Pathogenicity of human antibodies against myelin oligodendrocyte glycoprotein

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Abstract

Objective: Autoantibodies against myelin oligodendrocyte glycoprotein (MOG) occur in a proportion of patients with inflammatory demyelinating diseases of the CNS. We analyzed their pathogenic activity by affinity-purifying these Abs from patients and transferring them to experimental animals.

Methods: Patients with Abs to MOG were identified by cell-based assay. We determined the cross-reactivity to rodent MOG and determined the recognized MOG-epitopes. We produced the correctly folded extracellular domain of MOG and affinity-purified MOG-specific Abs from the blood of patients. These purified Abs were used to stain CNS tissue and transferred in two models of experimental autoimmune encephalomyelitis. Animals were analyzed histopathologically.

Results: We identified 17 patients with MOG Abs from our outpatient clinic and selected two with a cross-reactivity to rodent MOG; both had recurrent optic neuritis. Affinity-purified Abs recognized MOG on transfected cells and stained myelin in tissue sections. The Abs from the two patients recognized different epitopes on MOG, the CC' and the FG loop. In both patients these Abs persisted during our observation period of 2-3 years. The anti-MOG Abs from both patients were pathogenic upon intrathecal injection in two different rat models. Together with cognate MOG-specific T cells, these Abs enhanced T cell infiltration; together with MBP-specific T cells, they induced demyelination associated with deposition of C9neo, resembling a multiple sclerosis type II pathology.

Interpretation: MOG-specific Abs affinity purified from patients with inflammatory demyelinating disease induce pathological changes in vivo upon co-transfer with myelin-reactive T cells, suggesting that these Abs are similarly pathogenic in patients.

Introduction

High levels of antibodies to conformationally intact myelin oligodendrocyte glycoprotein (MOG) have initially been detected in pediatric patients[1] then also in a proportion of patients with different demyelinating diseases such as optic neuritis, myelitis, encephalomyelitis, brainstem encephalitis, acute disseminated encephalomyelitis (ADEM), anti-NMDA-R encephalitis, and in a few patients with multiple sclerosis (MS).[2-6] Patients with autoantibodies to MOG have distinct brain MRI characteristics.[7, 8] It is debated whether anti-MOG disease constitutes a separate entity.[9]

In animal models, some monoclonal antibodies (mAbs) to MOG induce demyelination provided the blood-brain barrier is breached giving the Abs access to the CNS (reviewed in [5, 10]). Only a proportion of anti-MOG Abs is able to induce demyelination in vivo, related to complement activation[11] and recognition of conformationally correct MOG.[12, 13] In rodents, pathogenic MOG-specific Abs mainly recognize the FG loop of MOG as the prototype mAb 8-18C5,[14] while patients with Abs to MOG recognize different loops of MOG, most frequently the CC' loop around the amino acid P42.[15]

Previous experiments to test the potential pathogenic activity of human anti-MOG Abs in vitro reported that sera of patients with Abs to MOG activated complement,[16] stimulated natural killer cell mediated toxicity,[17] induced cytoskeletal changes in oligodendroglial cells,[18] mediated myelin destruction in slice cultures[19] and facilitated MOG uptake by macrophages.[20] Peripheral injection of concentrated serum from MS patients in rats with experimental autoimmune encephalomyelitis (EAE) slightly enhanced demyelination and axonal loss.[21] Total IgG preparations pooled from 5 NMO patients were injected intracerebrally and induced myelin changes independent of complement, but no inflammation.[22] Intrathecal injection of IgG from a patient with MOG-Abs accelerated EAE in mice.[23] Peripheral injection of IgG from MS patients with Abs to MOG exacerbated EAE in mice.[24] Thus, there is evidence that human Abs to MOG are pathogenic, but one has to consider that patients with neuroinflammation may have multiple autoantibodies[25-27] which complicates the interpretation of transfer experiments with whole IgG preparations. Transfer experiments with human affinity purified Abs to MOG have not been done so far and therefore detailed pathogenic mechanisms of human Abs to MOG remain to be elaborated.

Patients with Abs to MOG have a pathology described as MS pattern II,[28-31] characterized by active demyelination along with deposition of C9neo suggesting an antibody-mediated demyelination.[32, 33] Transfer experiments with autoantibodies to MOG

from these patients were hampered by the fact that only a proportion of MOG-Abs from patients cross-react with rodent MOG,[15, 28] therefore the linkage of human MOG-Abs to a certain neuropathology is still speculative.

The aim of this study was to analyze which human Abs to MOG are pathogenic, to identify recognized epitopes of pathogenic autoantibodies, to test whether they can mediate MS type II pathology and to explore their pathogenic mechanisms. To this end we combined affinity purification of Abs that recognize cell-based MOG, epitope identification with mutants of MOG, staining of tissue sections and transfer experiments in two EAE models. This showed that Abs to MOG were pathogenic by two mechanisms: in synergy with MBP-specific T cells they mediate MS type II pathology; together with MOG-specific T cells they enhance T cell infiltration.

Subjects and Methods

Standard Protocol Approvals, Registrations, and Patient Consents

We analyzed sera from 260 patients with inflammatory CNS diseases for anti-MOG reactivity. The clinical characteristics of patients who scored positive in our cell-based assay detecting Abs to MOG are summarized in **Table 1**. All MOG-Ab positive patients were followed longitudinally. Informed consent was obtained from each donor according to the Declaration of Helsinki and the ethical committee of the medical faculty of the LMU approved this study.

Determination of anti-MOG Reactivity and Epitope Recognition

Patients positive for Abs to MOG were identified with a cell-based flow cytometry assay using viable cells and a serum dilution of 1:50, as described.[28, 34] isotype-specific secondary Abs were obtained from Southern Biotechnec (Birmingham, AL, USA). To identify the recognized epitopes, mutant variants of MOG were applied and the % binding compared to hMOG was calculated as described.[15] In some experiments, we used a recombinant variant of the mAb 8-18C5 (designated r8-18C5), which has the same antigen recognition site, but a human IgG1 Fc part.[35]

Production and Validation of recombinant human MOG

We aimed to produce a recombinant version of the extracellular domain (ECD) of human MOG that comes as close as possible to the conformation of MOG displayed in transfected cells. To this end, we produced the ECD of human MOG in HEK293-EBNA cells and added at the C-terminus instead of the first transmembranous region a HisTag and an AviTag using the pTT5 vector.[36] MOG was biotinylated by using the BirA biotin ligase Kit (Avidity, Aurora, Co, USA). Folding of the purified protein (0.2 mg/ml) was analyzed by circular dichroism using a Jasco J-810 Spectropolarimeter (JASCO Corporation, Tokyo, Japan). To further validate the anti-MOG binding activity of our recombinant MOG, we tested whether this MOG was bound by B cells from mice with a knock-in of the heavy chain of the anti-MOG 8-18C5.[37] To this end, we formed MOG-tetramers with our biotinylated MOG and fluorescently labeled streptavidin (Jackson ImmunoResearch, West Grove, PA, USA).

Affinity Purification of anti-MOG Abs

Biotinylated MOG was bound to a HiTrap Streptavidin HP column (GE Healthcare, Munich, Germany). Ig from plasma (obtained from EDTA-blood) was first enriched by ammonium sulfate precipitation and then loaded on this column. Bound Ig was eluted (100 mM Glycin, 150 mM NaCl, pH 2.5) and immediately neutralized with 1 M Tris-HCl pH 8.8. The eluates from both patients were separated by reducing and non-reducing SDS gel electrophoresis and stained by Coomassie. The excised gel bands were in-gel digested essentially as described.[35] Peptides were analysed by MALDI-TOF/TOF using a 4800 Analyzer (Applied Biosystem). The eluates were tested by ELISA for streptavidin reactivity using streptavidin-coated plates.

