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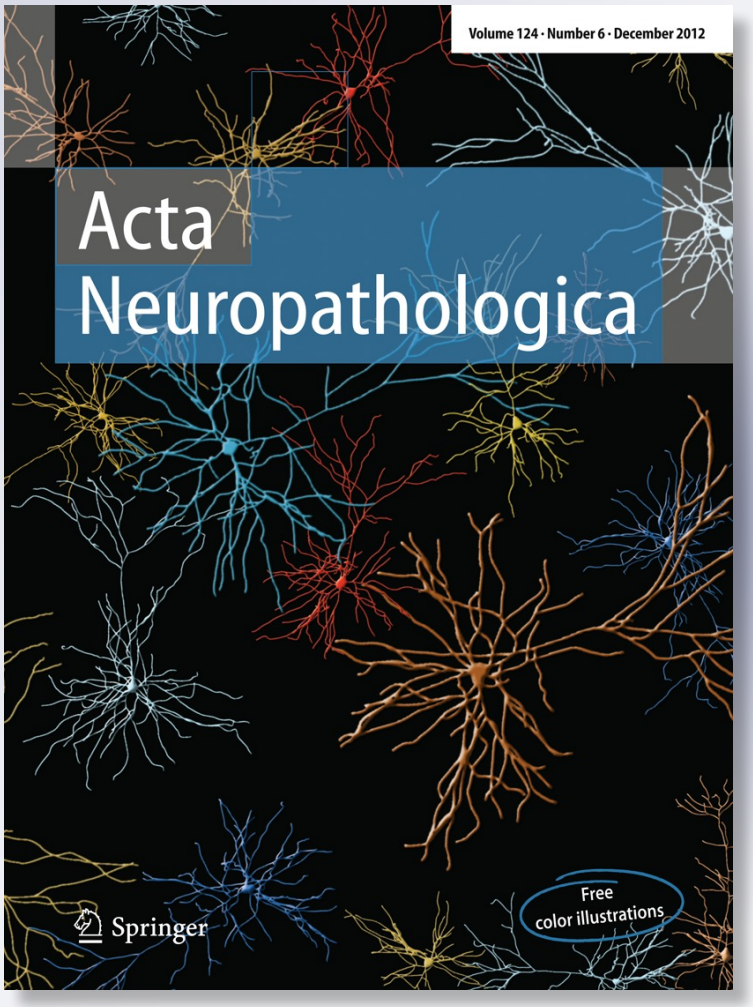

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ORIGINAL PAPER

Tertiary lymphoid organ development coincides with determinant spreading of the myelin-specific T cell response

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Abstract While the role of T cells has been studied extensively in multiple sclerosis (MS), the pathogenic contribution of B cells has only recently attracted major attention, when it was shown that B cell aggregates can develop in the meninges of a subset of MS patients and were suggested to be correlates of late-stage and more aggressive disease in this patient population. However, whether these aggregates actually exist has subsequently been questioned and their functional significance has remained unclear. Here, we studied myelin basic protein (MBP)–proteolipid protein (PLP)-induced experimental autoimmune encephalomyelitis (EAE), which is one of the few animal models for MS that is dependent on B cells. We provide evidence that B cell aggregation is reflective of lymphoid neogenesis in the central nervous system (CNS) in MBP–PLP-elicited EAE. B cell aggregation was present already few days after disease onset. With disease progression CNS B cell aggregates increasingly displayed the phenotype of tertiary lymphoid organs (TLOs). Our results further imply that these TLOs were not merely epiphenomena of the disease, but functionally active, supporting intrathecal determinant spreading of the myelin-specific T cell response. Our data suggest that the CNS is not a passive "immune-privileged" target organ, but rather a compartment, in which highly active immune responses can perpetuate and amplify the autoimmune pathology and thereby autonomously contribute to disease progression.

Keywords B cells · Determinant spreading · EAE · MS · T cells · TLO

Abbreviations

cLN	Cervical lymph node
CNS	Central nervous system
drLN	Draining lymph node
EAE	Experimental autoimmune encephalomyelitis
FDC	Follicular dendritic cell
HEV	High endothelial venule
MAdCAM	Mucosal addressin cell adhesion molecule
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MP4	MBP–PLP fusion protein
MS	Multiple sclerosis
OVA	Ovalbumin
PNAd	Peripheral node addressin
PLP	Proteolipid protein
TLO	Tertiary lymphoid organ

Introduction

The data concerning the role of B cell follicles in multiple sclerosis (MS) patients have remained controversial [1, 9,

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10, 20, 21, 28, 33, 35, 36]. A major unknown is whether the B cell follicles, even if they do exist in the CNS, are functional [1, 9, 20, 21, 33]. Here, we share our own results obtained in a novel animal model of MS, the myelin basic protein (MBP)–proteolipid protein (PLP) fusion protein MP4-induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice [11]. Unlike the majority of the commonly used EAE models, this model proved to be B cell/antibody dependent [13, 14], and is therefore ideally suited to further study the role of B cell follicles in CNS autoimmunity. We feel that our results will help clarify the ongoing controversy about the existence of B cell follicles in MS because they clearly demonstrate the presence of B cell aggregation already early on in this model of the disease and the transformation into tertiary lymphoid tissue as disease progresses to its chronic stage. In addition, our data support the concept that antigen presentation is occurring within these ectopic sites.

Accumulating evidence suggests that in MS, similar to other autoimmune diseases, B cells can aggregate into tertiary lymphoid organs (TLOs) in the target tissue, that is the cerebral leptomeninges [9, 21]. In patients with secondary-progressive MS, the presence of ectopic B cell follicles has been linked to a younger age at disease onset, irreversible disability, more pronounced demyelination, microglia activation and loss of neurites in the cerebral cortex [9, 21, 33]. In addition, a recent study of early-stage MS biopsies has suggested that an association between leptomeningeal B cell aggregates and cortical demyelination is not restricted to progressive disease, but can already occur initially [17].

In other autoimmune diseases, such as rheumatoid arthritis, autoimmune thyreoiditis, myasthenia gravis or Sjögren's syndrome chronic inflammation has been frequently associated with the formation of tertiary lymphoid organs [1, 2, 4, 7, 16, 19, 25, 32, 37]. This lymphoid reorganization of the inflamed tissue has been shown to be mainly directed by the expression of lymphotoxin ($LT\alpha_1\beta_2$) by activated B and T cells interacting with the lymphotoxin-β receptor on stromal organizer cells [1, 3–5, 31]. The structure of TLOs has been reported to be variable and there has been controversy as to the exact definition of a TLO. Overall, TLOs are thought to resemble lymph nodes [1, 7]. Major features of TLOs include T cell/B cell compartmentalization, the presence of lymphatic vessels and high endothelial venules (HEVs) as well as the expression of lymphoid chemokines such as CXCL13, CCL12, CCL19 and CCL21 [2, 8, 19, 22, 32]. In addition, follicular dendritic cell (FDC) networks have been associated with TLO formation and germinal centers have been found [6, 18, 20, 22, 27, 33]. While functionally TLOs may represent a site where pro-inflammatory mediators, autoantibodies and autoreactive T and B cells can be generated, their pathophysiological contribution to CNS autoimmunity remains to be investigated.

