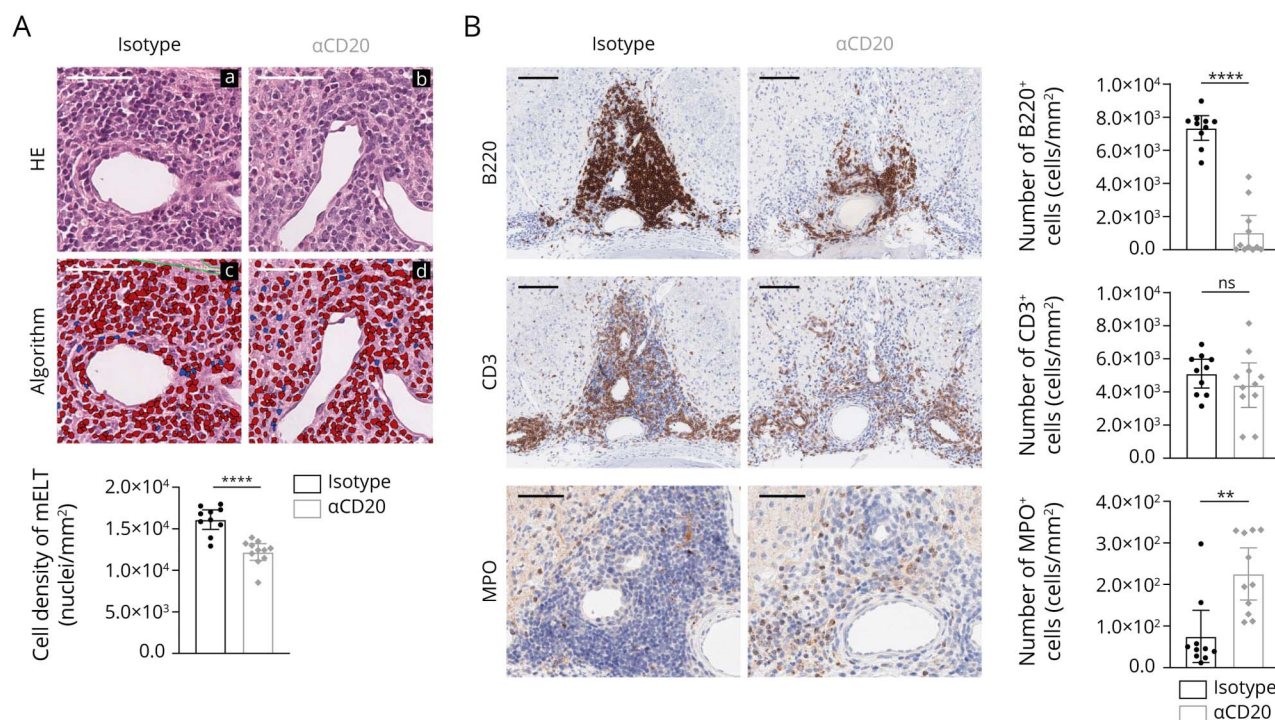
thoracolumbar part of the spinal cord and was comparable in size in both groups (figure 2D).

Our data indicate that the absence of B cells in preventatively treated 2D2xTh mice is not sufficient to prevent mELT formation and that not only mELT formation but also its retention may be independent of B cells. Moreover, mELT formation in our model occurs before developing clinical signs of EAE.

### αCD20 mAb Treatment Does Not Influence the Size of mELT

In a second experiment, more closely resembling a clinical scenario, mice were randomized into groups treated with αCD20 or control mAbs after disease onset, i.e., when having developed an EAE score of ≥3 (aged on average 47.6 days in the αCD20 mAb and 48.4 days in the isotype-treated group). As a baseline, we used mice which were euthanized without further treatment immediately after having developed a score of ≥3 (figure 3A). At that stage of disease (onset), all mice already exhibited extensive mELT with a distinct fraction of B220<sup>+</sup> B cells (figure 3C), confirming that mELT was sufficiently present before we started our intervention. After 4 weeks of treatment, distribution of mELT throughout the spinal cord was almost identical in-between groups, with all mice exhibiting mELT in at least 1 of 14–16 cross-sections analyzed (figure 3D). Again, we did not observe a difference in the mean area of mELT in both groups (figure 3E). Similar to what was observed in autopsy cases of MS, the extent of mELT correlated with disease severity in control mice. However, this was not statistically significant in αCD20 mAb-treated animals (figure 3F).