Staining of Tissue with Patient Abs

Rat brains were fixed in 4 % paraformaldehyde (PFA) for 1 hour, cryoprotected with 40 % sucrose and snap frozen. Seven micron thick sagittal sections were incubated with 0.3 % hydrogen peroxide for 20 minutes, with 10 % donkey serum in PBS for 1 hour, and then labeled with the Abs at 4 °C overnight. The next day, sections were labeled with a donkey-anti-human IgG (H+L) secondary antibody (Jackson ImmunoResearch) and visualized with an avidin-biotin-diaminobenzidine reaction.

Transfer EAE and Rat T Cell Lines

Antigen specific T cells were established from Lewis rats immunized with antigen emulsified in complete Freund's adjuvant as described previously.[38] The following antigens were used: recombinant MOG (amino acid 1-125), MBP purified from guinea pig brain, ovalbumin (OVA) purchased from Sigma Aldrich. To induce mild EAE, freshly restimulated 15 x 10^6 MOG-specific T cells or 1.2 x 10^6 MBP-specific T cells were injected intravenously in Lewis rats. Clinical scores were evaluated as follows: 0 = normal; 0.5 = loss of tail tonus; 1 = tail paralysis; 2 = gait disturbance; 3 = hind-limb paralysis. Two days after injection of T cells, 100 µg of the indicated Ab preparations were injected intrathecally into the cisterna magna to animals anesthetized by Fentanyl/Midazolam/Medetomidin. For the monitoring of clinical score, animals were followed until full recovery and were then sacrificed. For histopathological analysis, 72 hours after antibody injection, animals were perfused with PBS and 4 % PFA in PBS under terminal anesthesia with Fentanyl/Midazolam/Medetomidin; the spinal cord and brain were then postfixed with 4 % PFA in PBS at 4 °C. The procedures are approved by the government of Oberbayern.

Histological Examination of the EAE Rats

Brain, spinal cord and optic nerves were dissected and embedded in paraffin. Serial sections of all tissues were stained with hematoxylin / eosin (H&E), luxol fast blue (LFB) myelin stain and Bielschowsky silver impregnation for axons. Immunocytochemistry was performed on paraffin sections after antigen retrieval in a food steamer with EDTA buffer pH 8.5. Primary antibodies against the following targets were used in the following dilutions: CD3 (T-cells; rabbit monoclonal, Neomarkers (Fremont, CA, USA) RM-9107-5; 1:2.000), ED1 (phagocytic macrophages and microglia; mouse monoclonal, Serotec (Raleigh, NC, USA) MCA341R, 1:10.000), Iba 1 (pan microglia and macrophages; rabbit polyclonal, WAKO (Osaka, Japan) 019-19741; 1:3.000), cyclic nucleotide phosphodiesterase (CNP, oligodendrocytes; mouse monoclonal, Sternberger Monoclonals (BioLegend, San Diego, CA, USA) SMI 91; 1:2.000), glial fibrillary acidic protein (GFAP, astrocytes; rabbit polyclonal; DAKO (Santa Clara, CA, USA) Z0334; 1:3.000), human Ig (biotinylated species specific anti human Ig; donkey polyclonal, Jackson 709-065-149; 1:1.000) and activated complement (C9neo antigen, rabbit polyclonal; 1:2.000).[11] Bound primary antibodies were visualized with a biotin / avidin / peroxidase system. To quantify the inflammation, $CD3^+$ T cells / mm^2 were counted in a zone of 200 µm spanning from the ventral subpial surface into the tissue of the pons. To quantify demyelination, the distance of subpial demyelination from the ventral surface of the pons was

measured. To this end, macrophages were stained with ED1 and the distance from the pial surface in which we can see classical macrophages with degradation products was measured. This also represents the area of macrophages in LFB staining, which contain myelin degradation products.

Results

Anti-MOG Reactivity in Patients with inflammatory CNS Diseases and Cross-Reactivity to rodent MOG

We tested sera from 260 patients with different inflammatory CNS diseases, 17 of them had autoantibodies to MOG (Clinical details in **Table 1**). The highest anti-MOG reactivity was seen in patients with relapsing optic neuritis and NMO phenotype. The vast majority of patients with MS do not have antibodies to MOG, but Abs to MOG are detected in special cases with MS [34]. The five patients with MOG-Abs included in **Table 1** fulfill the diagnostic criteria of MS including MS-typical CSF and radiological features, but had a clinical phenotype that overlaps with NMO (severe myelitis, brainstem involvement and optic neuritis). These patients did not have Abs to NMDA-R or AQP4. Details of their clinical picture, their MRI and anti-MOG reactivity have been described in a previous paper.[34] We determined the cross-reactivity to rodent MOG of these patients (**Table 1**). Further analysis of the pathogenic features of Abs to MOG was performed with the patients #7 and #5, who showed a high reactivity towards MOG and cross-reactivity to rodent MOG (**Table 1; Fig. 1**). Both patients had a recurrent optic neuritis, one of the diseases associated with MOG-Abs [39, 40]. These patients were followed for a period of about 26 and 35 months and kept recognizing MOG (**Fig. 1A**). Their anti-MOG reactivity was so high that a reactivity could still be detected at serum dilutions of 1:3,000 to 1:10,000 (**Fig. 1B**). Both patients had anti-MOG of isotype IgG1. Patient #5 had in addition to IgG also persisting IgM to MOG (**Fig. 1A**).

The applications of mutant variants of MOG showed that the two patients recognized different epitopes on MOG (**Fig. 1C,D**). The binding to MOG of patient #5 was reduced by the mutation P42S indicating that this patients' Abs recognize the CC' loop on MOG; the MOG-Abs of IgG and IgM isotype showed similar reactivity to MOG mutants. Patient #7 showed a stronger reactivity to mouse MOG than to human MOG. Such a feature we had previously noted in 12/111 patients analyzed.[15] Consistent with the better recognition of mouse MOG, this patient also showed a stronger reactivity to the MOG mutant P42S, in which the serine present in murine MOG replaces the proline of human MOG. Another

mutation at the EF loop (H103A, S104E) greatly reduces the MOG binding of this patient (**Fig. 1D**). MOG residues important for binding of Abs from patients #5 and #7 are visualized in **Fig. 1E.**

From patient #5 we could also analyze CSF and this showed that anti-MOG IgG were present in this compartment, but there was no evidence that the anti-MOG IgG present in the CSF was produced intrathecally: after adjustment to equal IgG concentrations, similar anti-MOG reactivity was seen in CSF and serum (**Fig. 1F**).

Specificity of Affinity purified Abs to MOG

We produced the ECD of human MOG in HEK cells with an Avi-Tag at the C-terminus replacing the transmembranous and intracellular part. Then MOG was enzymatically biotinylated at the AviTag and bound to a streptavidin column, which puts the extracellular part of MOG on the beads in the same orientation as in the membrane. The confirmation with beta-sheet formation was seen by circular dichroism (**Fig. 2A**). To further validate this MOG preparation we formed MOG-tetramers and tested the binding to B cells from mice with a knock-in of the heavy chain of the anti-MOG mAb 8-18C5 and found that this stained about a third of the B cells from these mice, which is in line with their published MOG-binding activity (data not shown).[37] With this protein we could affinity purify MOG-specific Abs from both patients (**Fig. 2**). Starting from more than 600 ml blood, we eluted from the MOG-column 471 µg of IgG and 55 µg of IgM from patient #5 and 571 µg IgG, but no IgM from patient #7. Mass spectrometry showed that the eluates from patient #5 contained IgG, IgM, α-2 macroglobulin, fibrinogen and albumin, from patient #7 IgG and fibrinogen. Importantly, no MOG was detected in the eluates. The eluates did not bind to streptavidin as seen by ELISA using streptavidin coated plates. We could not obtain Abs that recognize MOG on transfected cells from donors who did not have a strong anti-MOG reactivity in their blood. This excludes that the anti-MOG-reactivity we observed in the purified fraction is an artifact due to the purification procedure.