Materials and methods

Mice

Female C57BL/6 mice (6–8 weeks old) were purchased from Janvier (France) and maintained at the Cologne animal facilities under specific pathogen-free conditions in IVC cages. All treatments complied with the institutional guidelines.

Induction and clinical assessment of EAE

The MBP–PLP fusion protein MP4 was obtained from Alexion Pharmaceuticals (Cheshire, CT). Incomplete Freund's adjuvant (IFA) was prepared as a 1:9 mixture of mannide monooleate (Sigma-Aldrich, St. Louis, MO, USA) and paraffin oil (EMScience, Gibbstown, NJ, USA). Complete Freund's adjuvant (CFA) was obtained by mixing *Mycobacterium tuberculosis H37 RA* (Difco Laboratories, Franklin Lakes, NJ, USA) at 5 mg/ml into IFA. For the different experiments, a total of 39 mice were immunized subcutaneously in both sides of the flank with a total dose of 200 µg MP4 emulsified in CFA. Pertussis toxin (PTX; List Biological Laboratories, Hornby, ONT, Canada) was given at 200 ng per mouse on the day of immunization and 48 h later. Mice that did not receive any treatment or were immunized with PBS in CFA were used as controls ($n = 10$). Clinical assessment of EAE was performed daily according to the standard EAE scale: (0), no disease; (1), floppy tail; (2), hind limb weakness; (3), full hind limb paralysis; (4), quadriplegia; (5), death. Mice that were in between the clear-cut gradations of clinical signs were scored intermediate in increments of 0.5. Overall, it should be noted, that the term "chronic EAE" used in this study needs to be considered with caution as 30–57 days of disease in mice naturally do not equate the chronic disease of MS patients.

Histological analysis

Histological analysis of CNS samples was done as described in detail previously [30]. Briefly, a total of 27 mice ($n = 10$, control mice; $n = 8$, mice with acute EAE; $n = 9$, mice with chronic EAE) were sacrificed with CO_2 and cerebrum, cerebellum, brain stem and spinal cord were carefully removed and snap-frozen in liquid nitrogen. Cryostat sections (7 µm thick) were obtained that covered the entire tissue of each specimen. For the staining of IFN-γ, IL-17 and reticulin we used paraffin-embedded cerebellum

sections. These were obtained from mice ($n = 4$) that had been transcardially perfused with 4 % paraformaldehyde (PFA). Every 10th section was stained with hematoxylin and eosin to screen for infiltration. The regions that showed infiltration were then subjected to immunohistochemical analysis. An overview of the antibodies used for the assessment of TLO formation and CNS histopathology is provided in Table 1. Specificity controls included the omission of primary and secondary antibodies. Cellular nuclei were stained with Hoechst 33342 (Thermo Scientific). Sections were incubated with Hoechst 33342 at 1:1,000 dilution in PBS for 10 min at room temperature. Reticulin fibers were stained by Novotny silver impregnation [26]. Sections were analyzed on a Zeiss Axioskop 50 epifluorescence microscope using Carl Zeiss Plan-NEOFLUAR 10×/0.30, 20×/0.5 and 40×/1.30 objectives. For fluorescence analysis Carl Zeiss filter sets No. 1 (excitation BP 365/12, emission LP 397), No. 10 (excitation 450–490 nm, emission 515–565 nm) and No. 15 (excitation BP 546/12 nm, emission 590 nm) were used, respectively. Digital images were acquired using a Leica DFC350FX camera and software. Light microscopic analysis was performed on a Leica DM LB2 microscope with a Zeiss AxioCam MRc camera and AxioVision 40 4.7 software (Carl Zeiss AG, Oberkochen, Germany).

ELISPOT

Spleen, draining and cervical lymph node and CNS cells were isolated from MP4-immunized mice on days 3.9 ± 0.9 (acute EAE) and 34.2 ± 4.9 (chronic EAE) after disease onset, respectively, with 9 individual mice in both groups. Low volume Whatman UNIFILTER plates (Whatman, Maidstone, Kent, UK) were coated with anti-mouse AN-18 (eBioscience, San Diego, CA, USA) at 3 μg/ml for capturing IFN-γ or rat anti-mouse TC11-18H10 at 4 μg/ml for capturing IL-17. Plates were blocked with 1 % BSA in sterile PBS for 2 h and subsequently incubated for 24 h at 37 °C and 7 % CO_2 with the cells isolated from the various organs. Cells were diluted in HL-1 (Lonza, Cologne, Germany) containing 1 % L-glutamine (Sigma, Schnelldorf, Germany), 1 % penicillin/streptomycin (Sigma) and plated at 5×10^5 cells/per well for spleen and lymph node testing and at 1×10^5 cells/well for testing of CNS-derived cells. CNS-derived cells were isolated from the spinal cord and cerebellum. MP4 (Alexion) was used at 60 μg/ml, MOG peptide 35-55 (Probiodrug AG, Halle/Saale, Germany) at 45 μg/ml and OVA (Sigma, Schnelldorf, Germany) at 30 μg/ml final concentration. The secondary antibodies used were biotin-conjugated rat anti-mouse R4-6A2 (eBioscience) at 1 μg/ml for the detection of IFN-γ and biotin-conjugated rat anti-mouse TC11-8H4.1 for the detection of IL-17. All plates were developed with Vector Blue substrate (Vector Laboratories, Burlingame, CA, USA) after incubation with Streptavidin-AP (Dako) at 1:1,000 dilution. Spots were counted and analyzed on an ImmunoSpot Series 5 Analyzer (Cellular Technology Limited, Cleveland, OH, USA).

Statistics

Statistical significance of the differences was evaluated using unpaired two-tailed Student's t test. Statistical significance was set at $P \leq 0.05$.

