

RESEARCH ARTICLE

Dose-dependent inhibition of demyelination and microglia activation by IVIG

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Introduction

Clinical trials have established Intravenous immunoglobulin (IVIG) as a well-tolerated, effective drug for the treatment of a wide variety of diseases, ranging from immunodeficiency to autoimmunity (for review see^{1–4}). In neurology, IVIG serves as a mainstay therapy in immune-mediated neuropathies^{5–8} and has been shown to be equally effective as plasmapheresis in Guillain-Barré Syndrome⁹ and myasthenic crisis.^{10,11} In multiple sclerosis, clinical

Abstract

Objective: Intravenous immunoglobulin (IVIG) is an established treatment for numerous autoimmune conditions. Clinical trials of IVIG for multiple sclerosis, using diverse dose regimens, yielded controversial results. The aim of this study is to dissect IVIG effector mechanisms on demyelination in an *ex vivo* model of the central nervous system (CNS)-immune interface. **Methods:** Using organotypic cerebellar slice cultures (OSC) from transgenic mice expressing green fluorescent protein (GFP) in oligodendrocytes/myelin, we induced extensive immune-mediated demyelination and oligodendrocyte loss with an antibody specific for myelin oligodendrocyte glycoprotein (MOG) and complement. Protective IVIG effects were assessed by live imaging of GFP expression, confocal microscopy, immunohistochemistry, gene expression analysis and flow cytometry. **Results:** IVIG protected OSC from demyelination in a dose-dependent manner, which was at least partly attributed to interference with complement-mediated oligodendroglia damage, while binding of the anti-MOG antibody was not prevented. Staining with anti-CD68 antibodies and flow cytometry confirmed that IVIG prevented microglia activation and oligodendrocyte death, respectively. Equimolar IVIG-derived Fab fragments or monoclonal IgG did not protect OSC, while Fc fragments derived from a polyclonal mixture of human IgG were at least as potent as intact IVIG. **Interpretation:** Both intact IVIG and Fc fragments exert a dose-dependent protective effect on antibody-mediated CNS demyelination and microglia activation by interfering with the complement cascade and, presumably, interacting with local immune cells. Although this experimental model lacks blood–brain barrier and peripheral immune components, our findings warrant further studies on optimal dose finding and alternative modes of application to enhance local IVIG concentrations at the site of tissue damage.

trials applying different dose regimens and study designs (for review see^{12–16}), yielded inconclusive results. More recently, IVIG has gained some attention as a treatment option for special forms of inflammatory CNS conditions, which are characterized by pathognomonic autoantibody signatures such as neuromyelitis optica,^{16,17} yet larger controlled trials are still lacking.

Mechanistically, a plethora of therapeutic modes of action have been attributed to IVIG (for review see^{3,4,18–21}). While a number of reports described effects of IVIG on

various cellular immune components, ranging from T cells,^{22–27} B cells,^{28,29} NK cells³⁰ and dendritic cells,³¹ other authors stress the influence of IVIG on humoral autoimmunity, suggesting anti-idiotypic antibodies in IVIG, FcR engagement, inhibition of complement deposition and others (for review see^{3,4,19,32}).

This study focused on IVIG effects on antibody-mediated immune mechanisms. We utilized murine organotypic cerebellar slice cultures (OSC) as an *ex vivo* model of the immune-CNS interface. As compared to primary cell cultures on the one hand and animal models on the other, the use of OSC has the advantage that the complex spatial microarchitecture of the CNS is maintained and effector mechanisms of CNS damage can be clearly defined, unobscured by the blood-brain barrier (BBB) and peripheral immune components.

Using transgenic mice, which express green fluorescent protein (GFP) in oligodendrocytes and myelin, allowed us to directly monitor demyelination in living OSC. Previously, we had demonstrated the usefulness of this model for the live imaging analysis of different immune effector mechanisms deemed relevant in CNS inflammation^{33,34} as well as the process of CNS myelination itself.³⁵ Now we systematically evaluated - in a “checkerboard” fashion - multiple variables potentially influencing IVIG-mediated therapeutic effects on demyelination induced by a myelin-specific antibody and complement.

We demonstrated that the addition of IVIG efficiently inhibits antibody-mediated demyelination and microglia activation in OSC of the CNS. This effect clearly depended on the Fc part rather than the antigen-binding Fab fragment, since IVIG-derived Fab fragments could neither protect OSC from demyelination nor prevent microglia activation, suggesting a possible direct effect on microglia via binding to SIGN-R1. Interestingly, monoclonal IgG was incapable of exerting protection in a demyelinating environment. While IVIG did not substantially inhibit the binding of the demyelinating antibody to target structures, IVIG-mediated protection was overruled by increasing concentrations of complement. Our data argue for a direct effect of IVIG on cells of the CNS and on the complement cascade, thereby protecting oligodendrocytes in an inflammatory environment.