These affinity purified Abs showed a highly enriched reactivity to human MOG in a cell-based assay (**Fig. 2B-E**); when plasma and affinity-purified Abs were adjusted to the same concentration of 12 µg/ml, we noted the following MCF ratios, which were calculated as described above: Patient #5: plasma 14.9, purified 190.3, flow through 8.1; Patient #7: Plasma 8.6, purified 207.5, flow through 3.5. We noted that in both patients the reactivity to our mutated variants was the same in the anti-MOG Abs from the starting material and the

eluates. We also compared the affinity-purified MOG-Abs from both patients with the prototype anti-MOG 8-18C5. For this comparison we used a recombinant variant of 8-18C5 with a human Fc-IgG1, so the same detection Ab could be used. Our dose responses show that these purified MOG-Abs recognized MOG in a cell-based assay still in the ng/ml range and came quite close to the intensity of MOG binding of the 8-18C5 (**Fig. 2 F**). The isotype of the anti-MOG response of the affinity-purified Abs of both patients was IgG1. We also analyzed the cross-reactivity of the patient-derived MOG-Abs to rat-MOG (**Fig. 2G**), since their pathogenicity will be tested in a rat model (see below). We noted that patient #7 recognized rat MOG stronger than human MOG (Figure **2 F,G**), which is consistent with our observation that this patient recognizes also mouse MOG stronger than human MOG (**Fig. 1D**); mouse and rat MOG are very similar although not identical.

The flow through of the column used for affinity purification of MOG-Abs from these two patients still contained anti-MOG reactivity as seen with MOG-transfectants. This was not due to a limited capacity of the column, since it could still bind the mAb 8-18C5. Along this line, from another patient (#14) we could obtain only a small amount of anti-MOG IgG with this column and the flow through still contained a similar reactivity to MOG as the starting material. Thus, the ECD of human MOG produced in HEK cells binds only a fraction of Abs to MOG.

Staining of Brain Tissue with Affinity purified Abs to MOG

The affinity purified Abs from both patients bound to myelin in tissue sections from the rat, r8-18C5 was used as a positive control (**Fig. 3**). We noted a stronger binding of the Abs from patient #7 (**Fig. 3 C,D**) than from patient #5, which is consistent with the dose response of these preparations to rat-MOG on the surface of rat transfectants (**Fig. 2G**). Since the MOG-reactivity of these patients was established by using native cells, while the tissue was fixed with PFA, we compared the recognition of live and PFA-fixed cells after MOG transfection. This showed that patients #5 and #7 recognized MOG also after PFA fixation of the transfected cells, but the background was much higher with fixed cells (data not shown).

Pathogenicity and histopathological changes induced by patient-derived Abs to MOG

We analyzed the pathogenic potential of patient-derived MOG-specific Abs in two models of T cell mediated EAE in the Lewis rat. In both models we injected the MOG Abs intrathecally two days after the injection of either MOG-specific T cells or MBP-specific T cells. Since the

amount of purified Abs from patients was limited, we first established the details of the transfer models with 8-18C5 and the humanized r8-18C5. These experiments showed that EAE can be enhanced, when 8-18C5 or r8-18C5 were injected two days later than the T cells. Under these conditions, the peak of disease was reached at day 5, the animals recovered largely until day 10. Therefore we sacrificed the EAE rats after injection with the patient-derived Abs at day 5.

The MOG-specific T cells alone did not induce a clinical effect in our Lewis rat model. However, when affinity purified Abs from both patients #5 and #7 were injected, a clinical disease was induced (**Fig. 4A**). As control, we used human ivIg and Ig obtained from a protein G column. This control human Ig did not induce disease, while the positive control 8-18C5 enhanced disease (**Fig. 4**). In contrast to the MOG-specific T cells, the MBP-specific T cells induced a clinical disease on their own in the absence of any added Ab, consistent with previous observations with MBP-specific T cells in this rat model.[41] One day after injection of r8-18C5 and the Abs from patient #7 the clinical disease was enhanced (**Fig. 4B**).

All animals shown in Fig. 4 were perfused at day 5 and analyzed by histopathology. A quantitative analysis of the T cell infiltration and of demyelination (**Fig. 4C,D**) in all 17 animals revealed the following: The patient-derived MOG-specific Abs massively enhanced the T cell infiltration in the subpial area of the pons when given together with cognate MOG-specific T cells, but not together with MBP-specific T cells (**Fig. 4C)**. Pathological analysis of animals injected with MOG-specific T-cells alone or together with control antibodies displayed a moderate inflammatory reaction in the spinal cord and less obvious in the brain and optic nerve, consisting of T-cell infiltrates in the meninges and CNS tissue and of $ED1^{+}$ macrophages, being restricted to the meninges (**Fig. 5** middle panel).

In combination with the injection of the MOG-specific Abs from patients #5 and #7 a massively enhanced T cell and macrophage infiltration in the meninges and the subpial CNS tissue was observed (**Fig. 4C** and **Fig. 5** left panel for patient #7) and this was similar to the pathology observed after injection of the 8-18C5 antibody (**Fig. 4C** and **Fig. 5** right panel). The enormous enhancement of the infiltration of T cells is already visible at a low magnification displaying cross-sections of the whole spinal cord (**Fig. 5** first and third row). Human immunoglobulin reactivity was seen on subpial myelin, but only traces of activated complement (C9neo antigen) and a slight perivascular demyelination were present (data not shown).

Following transfer of MBP-specific T-cells alone (which induced with the applied cell number a mild EAE on their own) or in combination with control Abs a different pathology was seen. It consisted of mild to moderate T-cell infiltration together with the dispersion of $ED1^+$ macrophages throughout the tissue (**Fig. 6**). In combination with patient-derived MOG-specific Abs human Ig was also seen on subpial myelin but this was associated with complement C9neo activation (**Fig. 6)**. This was accompanied by subpial demyelination (**Fig. 4D**), which was seen by LFB staining and by immunostaining for CNPase. Demyelination and complement activation was massive with the Abs from patient #7, less intense, but detectable with the Abs from patient #5, and absent after control Ab injection (**Fig. 4D** and **Fig. 6**). Due to injection into the cisterna cerebelli magna, the antibodies hardly reached the optic nerve.

Thus, in this model, we see an impressive effect of the MOG-Abs on the histopathology, but only a slight enhancement of the clinical disease. There are two reasons for this. First, the sensitivity to detect an enhanced clinical disease is lower if the control group is already sick (**Fig. 4B**) as compared to a model in which the control group is not sick at all (**Fig. 4A**). Second, the clinical score in this EAE model detects only motor functions. We have quantified the amount of LPS in the samples used for in vivo experiments and found that the contaminating amount of LPS was similar in control Ig and patient preparations, less than 10 ng were injected per animal. The same Ig preparations had different effects depending on the antigen-specificity of the co-injected T cells; the patient Abs enhanced microglia activation and T cell infiltration only together with MOG-specific T cells, but not in the context of MBP-specific T cells; a strong activation of terminal complement complex C9neo, on the other hand, was seen in the context of MBP-, but little in the context of MOG-specific T cells. We conclude from all this that the effects we describe were induced by the patient-derived Ig and not by LPS.