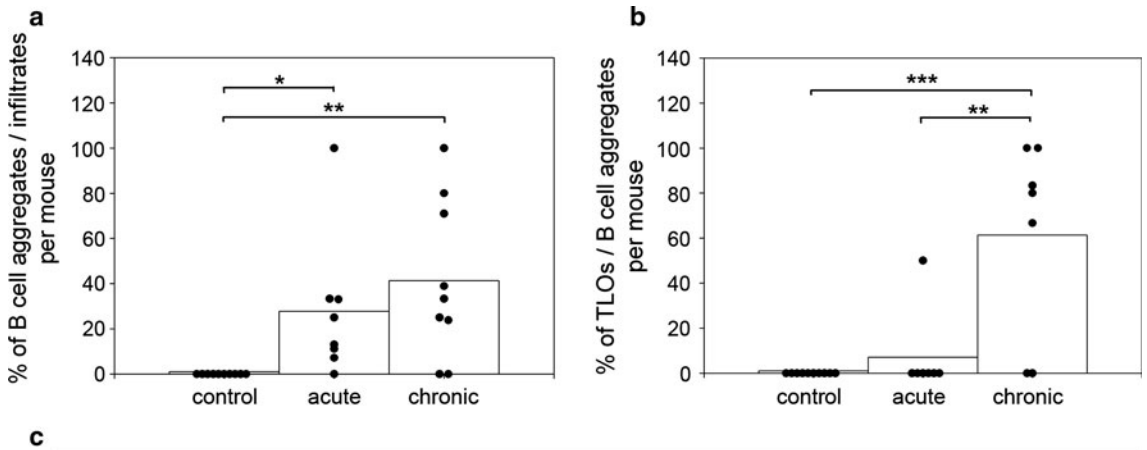
Results

TLOs develop in the CNS in the chronic stage of MP4-induced EAE

We screened CNS tissue of mice undergoing MP4-induced EAE for the hallmarks of TLO development. In each of the mice, the total numbers of cerebral, cerebellar, brain stem, and spinal cord inflammatory infiltrates were assessed on hematoxylin/eosin-stained sections spanning the entire organ. These infiltrates were stained for B cell aggregation. The percentage of infiltrates that displayed B cell aggregation was assessed per mouse and the mean value was calculated for each disease stage. A B cell aggregate was defined as an infiltrate with more than 20 aggregated B cells. Overall, 69 infiltrates were analyzed in acute EAE and 83 in chronic EAE. Already in acute EAE, between 1 and 17 days after disease onset B cell clustering/aggregation within the CNS infiltrates was observed in 7 of 8 mice analyzed with a mean value of 27.8 ± 31.6 %. In chronic EAE tested on days 30–57 after disease onset, when disease severity had reached a stable plateau, 7 of 9 mice had B cell aggregates in the CNS and the percentage increased to 41.1 ± 35.2 % (Fig. 1). B cell aggregates were found in or in association with the spinal cord meninges as well as in the perivascular space and parenchyma of brain and spinal cord. Overall, there was no correlation between the number of B cell aggregates and either the time point after EAE onset ($r^2 = 0.012$) or disease severity ($r^2 = 0.001$). Next, we evaluated the presence of TLOs in the CNS of MP4-immunized mice displaying acute or chronic EAE. For a B cell aggregate to classify as TLO, B cell/T cell compartmentalization and the presence of $PNAd^+$ and/or $MAdCAM^+$ HEVs with the typical cuboidal morphology was required as well as at least one of the following features such as the presence of reticulin fibers, follicular dendritic cells (FDCs), $CD138^+$ plasma cells, proliferative activity in B and/or T cells (Ki67 staining) and activation-induced cytidine deaminase (AICD) expression indicating somatic hypermutation (Fig. 2) [1, 3, 9, 10, 14, 16]. In particular

Table 1 Combination of antibodies used for immunofluorescence staining

Primary Ab (clone) [specificity]	Host	Source	Dilution	Secondary Ab	Host	Source	Tertiary reagent	Source
AICD [somatic hypermutation]	Rat	eBioscience	1:50	IgG-biotin	Rabbit	Dako	Streptavidin-HRP/DAB	Dako
B220 (RA3-6B2) [B cells]	Rat	eBioscience	1:1,000 (1:500 when used with Ki67)	IgG-biotin	Rabbit	Dako	Neutravidin-Dylight549 (Neutravidin-Dylight488 when used with Ki67)	Thermo scientific
CD4 (GK1.5) [$CD4^+$ T cells]	Rat	eBioscience	1:500	IgG-biotin	Rabbit	Dako	Neutravidin-Dylight549 (Neutravidin-Dylight488 when used with Ki67)	Thermo scientific
CD138 (281-2) [plasma cells]	Rat	BD Pharmingen	1:200	IgG-biotin	Rabbit	Dako	Neutravidin- Dylight549	Thermo scientific
FDC (FDC-M1)	Rat	BD Pharmingen	1:50	IgG-Cy3	Goat	Jackson ImmunoResearch		
IFN-γ [expressed by T_H1 cells]	Rat	Abcam	1:100	IgG-biotin	Rabbit	Dako	Neutravidin-Dylight549	Thermo scientific
IL-17 [expressed by T_H17 cells]	Rabbit	Abcam	1:100	IgG-Dylight549	Goat	Thermo scientific		
Ki67 [proliferation]	Rabbit	Abcam	1:1,000	IgG-Dylight549	Goat	Thermo scientific		
PNAd (MECA-79) [HEV]	Rat	BD Pharmingen	1:100	IgG-Cy2	Goat	Jackson ImmunoResearch		
MAdCAM-1 (MECA-364) [HEV]	Rat	BD Pharmingen	1:100	IgG-biotin	Rabbit	Dako	Neutravidin-Dylight549	Thermo scientific
SMI-99 [MBP]	Mouse	Covance	1:800	IgG-biotin	M.O.M. kit	Vector laboratories	Neutravidin-Dylight549	Thermo scientific
SMI-312 [hyperphosporylated neurofilament H]	Mouse	Covance	1:800	IgG-biotin	M.O.M. kit	Vector laboratories	Neutravidin-Dylight549	Thermo scientific
Hoechst 33342 [nuclear DNA dye]		Thermo scientific	1:1,000					



c

# of mice tested	Disease stage	Days after disease onset[a]	Clinical score	% of CNS lesions with B cell aggregates[b]	% of B cell aggregates that qualified as TLO[b,c]
8	**Acute**	1-17	2.1 ± 0.7	27.8 ± 31.6	7.1 ± 18.9
9	**Chronic**	30-57	1.9 ± 0.5	41.4 ± 35.2	61.4 ± 43.5
P		< 0.01	0.524	0.417	0.010

[a]Disease onset occurred on day 11.8 ± 3.4 after immunization.

[b]Statistical analysis was based on the number of animals in each group.

[c]TLOs were defined as B cell aggregates containing B cell/T cell compartmentalization, $PNAd^+$ and/or $MAdCAM^+$ HEVs and at least one additional characteristic feature such as FDCs, plasma cells, proliferative activity in B and/or T cells, reticulin fibers and AICD expression.

Fig. 1 Kinetics of B cell aggregate and TLO development in MP4-induced EAE. C57BL/6 mice were immunized with 200 µg MP4 in CFA. Pertussis toxin was given on the day of immunization and 48 h later. CNS tissue was obtained in acute EAE (at the peak after disease onset, i.e., days 1–17 after disease onset) and in chronic EAE when the disease had been fully established for at least 30 days (days 30–57 after disease onset). The number of inflammatory infiltrates was identified in cerebrum, cerebellum, spinal cord and brain stem by HE staining and the percentage of infiltrates that classified as B cell aggregates was then determined by immunohistochemistry using anti-B220 antibody (see "Materials and methods"). Subsequent identification of B cell aggregates that classified as TLOs was conducted as depicted in detail in Fig. 2. **a**, **c** Percentage of B cell aggregates in non-immunized controls ($n = 10$) and MP4-immunized mice displaying acute ($n = 8$) or chronic ($n = 9$) EAE. The individual data points represent the percentages obtained from individual mice. **b**, **c** Each B cell aggregate was analyzed for TLO markers. Again, the individual data points presented represent the percentages obtained from individual mice. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. The percentages in (**c**) are given as mean values ± standard deviation

FDC networks, which have been suggested to be highly associated with B cell follicle formation in previous publications [1, 9, 20, 21], were evident in about 80 % of the TLOs. As above, the percentage of B cell aggregates that qualified as TLOs was assessed per mouse and the mean value was calculated for each disease stage. In acute EAE, this mean value was 6.3 ± 17.7 %. However, it should be noted that only one mouse was TLO positive (with a disease duration of 8 days post-EAE onset). In chronic EAE, 5 of 7 mice displayed B cell aggregates that qualified as TLOs and the mean value increased to 61.4 ± 43.5 % ($P = 0.010$) (Figs. 1, 3). Again, there was no correlation between the number of TLOs and the clinical disease severity ($r^2 = -0.014$). This absence of correlation between B cell aggregates/TLOs and the clinical score can be explained by the fact that most B cell aggregates and TLOs were found in the cerebellum, while the clinical severity in EAE is mainly caused by spinal cord pathology.