**Figure 5**  $\alpha$ CD20 mAb B-Cell Depletion in Spontaneous Chronic EAE Alters Cellular Density and Composition of mELT



2D2xTh mice received 4 doses of  $\alpha$ CD20 mAb ( $n = 11$ ) or isotype control ( $n = 10$ ) as soon as they reached an EAE score of  $\geq 3$ . (A) HE staining of representative spinal cord sections of (a) isotype-treated and (b)  $\alpha$ CD20 mAb-treated mice visualizing the cellular density of mELT (c) and (d) show representative samples of the computer-aided quantification of nuclei in mELT with the corresponding graphs. (B) Quantification of B cells (B220), T cells (CD3), and neutrophil granulocytes (MPO) in spinal cord mELT. mELT of 1–4 randomly chosen cross-sections per mouse were analyzed. Scale bars: 50  $\mu$ m (A) and 100  $\mu$ m (B). Data are presented as individual data points and mean  $\pm$  95% CI. \* $p \leq 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ; statistical significance between groups was analyzed using the Student *t* test.  $\alpha$ CD20 mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; HE = hematoxylin & eosin; mELT = meningeal ectopic lymphoid tissue; MPO = myeloperoxidase; ns = not significant.

### Persisting High Titers of Anti-MOG Antibodies and Extensive Demyelination in B-Cell-Depleted Mice Reflects the Lasting Severe and Chronic Course of EAE

Although B cells were effectively reduced not only in the periphery but also in the meningeal compartment, MOG-specific total IgG titers remained high even after 4 weeks of B-cell depletion (figure 4A). Correlating with the clinical disease course, the spinal cord was similarly affected by demyelination in  $\alpha$ CD20 mAb-treated mice compared with controls (figure 4B). Together with persisting B-cell-free mELT, these observations may contribute to the lack of a clinical benefit of B-cell depletion in our model.

### $\alpha$ CD20 mAb Treatment Changes the Density and Composition of the Cellular Content of mELT

When we analyzed the cellular composition of mELT more closely, we found that, within the treatment paradigm, mELT in  $\alpha$ CD20 mAb-treated mice was populated with 24.2% less cells (figure 5A). The count of B220<sup>+</sup> B cells, which was the largest immune cell fraction in control mice, was drastically reduced on  $\alpha$ CD20 mAb treatment, suggesting that the absence of B cells led to this reduced cellular density in mELT (figure 5B, upper panel). We did not notice a relevant change

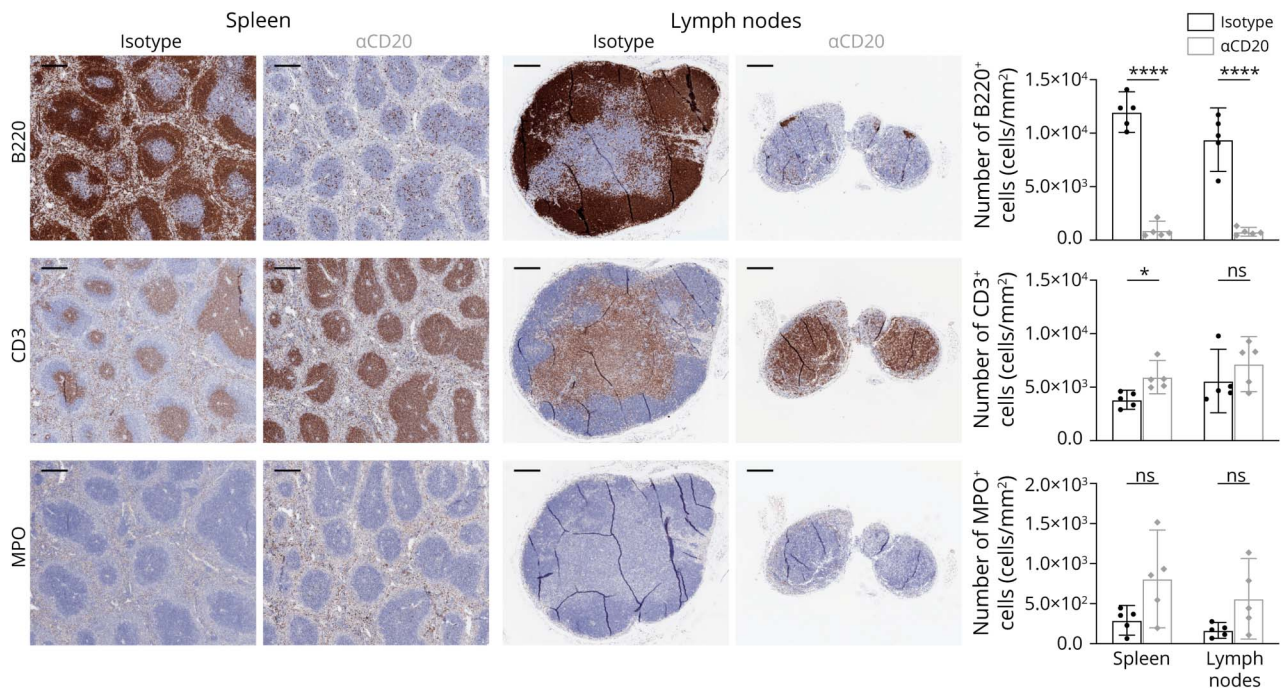
in CD3<sup>+</sup> cells (figure 5B, middle panel), whereas the number of MPO positive neutrophil granulocytes was significantly increased under  $\alpha$ CD20 mAb therapy (figure 5B, lower panel). Comparing these changes in mELT with SLOs, we observed that in the spleen and inguinal lymph nodes, B220<sup>+</sup> B cells were also largely eliminated. MPO<sup>+</sup> cells seemed to be increased, but this did not reach statistical significance. In contrast to mELT, however, the amount of CD3<sup>+</sup> T cells was slightly increased in B-cell-depleted lymph nodes (figure 6).