In this project we had tested three different human Ig control preparations, namely ivIg, human IgG not specific for MOG obtained from a protein G column and recombinant IgG with human Fc part. None of these human Ig variants recognized MOG and none of them had any effect on enhancement of the disease. As a further control experiment, we injected OVA-specific T cells in the absence or presence of an intrathecal injection of r8-18C5. In this context, no induction of clinical disease and no demyelination or complement activation was present (data not shown).

Discussion

Our study shows that Abs to MOG affinity purified from the blood of patients with inflammatory demyelination are pathogenic in transfer experiments to rodents. We found that these patient-derived MOG-specific Abs mediate damage to the CNS by different mechanisms. In synergy with T cells that induce clinical EAE, associated with profound blood-brain barrier damage and activation of macrophages (MBP-specific T cells in the Lewis rat in our model), human Abs to MOG mediate MS type II like pathology, characterized by active demyelination (phagocytes containing myelin in the lesion) and local activation of the terminal complement complex, visible as deposition of C9neo.[32, 33] We show here that these features are induced by the patient-derived MOG-specific Abs. This suggests that in patients with MOG-Abs and MS type II pathology,[28-31] MOG Abs are responsible for this part of the pathology. Remarkably, most patients with MOG-Abs and a MS type II pathology described so far, do not have a typical MS[42], but rather an encephalomyelitis overlapping with MS and NMOSD. It is discussed whether this should be grouped as MOG-Ab disease. On the other hand, most patients with clinical MS and a MS type II pathology do not have Abs to MOG,[30, 34] suggesting that these patients recognize other not yet identified autoantigens.

In our second model, in synergy with cognate MOG-specific T cells, which by themselves do not induce clinical disease, but only mild, predominantly meningeal inflammation in our rat model, the same affinity purified Ab preparations induced clinical disease with other pathological features, namely a massively enhanced T cell infiltration. An enhancement of T cell activation by mAbs to MOG has been shown in two recent studies and suggested to be mediated by opsonization of the antigen.[20, 23] We found that the patient derived anti-MOG Abs not only enhanced T cell infiltration induced by MOG-specific T cells, but also stimulated microglia/macrophage infiltration in the subpial gray matter. This indicates that human anti-MOG Abs in the CSF might also participate in the development of gray matter pathology together with MOG-specific T cells. MOG-specific T cells have been observed in patients with demyelination and their recognized epitopes were identified.[43] Further studies are needed to analyze MOG-specific T cells in patients with Abs to MOG.

Our two EAE transfer models show that the human Abs to MOG mediate tissue destruction via two different mechanisms. This could be revealed because in our models the two different T-cell lines showed different intensities of T cell re-activation in the CNS.[41, 44] In the model with MBP-specific T cells, strong T cell activation in the CNS was associated with blood-brain barrier disruption and the diffuse infiltration of the CNS tissue by recruited

ED1$^+$ macrophages. Therefore, the incoming Abs find a good environment to mediate demyelination via antibody-dependent cell-mediated cytotoxicity and complement activation which results in a pathology similar to MS type II. In the model with MOG-specific T cells, T cell activation in the CNS is not optimal and recruitment of ED1$^+$ macrophages is sparse and largely restricted to the meninges. Here, the entering MOG-specific Abs massively enhance the T cell recruitment and activation, because they recognize the same antigen; this then promotes infiltration of ED1$^+$ macrophages, which is associated with clinical disease but may be too low to effectively induce demyelination. Our observation that the patient derived Abs perform tissue destruction by two different mechanisms, demyelination and enhanced inflammation, is consistent with a previous study transferring sera from immunized non-human primates.[45]

Our EAE experiments indicate further that the anti-MOG Abs are not pathogenic on their own, since together with irrelevant T cells no pathology was induced. This is consistent with previous observations in other EAE models [37] or after intrathecal injection of the 8-18C5 antibody[46] and supports the concept that the anti-MOG Abs perform a second hit to enhance pathology. Thus, human MOG antibodies are pathogenic, but the precise pathological effects depend on their interactions with T cells; the human anti-MOG Abs can mediate MS type II pathology and gray matter injury upon transfer.

Experiences with mAbs in animals have shown that recognition of conformational MOG is required for pathogenicity.[12, 14] The secondary structure of MOG is characterized by two antiparallel beta-sheets that form an immunoglobulin-like beta-sandwich fold.[47] In rodents pathogenic MOG-specific Abs mainly recognize the FG loop of MOG as the prototype mAb 8-18C5.[14] While the epitope specificity of human anti-MOG antibodies was previously dissected by ELISA [48] and transfection of mutated variants of MOG [15], epitope-specificity of pathogenic Abs from patients was unknown. The pathogenic MOG-specific autoantibodies from the two patients recognize different epitopes and both are different from the one recognized by 8-18C5. Patient #5 recognized the CC'-loop, since its binding was reduced by the mutation P42S; this is the most frequently recognized part of human MOG.[15] This patient nevertheless strongly recognized mouse MOG, although the mouse MOG contains P42S. These two characteristics of MOG recognition (reduced reactivity to P42S, but strong recognition of mouse MOG) we had observed before in 5/111 patients.[15] Patient #7 recognized the FG loop of MOG, since its binding was completely abrogated by the mutation H103A+S104E. This resembles the recognition of 8-18C5, which is also abrogated by the

double mutation H103A+S104E. A closer look at the reactivity of patient #7 to other mutants of MOG points to epitopes that are discontinuous like the one recognized by the mAb 8-18C5,[47] but that differ from the 8-18C5 epitope as they are influenced by P42 positioned in the CC'-loop and/or the glycosylation site at N31 in addition to binding to the FG-loop. The observed binding pattern of patient #7 would therefore be consistent with the recognition of an ensemble of epitopes that include the FG-loop and are located at the top, membrane-distal part and/or at the five-stranded front β-sheet of MOG (Fig.1E).

Together, this part of our analysis shows that pathogenic MOG Abs from patients recognize different loops on MOG.

The anti-MOG response of the patients with recurrent optic neuritis persisted for the observation period of 26 and 35 months. This extends our knowledge of kinetic of MOG-Abs: In children with ADEM, the Abs to MOG appeared only transiently and were rapidly lost, while in children with MS the MOG-Abs persisted for years.[49] One of our analyzed patients had the unusual feature of having both an anti-MOG IgG and an anti-MOG IgM response. Both reactivities were directed against the same epitope of MOG. The co-occurrence of anti-IgG and anti-IgM to MOG is rare, but was noted in a previous study in 3/19 children with ADEM and Abs to MOG.[17] The long-term persistence of an anti-MOG IgM response might be surprising, but it is consistent with recently described human IgM memory B cells that have passed the germinal center.[50] Our study shows that rarely also an IgM response to MOG may persist.

Our study has the following limitations. First, we injected the patient-derived Abs intrathecally, not systemically, although MOG-Abs are typically detected in the blood. In pilot experiments with mAbs we noted that EAE can be enhanced both by peripheral and intrathecal injection, but that higher amounts of Abs were needed when the Abs were injected systemically. Since the amount of patient-derived Abs was limited, we chose intrathecal injection. We feel this is justified, since we found MOG-Abs also in the CSF: From patient #5 we could analyze CSF and found strong anti-MOG reactivity without evidence for intrathecal Ab production to MOG. Second, we analyzed the pathology only at one time-point after injection because we could inject only a limited number of animals with precious patient-derived Ig material. Compared to recombinant antibodies, however, patient-derived Abs more closely reflect the human in vivo situation. This is important when evaluating the pathogenic potential of the MOG-Abs present in the blood of patients, since the effector function of IgG is regulated by its glycosylation[51] and there is evidence that IgG glycosylation is altered in

MS patients.[52] Third, we show that human MOG-Abs identified in a cell-based assay include pathogenic Abs, but it remains unclear if all of the MOG-Abs are pathogenic and which features of the human antibodies would allow predicting their pathogenicity. Our approach displaying the correctly folded extracellular part of MOG on a column purified only a proportion of MOG-Abs. To affinity-purify and subsequently test the pathogenic activity of the other MOG-Abs, MOG might have to be displayed in a membrane-bound environment. Our observation that the extracellular part of MOG purifies only part of the MOG-Abs is consistent with the previous observation that in a cell-based assay a short construct of MOG lacking the intracellular part is less sensitive to detect anti-MOG Abs than full-length MOG.[53] Possible reasons for the differential reactivity to the two MOG variants with the same ECD include oligomerization or yet unidentified effects of the intracellular part of MOG on the conformation of the extracellular part. Further, human MOG-Abs are heterogeneous with respect to cross-reactivity to rodents. To address the pathogenicity of MOG-Abs not cross-reactive with rodent MOG, mice with a knock-in of human MOG or even transfers to nonhuman primates might have to be used.