The topography of TLO formation in MP4-induced EAE

We also determined the topography of CNS TLO formation screening the spinal cord, brain stem, cerebellum and

cerebrum. Figure 4 shows that TLOs were most frequently found in the cerebellar parenchyma. TLOs were also located in the cerebral periventricular region as well as in the spinal cord parenchyma, with frequent association with the meninges in the latter. Parenchymal infiltration was typically perivascular. TLOs were absent in the brain stem. TLOs of purely leptomeningeal localization were also absent.

The overall distribution of TLOs corresponded to our previously published results on the lesion distribution in MP4-induced EAE [12]. While spinal cord and cerebral involvement was evident directly after disease onset, infiltrates in the cerebellum developed only subsequently starting around day 25 after immunization (corresponding to day 15 after EAE onset). In the present study, the majority of TLOs were found in the cerebellum. Sixty percent of all cerebellar infiltrates qualified as TLO compared to 33 % in the cerebrum and 36 % in the spinal cord. It should also be noted that only six infiltrates were found in the cerebrum in chronic EAE, which corresponds to our previous data that cerebral involvement vanishes over time.

Finally, we also assessed the extent of demyelination and axonal damage in animals that did and did not display TLO formation. However, we did not observe any differences between the two groups. Representative images of diffuse infiltration, a B cell aggregate and a TLO in relation to demyelination and axonal damage are shown in Fig. 5.

The antigen-specific T cell response shows determinant spreading to myelin oligodendrocyte glycoprotein (MOG) in chronic MP4-induced EAE

The presence of organized lymphoid tissue in which proliferation of lymphocytes and somatic hypermutation of B cells are evident suggests that the TLOs represent sites of local immune activity in chronic disease. Determinant spreading is a phenomenon during which a first wave of effector T cells engages in a second wave of autoimmunity against unrelated autoantigens of the same target organ [15, 24]. In previous studies, it has been shown that determinant spreading to additional myelin antigens is first observed in the CNS itself [23, 34]. We tested whether MOG peptide-specific T cells could be detected in the CNS of mice immunized with MP4. In addition to MP4-specific T cells secreting specific IFN-γ/IL-17 in acute and chronic EAE (Fig. 6a, c, upper row), MOG peptide-specific IFN-γ/IL-17 producing T cells were regularly detected in the CNS in the chronic stage of the disease while, with the exception of one mouse, they were absent during acute disease (Fig. 6a, c, middle row). The MOG peptide-specific IFN-γ/IL-17 response was restricted to the CNS and absent in spleen, draining and cervical lymph nodes (Fig. 6c, middle row). There was no antigen-specific IFN-γ/IL-17 response specific for third-party antigens such as ovalbumin (OVA) (Fig. 6a, c, lower row).

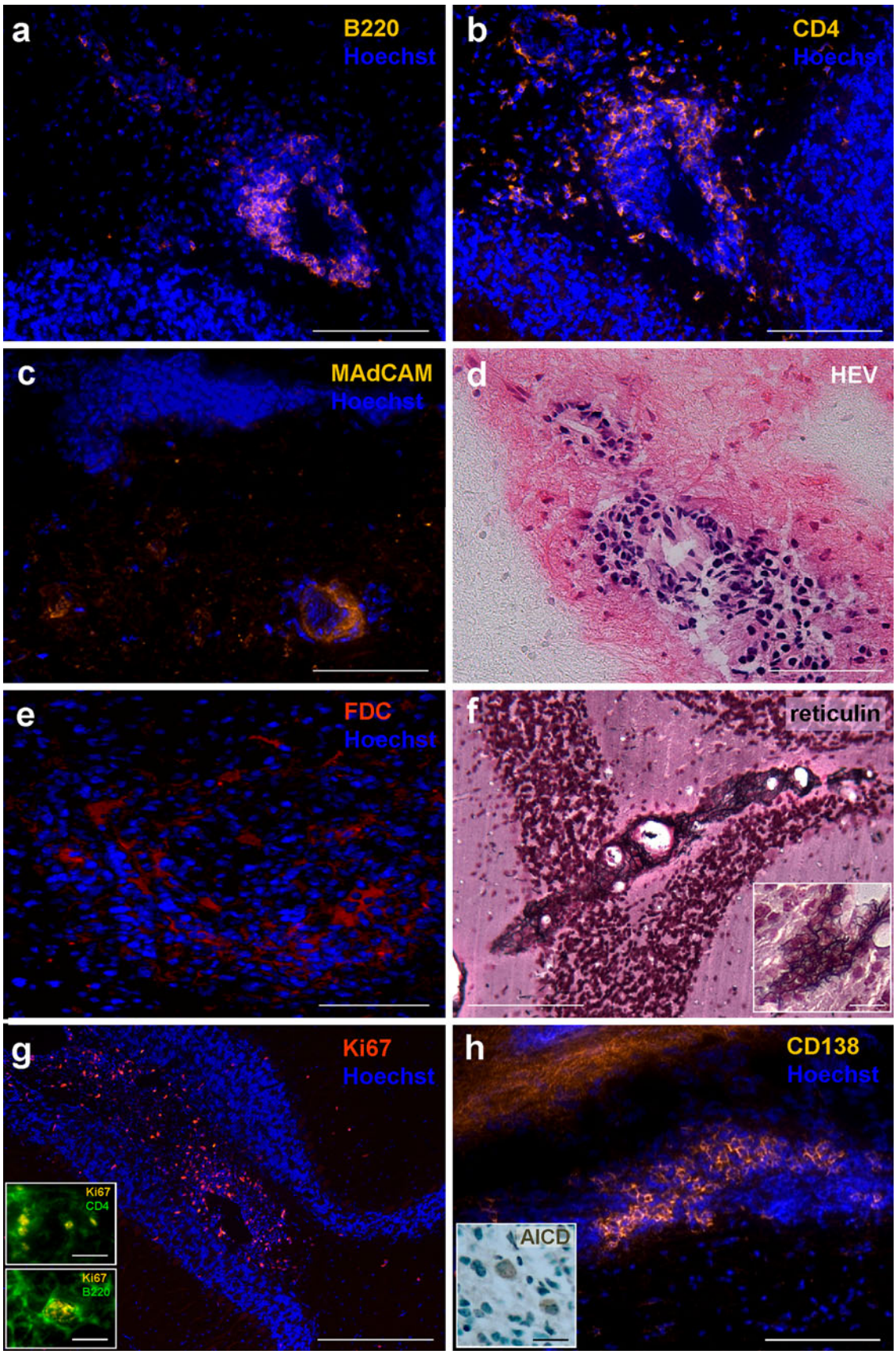
Fig. 2 Characteristic histological features of TLOs in MP4-induced EAE. In the same mice as used in Fig. 1, TLOs were identified as B cell aggregates showing $B220^+$ B cell/$CD4^+$ T cell compartmentalization (**a**, **b**), $MAdCAM^+$ or $PNAd^+$ HEVs (**c**, **d**) and additional characteristic features, which comprised the presence of FDCs (**e**), reticulin fibers (**f**), proliferation (Ki67) of B and T cells (**g**), $CD138^+$ plasma cells (**h**) and AICD expression (*inset* in **h**) indicating somatic hypermutation. Cellular nuclei were stained with Hoechst 33342. Main panel *scale bars* 100 μm, except for **e** (50 μm); inset *scale bars* 12 μm (**f**), 25 μm (**g**, *upper inset*), 6 μm (**g**, *lower inset*) and 20 μm (**h**). Details on the antibodies used for immunohistochemistry are provided in Table 1 ▶