Together, our findings show that  $\alpha$ CD20 mAb, to some degree, depletes B cells in mELT in mice with spontaneous chronic EAE, yet mELT persists. The role and function of mELT devoid of B cells remains unclear.

### Discussion

Although its exact role in MS pathogenesis remains unclear, meningeal inflammation has gained widespread attention in recent years. Because B cells are a major component of mELT and B-cell-depleting mAbs are among the most successful MS therapeutics today, the question how B-cell-depleting therapies affect mELT is obvious. In this study, we systematically addressed this question in a murine model of MS.

**Figure 6** αCD20 mAb Efficiently Depletes B Cells in SLOs



2D2xTh mice received 4 doses of αCD20 mAb (n = 11) or isotype control (n = 10) as soon as they reached an EAE score of ≥3. Quantification of B cells (B220), T cells (CD3), and neutrophil granulocytes (MPO) in spleens and inguinal lymph nodes. One randomly chosen cross-section per organ (n = 5 mice per group) was evaluated. Scale bars: 250 μm. Data are presented as individual data points and mean ±95% CI. \**p* ≤ 0.05; \*\*\*\**p* < 0.0001; statistical significance between groups was analyzed using the Student *t* test. αCD20 mAb = anti-CD20 monoclonal antibody; EAE = experimental autoimmune encephalomyelitis; LN = lymph node; MPO = myeloperoxidase; ns = not significant; SLO = secondary lymphoid organ; SPL = spleen.

Not unexpectedly, B-cell depletion did not change the disease course in our spontaneous chronic EAE model. It has previously been reported that αCD20 mAb did not significantly change the course of EAE in the same model because high titers of MOG-binding antibodies in conjunction with MOG<sup>TCR</sup> 2D2 T cells could support clinical disease in the absence of B cells.<sup>26</sup> Importantly, αCD20 mAb does not target antibody-producing plasma cells. It could be debated that spontaneous chronic EAE in 2D2xTh mice, because of the MOG specificity of both T and B cells, is not dynamic enough to allow being modified by pharmaceutical interventions. However, laquinimod ameliorated spontaneous chronic EAE and reduced meningeal B-cell aggregates and thereby demonstrated its plasticity.<sup>27</sup> Thus, other factors are likely to contribute.

Although clinical effects were neglectable, we found that αCD20 mAb depleted virtually all B cells not only from SLOs and blood but also from mELT. It has been shown before that B cells infiltrating the meninges in EAE could be efficiently depleted by αCD20 mAb administered either intraperitoneally or intrathecally.<sup>22</sup> This study used a model, however, which does not feature large meningeal B-cell aggregates such as mELT. A case report of a patient with gastrointestinal mantle cell lymphoma, who developed progressive multifocal leukoencephalopathy after rituximab therapy, showed that 8 months after the last dose, no B cells

were found in perivascular spaces.<sup>28</sup> By contrast, a case report of a young man with aggressive MS treated with rituximab and with no detectable peripheral B cells found some B cells in the parenchyma and in perivascular cuffs on CNS autopsy.<sup>29</sup> Regarding B-cell-depleting antibodies used to treat MS, it is an encouraging finding that αCD20 mAb could deplete B cells present in mELT and prospectively prevent colonization of the meningeal compartment by B cells. Yet, open questions remain.