Together, we show here that Abs to MOG, which were affinity purified from the blood of patients and recognize different epitopes on MOG, synergize with T cells in transfer experiments to rodents; they induce MS type II pathology and trigger T cell infiltration with microglia/macrophage activation in the subpial parenchyma. We conclude that MOG-Abs contribute to the pathology of patients with inflammatory demyelinating diseases by these mechanisms.

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Author Contributions

MS, SW, EB, CM, RH, RG, MM, FK, HL, NK conducted experiments, acquired and analyzed data. ES, FST, L-AG, SL, GK, DJ, SB, KD, MK, RH, TK, SW, CB analyzed data and contributed to manuscript preparation. MS, HL, TK, RH, NK, EM designed the study and wrote the manuscript.

Potential Conflicts of Interest

None of the authors has a conflict of interest related to this study.

Figure Legends

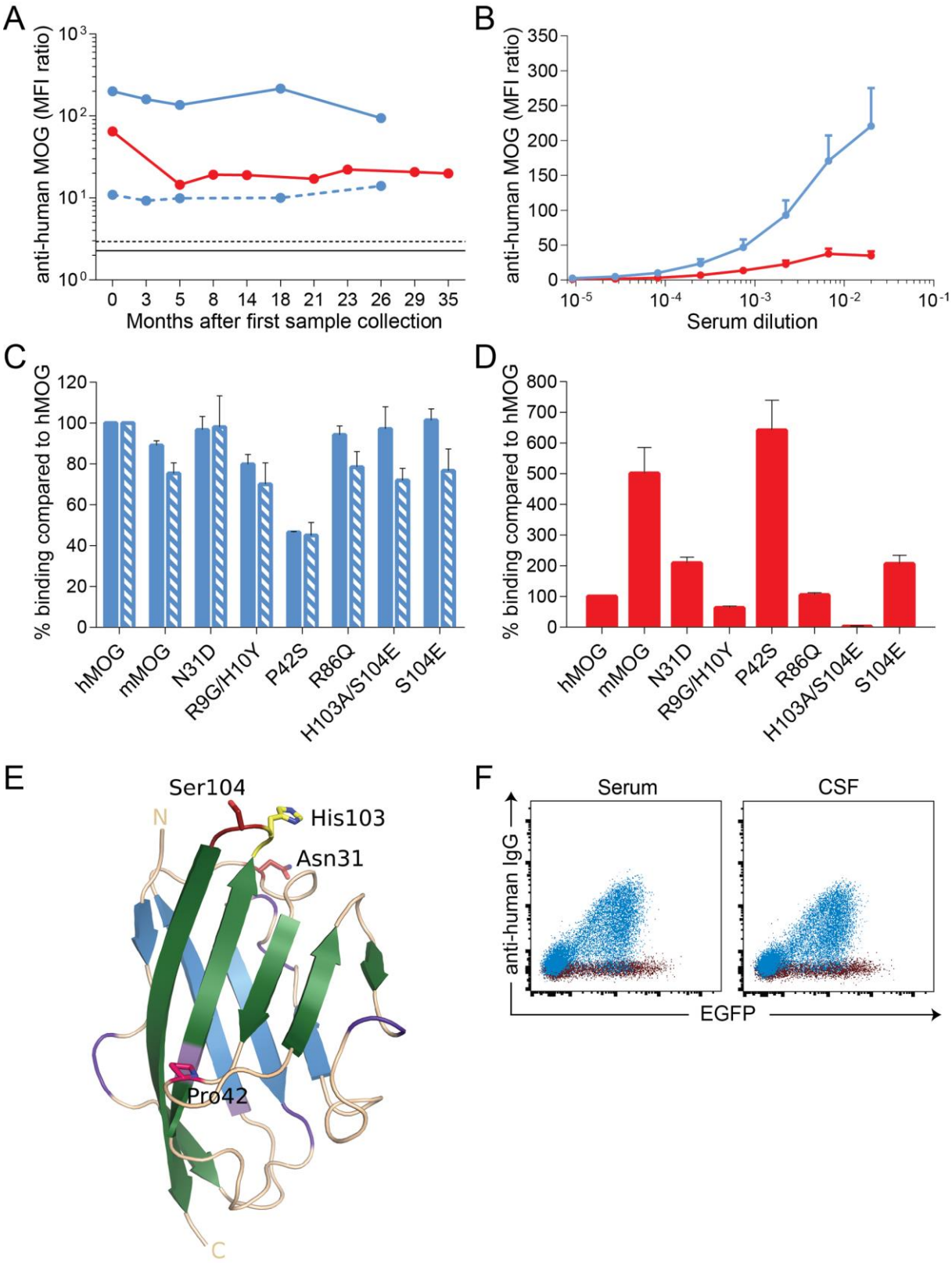

Figure 1. Anti-MOG reactivity in the two patients selected for transfer experiments. The anti-MOG reactivity in serum and plasma of patient #5 (blue) and patient #7 (red) was

determined with transfected cells as described in materials and methods. (**A**) Longitudinal analysis. Closed lines indicate anti-MOG IgG, the dotted bluish line shows persisting anti-MOG IgM in patient #5. The black closed line shows the cut-off for anti-MOG IgG, the dotted black line the cut-off for anti-MOG IgM. (**B**) Anti-MOG-reactivity in serum dilutions. (**C,D**) Reactivity to human MOG (hMOG), mouse MOG (mMOG) and the indicated mutations of MOG. The IgG responses are indicated in closed bars, the anti-MOG IgM response from patient #5 in hatched bars. (E) The structure of the human MOG model [15] is shown as ribbon representation with residues influencing antibody binding depicted as stick models. In addition, residues that differ between mouse and human MOG are colored pink (Pro 42), light violet (two conservatively mutated interior 13-strand residues) and violet (remaining non-identical residues). (**F**) Anti-MOG in CSF of patient #5. CSF (IgG 0.022g/l) was used undiluted and serum was diluted 1:377 to obtain the same IgG concentration as in the CSF. The calculated MFI ratio (MOG-EGFP/EGFP) of the CSF was 72.44, while that of the serum sample was 86.34. Control EGFP transfectants are shown in gray, the MOG-EGFP transfectants in blue. Error bars indicate SEM of 2-3 experiments.