T_H17 cells are present in CNS TLOs, while T_H1 cells are absent

It has recently been suggested in an adoptive transfer model of MOG:35-55 EAE that T_H17 cells are the major T effector cell subset involved in CNS TLO formation [29]. A similar role for T_H17 cells has been proposed for the context of transplantation [6]. Here, we provide functional evidence that both T_H1 and T_H17 cells show determinant spreading to other myelin antigens (Fig. 6). To determine the potentially differential involvement of T_H1 and T_H17 cells in B cell aggregation and TLO formation, we performed histological lesion analysis by staining the TLOs for IFN-γ and IL-17. The results are shown in Fig. 7. While T_H17 cells were frequently found in association with the TLOs (Fig. 7c, e), T_H1 cells were absent (Fig. 7d, f).

Discussion

The data presented here show that CNS TLO development is a characteristic feature of chronic MP4-induced EAE. The data are in line with previous studies that demonstrated B cell aggregation in the meninges of MS patients and in two additional murine model of MS, the PLP peptide 139-151-induced EAE of SJL mice and the spinal cord homogenate-induced EAE of Biozzi AB/H mice [20]. In the murine study, CXCL13 and FDCs were colocalized with the B cell aggregates. However, it should be noted that there were no data on the coexpression of both CXCL13 and FDCs within the same B cell aggregates. Data on other typical features of B cell follicles were also not included. In another murine study, B cell follicle-like structures were morphologically defined as B cell clusters surrounded by T cells and collagen-positive fibers [29]. In the studies of MS tissue samples, B cell-rich lymphocytic aggregates were screened for the presence of FDCs, proliferating B cells and plasma cells to characterize B cell follicular-like structures [9, 20].