The most unexpected finding in our study was that mELT formed or remained in the absence of B cells when mice were B-cell-depleted after EAE was fully established and even when treated before EAE onset. The size and extent of mELT was even comparable whether they contained B cells or not. This shows that, in our model, B cells are not essential for the induction or maintenance of mELT development. Yet, in the absence of B cells, mELT was less densely packed with cells and the cellular composition was altered. αCD20 mAb-treated mice had drastically fewer B220<sup>+</sup> B cells, comparable amounts of CD3<sup>+</sup> T cells, and significantly more MPO<sup>+</sup> neutrophil granulocytes in mELT. A recent study showed that neutrophil granulocytes pioneer mELT formation and precede B cells in the meningeal compartment.<sup>30</sup> In addition, several other cell types have been proposed as candidates to induce ELT formation, including IL-22 or IL-17 secreting immune cells, dendritic cells, and CD3<sup>-</sup>CD4<sup>+</sup>CD45<sup>+</sup>



lymphoid tissue inducer cells.<sup>16,31-35</sup> Our own data showed that shortly after EAE onset, B220<sup>+</sup> B cells were already present in mELT, but after 4 weeks of isotype control treatment, their amount had roughly doubled. Thus, B cells seem to fully populate mELT at later stages. This raises the question, which role B cells play in (later stage) mELT and whether mELT without B cells still has a pathogenic role. Generally, our mice would be expected to benefit from B-cell depletion, like it has been shown in another B-cell-dependent model without mELT or in MS itself.<sup>1-3,36</sup> If mELT lacking B cells still contribute to autoimmunity, this could explain why the EAE disease course is unchanged in αCD20 mAb-treated 2D2xTh mice. As discussed above, other inherent properties of the model could contribute, such as the presence of high titers of MOG-binding antibodies and MOG<sup>TCR</sup> T cells with a potentially altered activation status under αCD20 mAb therapy. However, the notion that mELT without B cells persist in EAE and its persistence correlates with sustained severe disease, may give rise to interesting considerations: B-cell-depleting therapies are disproportionately more effective in RRMS than PMS. It could be speculated that future therapeutic approaches targeting mELT directly, instead of B cells, may be more effective in treating PMS.

We observed that approximately one-third of 2D2xTh mice without clinically manifest EAE develop a minor degree of mELT both in the presence or absence of B cells. What does this imply regarding the role of mELT? Naturally, it remains unknown whether for these mice disease was imminent and the occurrence of mELT only preceding clinical signs. Alternatively, it is possible that 2D2xTh mice live with mELT without developing a phenotype. The latter would suggest that mELT may be a rather irrelevant epiphenomenon of encephalitogenic B and T cells being present in this model or that mELT may even have a regulatory role.

One limitation of our study is that we depleted B cells relatively soon, i.e., before the onset of EAE or at an early stage of disease, where B cells are already present in mELT but to a lesser extent. We believe that it is reasonable to conclude from our results that B cells already present in mELT were depleted by αCD20 mAb. However, we could not formally assess the respective contributions of preventing B-cell influx from the periphery or direct depletion in the CNS itself to our finding that mELT was devoid of B cells after several weeks of treatment with αCD20 mAb.

It is very challenging to study meningeal pathologies in patients in vivo or even postmortem. Thus, animal models are instrumental, and yet, the transferability of any findings in such models to MS must be questioned continuously. Our model of spontaneous chronic EAE has several characteristics that make it a suitable tool. EAE develops without an external stimulus, such as immunization or an adjuvant, and mice form meningeal inflammatory aggregates. Furthermore, mELT is readily available in high quality and quantity. However, it is a highly artificial model which is genetically determined

through B-cell-mediated and T-cell-mediated autoimmunity against MOG. MS, by contrast, is not known to have a single autoantigen and certainly does not feature such a highly polarized adaptive immune system. Therefore, studies in human subjects treated with αCD20 mAb are required to confirm our results.

In conclusion, we provide evidence that systemically applied αCD20 mAbs efficiently deplete B cells in meningeal inflammatory aggregates, and yet, mELT still form and persist with an altered cellular composition. The unchanged severe course of EAE, despite near complete B-cell depletion, both in the periphery and the meningeal compartment, may point to a yet unknown function of mELT, which is independent of B cells. Future studies are required, which aim to better understand the role and pathogenic relevance of mELT with and without B cells.

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## Disclosure

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