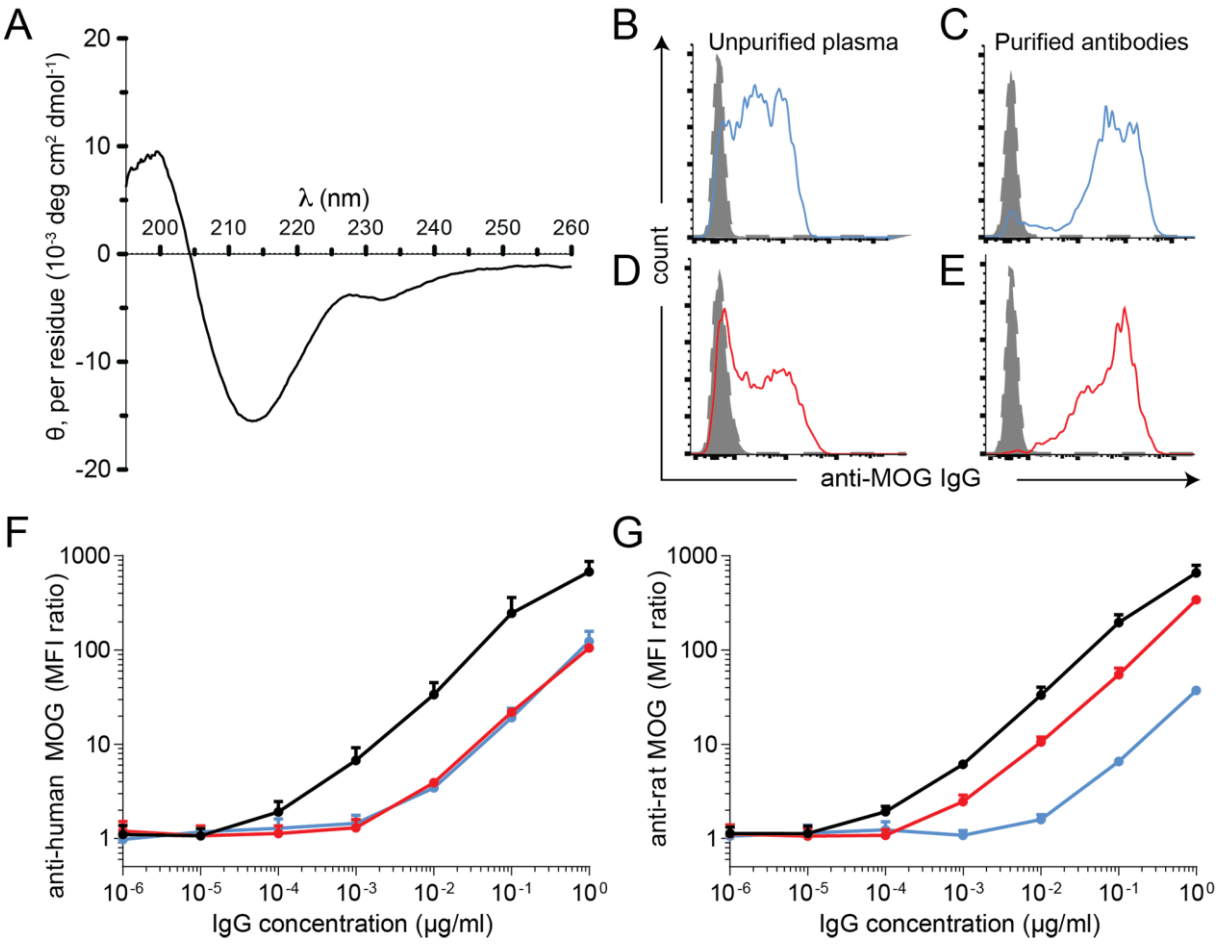

Figure 2. Affinity purified Abs to MOG. (**A**) Circular dichroism spectrum of MOG (0.2 mg/ml). The beta-sheet formation is indicated by the negative band at 213 nm. (**B-E**) Comparative analysis of plasma and affinity purified MOG-Abs to cells transfected with MOG of patient #5 (blue) and patient #7 (red). Plasma and purified Abs were used at an IgG concentration of 12 µg/ml. Closed graphs indicate the recognition of EGFP-transfected cells, open graphs of MOG-EGFP transfectants. (**F-G**) MOG recognition of the affinity purified Abs from patients #5 (blue) and #7 (red) in comparison with the recombinant humanized mAb 8-18C5 (black) on transfected cells (**F,G**). Error bars indicate SEM of 2-3 experiments.

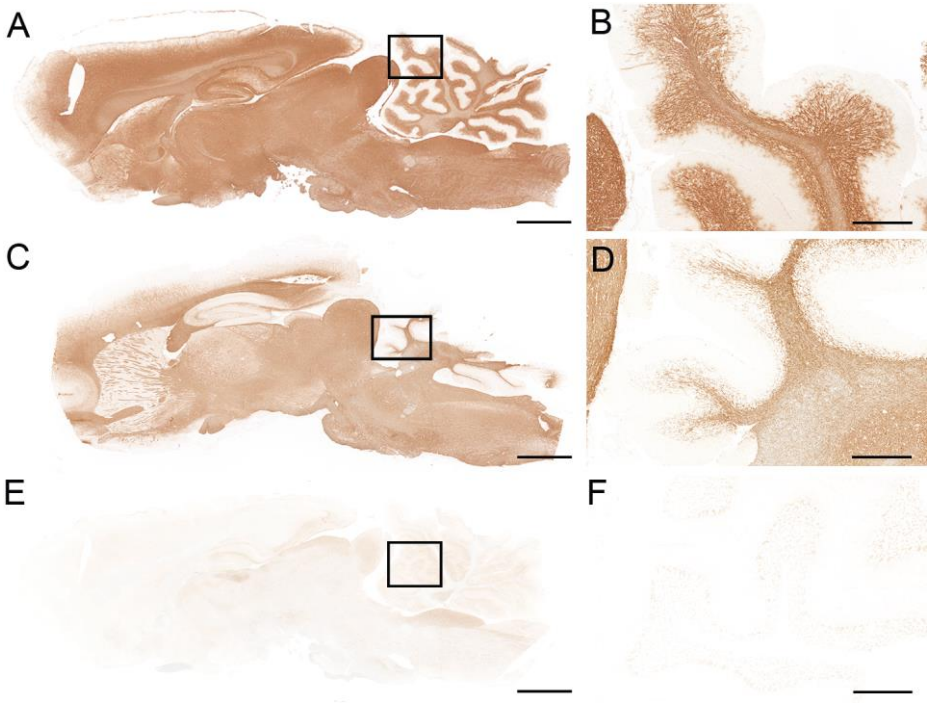

Figure 3. Myelin staining of affinity purified MOG-specific Abs. Samples were stained on sagittal rat brain sections. The humanized r8-18C5 was used as positive control (**A**) and showed a specific myelin staining throughout the cerebrum and cerebellum (**B**; rectangle in **A** enlarged). The affinity purified MOG-specific Ab from patient #7 (**C,D**) showed a strong binding to myelin, a recombinant human IgG which does not bind MOG on transfected cells (r#7_D7) was negative (**E,F**). All Abs were used at a concentration of 3 µg/ml. Scale bars: (**A, C, E)**: 2 mm; (**B, D, F)**: 300 µm.