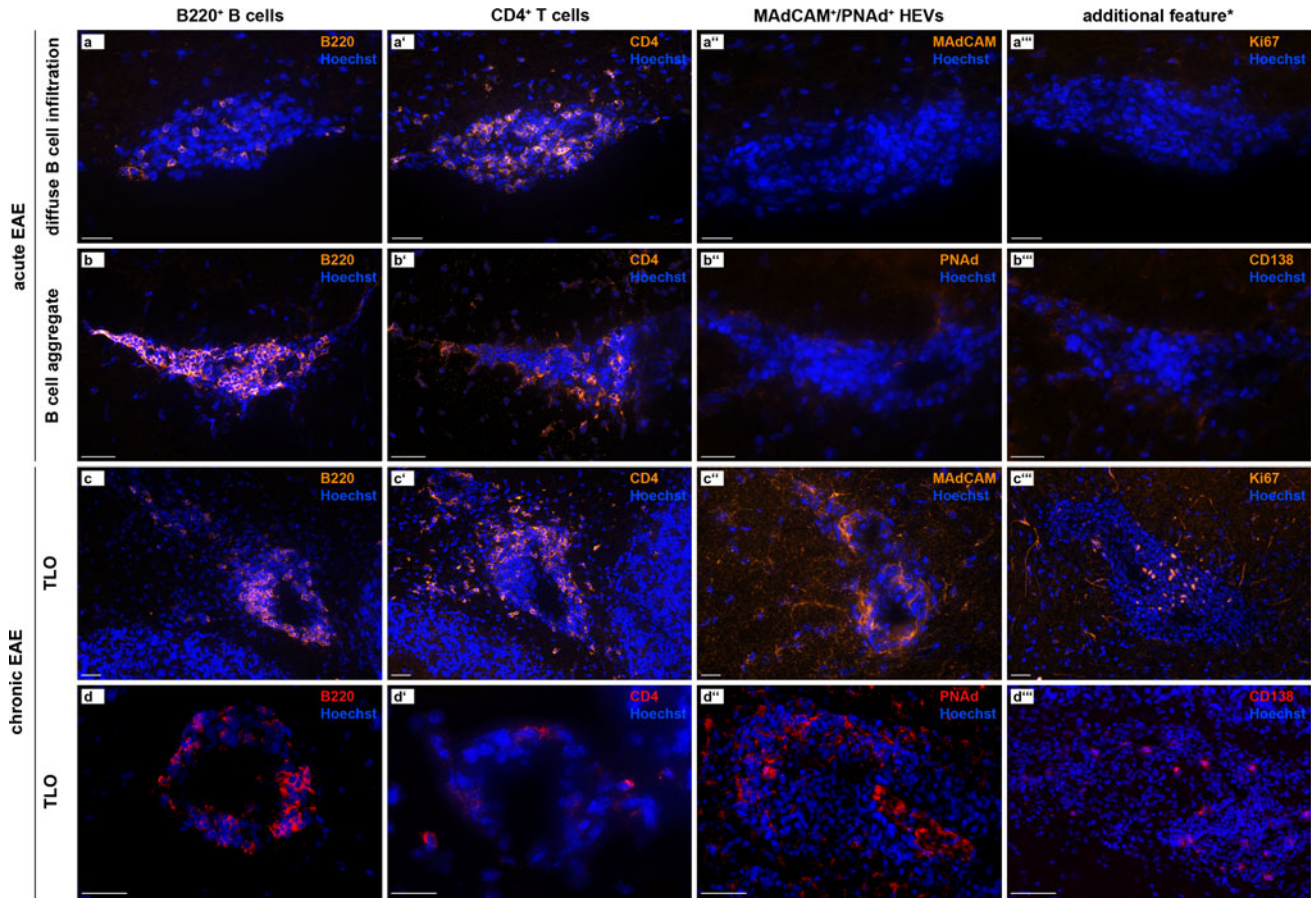


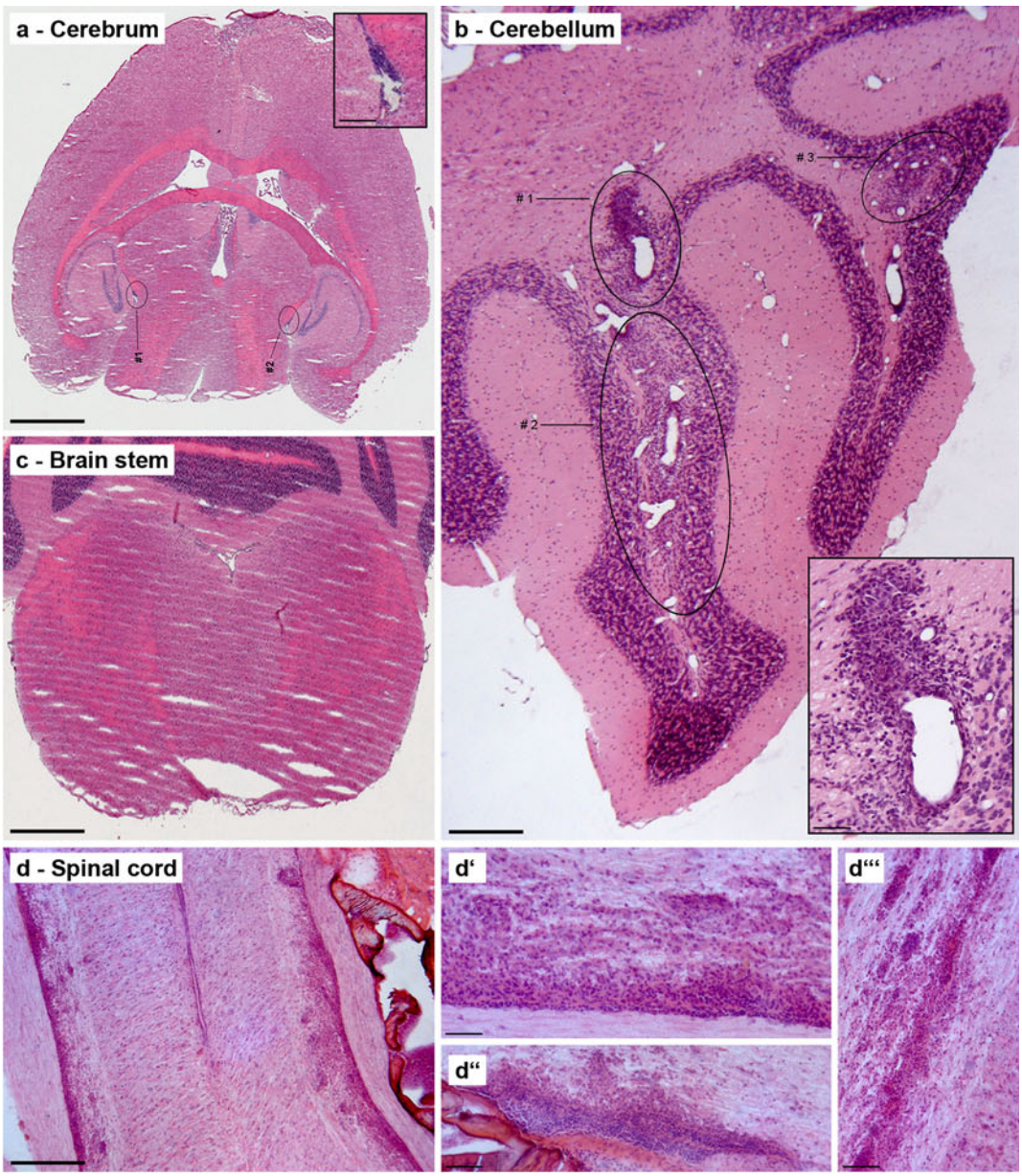
Fig. 3 Patterns of B cell infiltration in MP4-induced EAE. **a**–**a**‴ Representative diffuse B cell infiltrate. **b**–**b**‴ Representative B cell aggregate that did not classify as TLO. **c**–**c**‴ and **d**–**d**‴ Representative B cell aggregates that classified as TLOs. All *scale bars* represent 50 μm

In the present study, we more rigidly defined a B cell follicle or TLO as a B cell aggregate that contained B cell and T cell compartmentalization, HEVs as well as one additional characteristic feature of lymphoid tissue. We supposed that in particular HEVs are highly specialized adaptations of lymphoid tissue that enable naïve lymphocytes to egress from the circulation to engage in adaptive immune activation. Similarly, we considered B cell and T cell compartmentalization to be unique to lymphoid tissue.

Studying the kinetics of TLO formation, our data are in line with the previous studies on murine and human tissue demonstrating that TLOs or B cell follicles are a characteristic feature of chronic disease [9, 20]. However, differences applied to the topography of TLO formation. It has previously been suggested that in CNS autoimmunity B cell follicles can develop in the meninges, but not in white-matter lesions [9, 20, 21, 33]. Tissue fibroblasts were considered to be important stromal components needed for TLO formation, providing the functional scaffold for the retention and compartmentalization of lymphocytes after extravasation into the inflamed tissue [1]. Fibroblasts are present in the meninges, but absent in the neural parenchyma. The current study points to the fact that TLOs can essentially develop at any site at which chronic inflammation occurs and provides a permissive environment, thus also including the CNS parenchyma. Thus, this study challenges the conclusion drawn from the study of human tissue that a fibroblast-rich setting is an obligatory condition for TLO formation [1]. It is possible that further in-depth studies on human tissue with focus on the parenchyma rather than the meninges will reveal the additional presence of parenchymal B cell follicles.

Fig. 4 Topography of B cell aggregation and TLO formation in MP4-induced EAE. In the same mice as used in Fig. 1, the topography of B cell aggregates and TLOs was determined by screening HE-stained cerebrum (**a**), cerebellum (**b**), brain stem (**c**) and spinal cord (**d**) sections. **d**′–**d**‴ Higher magnifications of typical spinal cord infiltrates. Typical regions that were subjected to further immunohistochemical analysis are *circled* in the panels. The *scale bar* represents 1.65 mm in the cerebrum (50 μm in the *inset*), 200 μm in the cerebellum (50 μm in the *inset*), 750 μm in the brainstem and 350 μm in the spinal cord (70 μm in the *insets*). The data are summarized in **e** ▶

Furthermore, our data demonstrate T cell determinant spreading to MOG in chronic MP4-induced EAE, which



e

Disease stage	CNS region	# of meningeal B cell aggregates that qualified as TLO	# of parenchymal B cell aggregates that qualified as TLO	Total # of TLO[a]	Regional distribution of the 5 TLO positive animals
Chronic	Cerebrum	2/2 (peri-/intra-ventricular)	0/0	2 (11%)	2/5 (40%)
	Cerebellum	0/0	11/15	11 (61%)	4/5 (80%)
	Brain stem	0/0	0/0	0	0/5 (0%)
	Spinal cord	4/5 (infiltrating the parenchyma)	1/1	5 (28%)	4/5 (80%)

[a]The percentage refers to the number of TLOs found in the specified tissue in relation to the total number of TLOs present in chronic EAE.