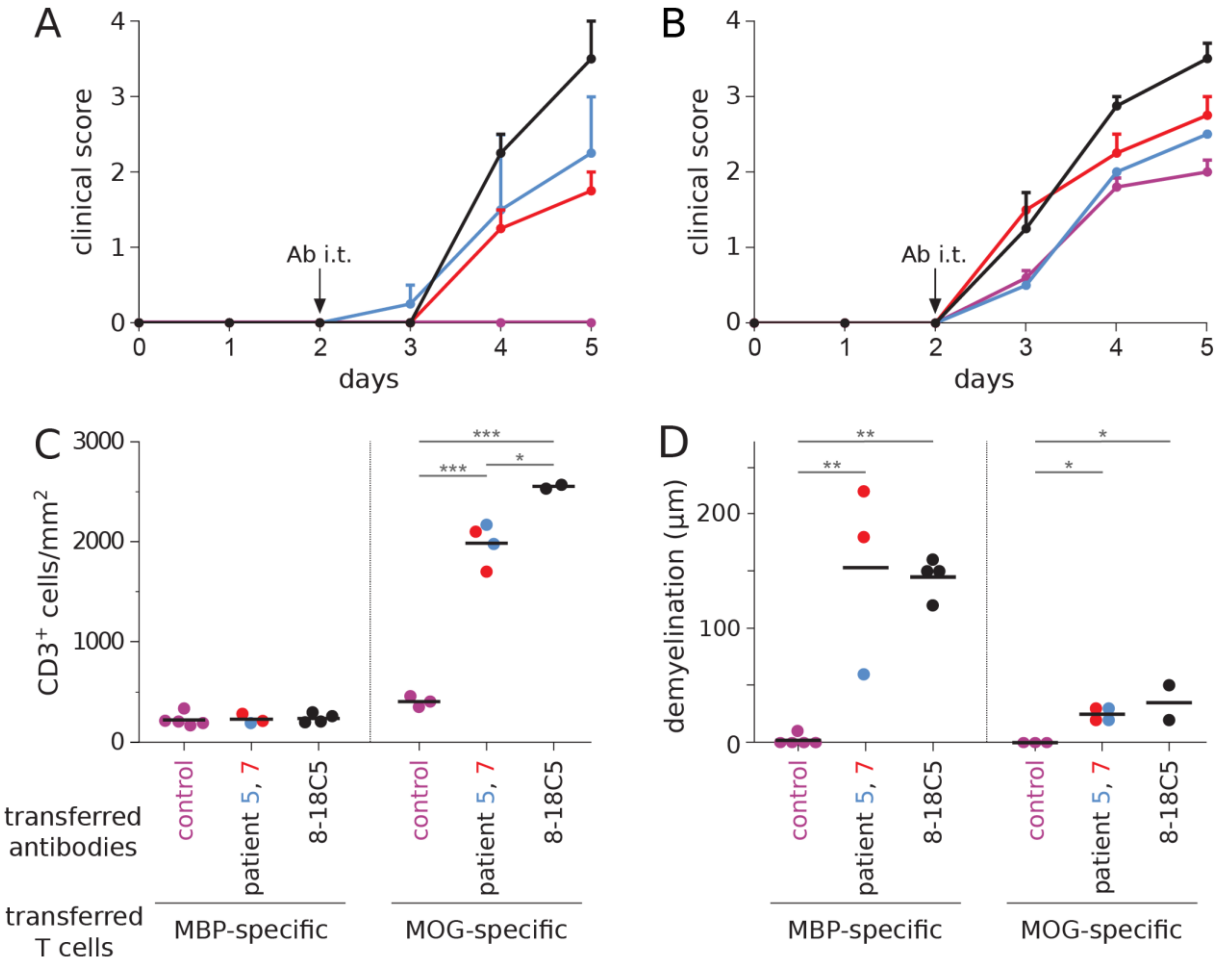

Figure 4. Pathogenicity of affinity-purified patient derived MOG-specific Abs. Lewis rats were injected with MOG-specific (**A**) or MBP-specific T cells (**B**). Two days later 100 µg of affinity purified MOG-specific Abs from patient #5 (blue), #7 (red), control IgG (purple) or 8-18C5 (black) were injected intrathecally (i.t.) into the spinal fluid (cisterna magna). (**A**) Three animals received human control IgG, two Abs from patient #7 and two from patient #5. Since the animals with the control IgG did not show any clinical disease, the induction of the clinical EAE with MOG-specific Abs from patients (data from the patients pooled) reached statistical significance at day 4 ($P < 0.05$) and day 5 ($P = 0.005$) using the unpaired two-tailed t-test. (**B**) Together with MBP-specific T cells, one animal received Abs from #5, two animals Abs from patient #7, five control IgG. As positive control r8-18C5 (**A,B**) and 8-18C5 (**A**) were used. Error bars indicate SEM. All animals were perfused at the end of the observation period and analyzed for histopathology. (C,D) Quantification of inflammation and demyelination of animals shown in A and B. (C) The T cell infiltrates in the subpial region at the basis of the pons were counted with a 40x objective and the number of $CD3^+$ T cells/mm^2 was calculated. (D) The distance of subpial demyelination at the basis of the pons was

measured. (C, D) We performed ANOVA testing followed by Tukey's honest significant difference test.

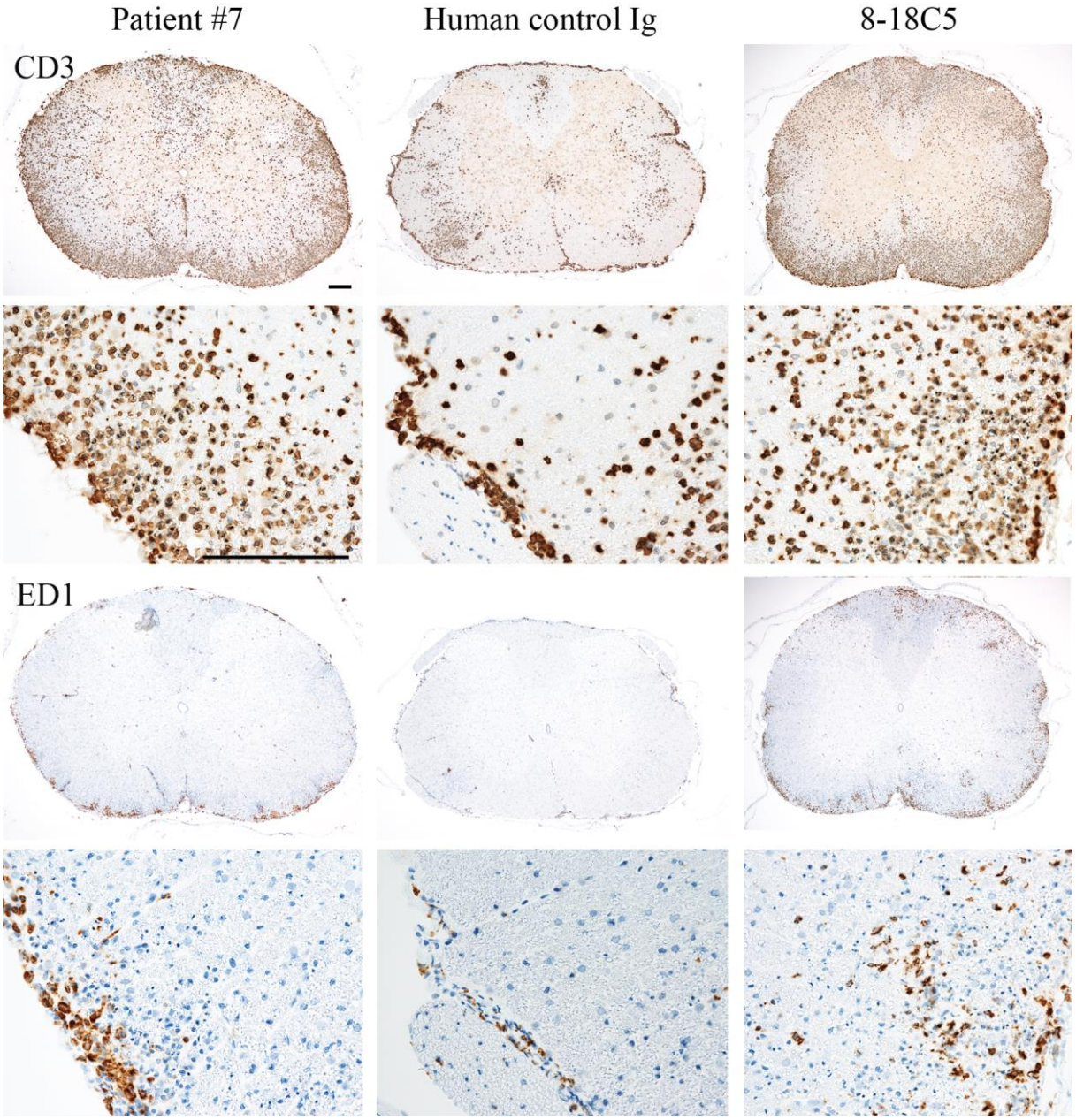

Figure 5. Affinity purified Abs to MOG enhance T cell activation and promote microglia activation in the subpial parenchyma together with MOG-specific T cells

Spinal cord pathology following passive co-transfer of MOG specific T-cells with control IgG or anti-MOG Abs. EAE after injection of control Abs is characterized by T-cell infiltration in the meninges and diffusely in the spinal cord parenchyma, but $ED1^+$ macrophages are largely restricted to the meningeal space (middle panel). After injection of patient #7 Ab (left panel) or 8-18C5 (right panel) there is a massive enhancement of subpial T-cell infiltration and $ED1^+$

macrophages pass the astrocytic glia limitans and infiltrate the CNS parenchyma. Scale bars: 100 µm.

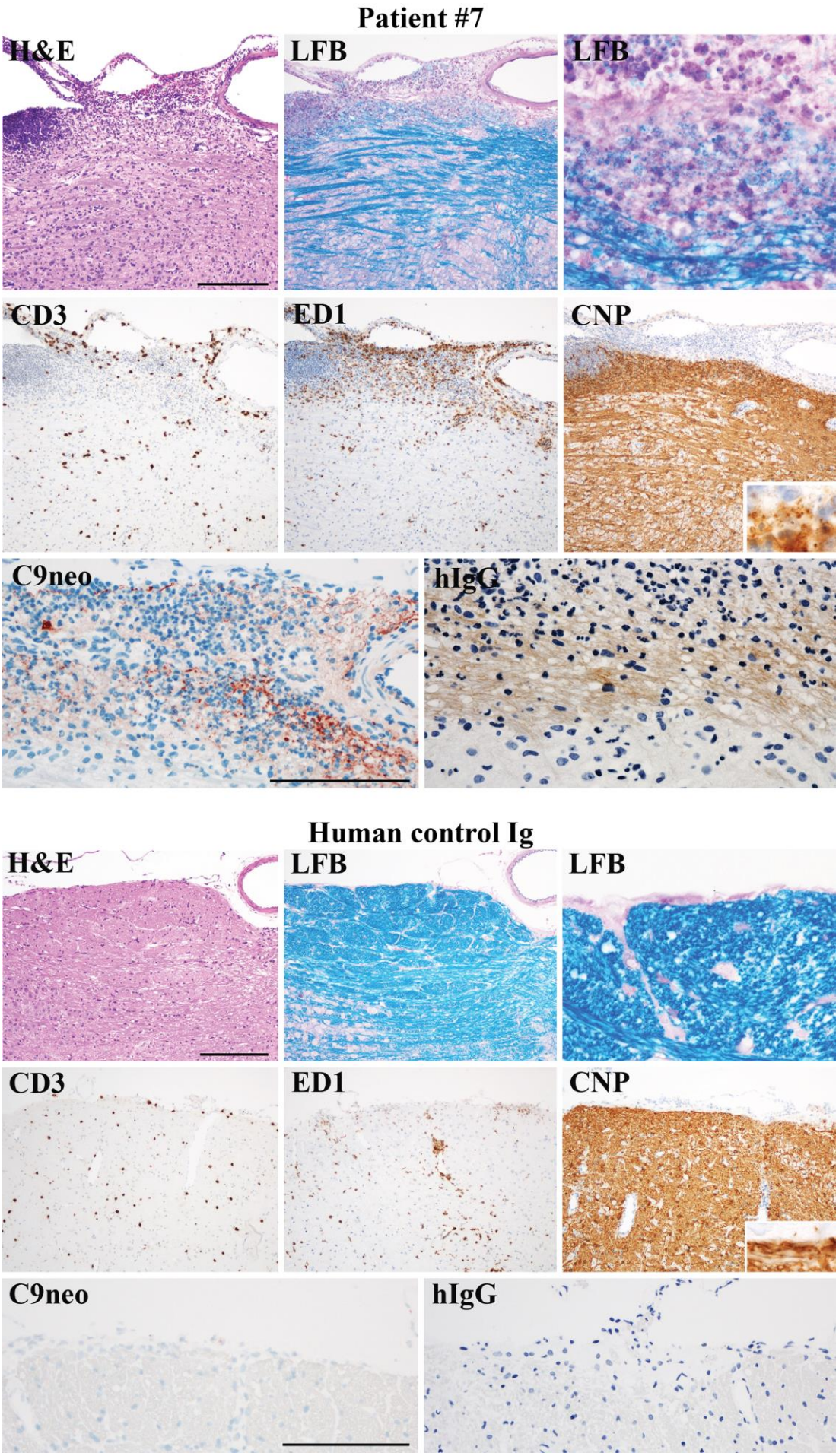

Figure 6. Affinity purified Abs to MOG induce complement activation and demyelination enhance T cell activation

EAE was induced with MBP specific T-cells. After two days either MOG-specific affinity purified Abs from patient #7 (upper panel) or human control Ig (lower panel) was injected. When human control Ig was injected, there is a diffuse infiltration of the tissue by $CD3^+$ T-cells and $ED1^+$ macrophages, but there is no deposition of human IgG on myelin or activation of complement (C9neo) (lower panel). However, when anti-MOG Ig from patient #7 was co-transferred, inflammation is massively enhanced and $ED1^+$ macrophages are concentrated at sites of active myelin destruction, associated with immunoglobulin deposition on myelin and complement activation (C9neo antigen deposition; lower left in the upper panel). Scale bars: 100 µm. CNP, CNPase; LFB, luxol fast blue.

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Table 1. Features of patients with anti-MOG reactivity

ID	Current Diagnosis	Gender	Age at first MOG^+ sample (years)	Reactivity to human MOG (MFI ratio)	Reactivity to mouse MOG (MFI ratio)
#5	Relapsing bilateral ON	f	42	220.7	212.9
#14	Relapsing bilateral ON	m	54	44.9	20.6
#8	NMOSD	m	37	38.3	3.0
#7	Relapsing unilateral ON	m	46	34.7	216.8
#16	NMOSD	m	30	18.6	5.6
#17	Relapsing bilateral ON	f	31	18.2	2.1
#6	Monophasic encephalitis	f	31	17.7	2.3
#10	RRMS	f	37	11.9	8.3
#13	Relapsing encephalomyelitis	m	34	8.6	5.5
#1	NMOSD	m	40	6.1	1.7
#3	Relapsing encephalomyelitis	m	26	5.4	1.8
#4	RRMS	f	55	4.6	7.1
#11	RRMS	f	50	4.1	1.5
#2	Relapsing encephalomyelitis	f	66	4.0	0.9
#9	RRMS	m	32	3.9	3.1
#12	RRMS	f	23	2.9	3.9
#21	NMOSD	f	33	2.7	1.8

ON = optic neuritis; NMOSD = neuromyelitis optica spectrum disorders; RRMS = relapsing/remitting multiple sclerosis; MFI = mean fluorescence intensity; f = female; m = male. Details about #10, #4, #9, #12, #11 are reported in[33], about #2 in[27]. Patients with MOG-Abs might constitute a condition called MOG-antibody disease. The cut-off for recognition of human MOG was 2.27 (mean + 3SD of controls). The MFI ratio was calculated as the mean of 2-5 experiments