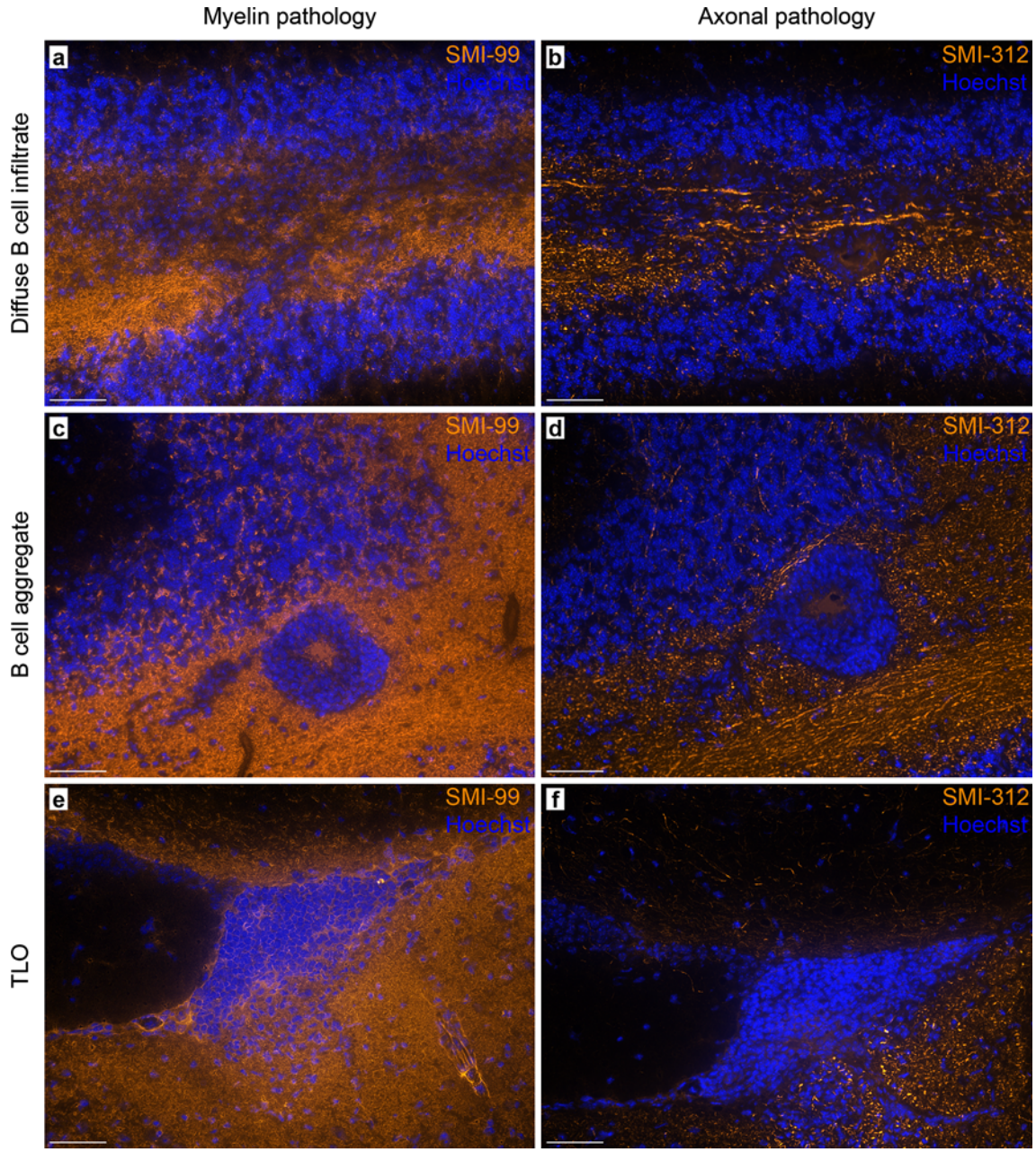


Fig. 5 For analysis of myelin and axonal pathology tissue sections were stained with antibodies against SMI-99 and SMI-312 (for antibody details see Table 1). Cellular nuclei were stained with Hoechst 33342. The *panels* show representative images of the staining patterns in diffuse B cell infiltrates (**a**, **b**), in a B cell aggregate that did not classify as TLO (**c**, **d**) and in B cell aggregates that qualified as TLO (**e**, **f**). *Scale bars* represent 50 μm

coincided with the formation of TLOs. We acknowledge that direct evidence for the determinant spreading response within the TLOs is missing. However, we suggest that the data support the notion that in MP4-induced EAE naïve T cells enter the CNS, where they are primed to new antigens, including MOG. Supporting this concept, MOG-specific IFN-γ and IL-17 producing T cells were present in the CNS, but absent in cervical and inguinal lymph nodes or spleen (Fig. 6). In addition, we found approximately 20 % of T cells within the TLOs in an actively proliferating state suggesting that the TLOs are foci for generating de novo T cell responses to CNS antigens.

Our data also support the concept that T_H17 cells are closely associated with TLO formation, while at this point the functional relationship between T_H17 cells and TLOs remains unclear and clearly needs to be addressed in future studies. In the light of these data, it was interesting to observe that also the T_H1 response displayed determinant

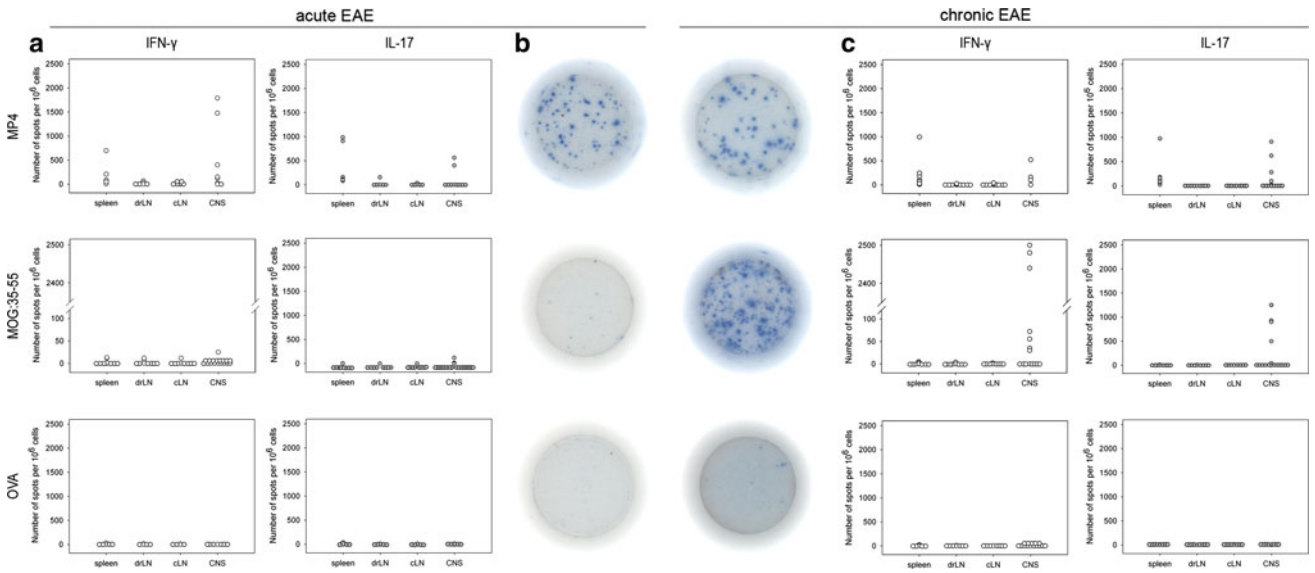


Fig. 6 Determinant spreading of the autoreactive T cell response coincided with TLO development. **a**, **c** Spleen, draining (drLN) and cervical lymph node (cLN) as well as CNS-derived cells from MP4-immunized mice displaying acute (**a**) or chronic (**c**) EAE were stimulated with MP4 (*upper row*), MOG:35-55- (*middle row*) or ovalbumin (OVA, *lower row*) in IFN-γ or IL-17 ELISPOT assays. **b** Representative well images of the antigen-specific IFN-γ response of cells isolated from the CNS after restimulation with MP4, MOG:35-55 or OVA in acute (*left row*) and chronic EAE (*right row*). The data are representative of eight independent experiments

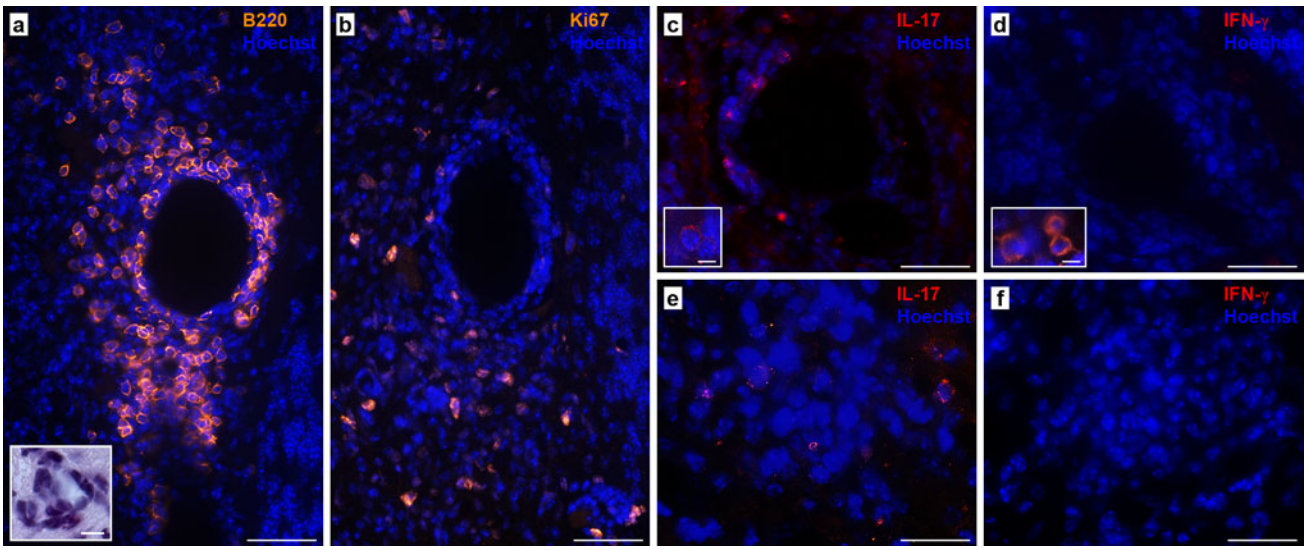


Fig. 7 Analysis of the T_H1/T_H17 distribution in TLOs of MP4-immunized mice. EAE was induced as described in Fig. 1. The tissue was formalin-fixed and paraffin-embedded. Four micrometer thick sections were obtained from the cerebellum of $n = 4$ mice and infiltrates that identified as TLOs as previously described were stained with antibodies against IL-17 and IFN-γ (for antibody details see Table 1). Cellular nuclei were stained with Hoechst 33342. Representative images of a TLO displaying $B220^+$ B cell aggregation (**a**), proliferation (**b**), the presence of HEVs (*inset* in **a**) and IL-17^+ (**c**, **e**, *inset* in **c**), but not IFN-γ^+ (**d**, **f**) cells. The *inset* in **d** is meant as a positive staining control, showing IFN-γ^+ cells in the spinal cord, which were not associated with a TLO. Main panel *scale bars* 30 μm; inset *scale bars* 7.5 μm (**a**), 5 μm (**c**, **d**)

spreading to MOG:35-55 in chronic EAE. According to our model HEVs are present in TLOs, connecting these organs to the recirculating naïve T cell pool and attracting naïve T cells to the TLO sites. Naïve T cells that enter the TLOs are still uncommitted in respect to cytokine production and do not produce cytokines when encountering antigen for the first time. In the TLOs these cells recognize antigen and undergo proliferation and differentiation from which they emerge as committed cytokine expressing $CD4^+$ effector cells of the T_H1 type producing IFN-γ and of the T_H17

lineage capable of producing IL-17. When such cells are isolated from the CNS, restimulation with antigen makes both cell types visible. In situ we could detect IL-17 producing cells only. On the one hand, this may be a consequence of T_H17 cells staying resident in the TLOs, while IFN-γ producing cells disseminate in the tissue (i.e., both T_H1 and T_H17 cells follow different recirculation pathways). On the other hand, the activation threshold for T_H17 cells may be lower than that of IFN-γ producing T_H1 cells so that T_H17 cells become locally activated in the CNS lymphoid follicle (and thus visible by immunohistochemistry), while the activation of T_H1 cells occurs outside the CNS or in non-TLO tissue.

In conclusion, our results not only strongly support the concept that B cell aggregates can acquire the phenotype of TLOs, but also suggest that these TLOs take over relevant functions in the disease process. In the present study, the spreading of the autoreactive T cell response coincided with the formation of TLOs in chronic EAE. The data imply that TLOs are not solely epiphenomena that emerge in the context of chronic inflammation, but rather ectopic lymphoid sites, which are involved in the diversification and progression of the disease process and detached from the immune processes that take place in peripheral lymph nodes. Considering what has already been published [9, 21], it is conceivable that the formation of TLOs in MS patients is associated with the transition from a remitting–relapsing to a secondary-progressive disease outcome. The notion that highly active immune responses can perpetuate and amplify autoimmune pathology in the "immune-privileged" CNS itself, provides a paradigm shift with broad diagnostic and therapeutic implications. On the one hand, it will be worthwhile to consider the disruption of TLOs as a therapeutic strategy aimed at preventing or delaying disease progression in MS and other autoimmune diseases. On the other hand, better options to longitudinally monitor the development of TLOs by MRI or other imaging techniques will offer new possibilities of disease prediction and early intervention. This concept is supported by the fact that these structures can reach a considerable size (up to 0.16 mm^2) and diameter (up to 1.1 mm), which make them a reasonable target for neuroimaging strategies.

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Conflict of interest The authors declare that they have no conflict of interest.

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