Material and Methods

Animal husbandry

Mice were bred at the animal facility of the Heinrich-Heine-University Duesseldorf under specific pathogen-

free conditions. B. Zalc kindly provided PLP-GFP mice.^{36,37} In these mice, GFP is expressed under regulatory elements of the PLP gene in oligodendrocytes and located in the cytosol as well as in the myelin sheath (B. Zalc, personal communication).

Media

Dissecting medium

A dissecting medium was used to dissociate the chopped cerebellar OSC from each other and consisted of Hank's Balanced Salt Solution with calcium and magnesium (HBSS, Invitrogen, Darmstadt, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin (P/S, both Invitrogen), 5 mg/mL glucose (Sigma-Aldrich, Seelze, Germany) and 1 mmol/L kynurenic acid (Sigma-Aldrich). The pH was adjusted to 7.2–7.4.

Washing medium

A quantity of 50% HBSS, 50% MEM (Life-Technologies, Darmstadt, Germany), supplemented with P/S and 25 mmol/L HEPES (Sigma-Aldrich).

Culture medium

A quantity of 50% MEM supplemented with P/S, 25% HBSS supplemented with P/S, 25% horse serum (Sigma-Aldrich), 5 mg/mL glucose and 2 mmol/L glutamine.

OSC

OSC were prepared according to a modified protocol published by Stoppini *et al.*³⁸ Shortly, P9-P11 pups were anesthetized, killed, the cerebellum was removed and cut into 400 µm thick slices using a McIlwain tissue chopper. OSC were then dissected manually in ice-cold dissecting medium and transferred to washing medium for 10 min on ice. OSC were cultured on cell culture inserts with a pore diameter of 0.4 µm (Millipore, Billerica, Massachusetts, USA) at 37°C and 5% CO₂ for 3–5 days and subsequently at 33°C and 5% CO₂ for the duration of the experiment.

Fab fragment preparation

Fab fragments were generated from whole IVIG by papain cleavage using a Fab Preparation Kit (Thermo Fisher Scientific GmbH, Darmstadt, Germany), according to the manufacturer's instructions. Fab fragments and IVIG were dialyzed against HBSS using a dialysis cassette (Thermo Fisher Scientific GmbH) prior to use.

Demyelination

Pooled, lyophilized normal baby rabbit serum (BRS) reconstituted in 1 mL of water was used as a source of complement (Cedarlane, Ontario, Canada). For clarity of this manuscript, BRS is referred to as “complement”. Three to twelve percent (vol/vol) of complement were used together with 5–40 $\mu\text{g}/\text{mL}$ recombinant humanized 8-18c5 (hu8-18c5 IgG1, kappa^{39,40}), directed against MOG, for the duration indicated to induce demyelination.³³ IVIG (Privigen[®], CSL-Behring, Hattersheim, Germany; 1–12 mg/mL), monoclonal IgG (Rituximab[®], Roche, Basel, Switzerland; 6 or 12 mg/mL), IVIG-derived Fab fragments (4 mg/mL), bovine serum albumin (BSA, Sigma; 6–12 mg/mL) or human serum albumin (HSA, CSL-Behring; 12 mg/mL) were added at the beginning of demyelination for the duration of the experiment as indicated. Prior to use, Fc fragments, IVIG, HSA, Rituximab, and Fab fragments were dialyzed against HBSS. BSA was dissolved in HBSS.

Flow cytometry

To better quantify the fraction of living oligodendrocytes and microglia, single-cell suspensions were prepared by enzymatic digestion of OSC in HBSS + 0.5% BSA using the Neural Tissue Dissociation Kit-Postnatal Neurons (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations, stained with Fixable Viability Dye (eFluor 450, eBioscience, San Diego, California, USA) and anti-CD11b PerCP-CyTM5.5 (eBioscience), fixed with 4% PFA/PBS and analyzed by flow cytometry (BD FACS CantoTM II). GFP positive cells not stained by eFluor 450 were quantified as “living oligodendrocytes”. CD11b positive cells not stained by eFluor 450 were quantified as “living microglia”. Living oligodendrocytes and living microglia were expressed in percent of all living and gated cells.

Quantification of fluorescence

For the quantification of the relative myelin content of OSC during demyelination, we assessed the intensity of GFP expression in living OSC at different time points during each experiment: Using ImageJ software, we assessed the area of fluorescence signal exceeding a defined threshold in digital images acquired with an Olympus BX51 microscope (Hicksville, NY) at 4 \times magnification. The threshold was chosen according to the background intensity, and only the specific GFP expression was quantified. For each individual OSC, results are depicted relative to time point 0, prior to the beginning of the experiment.

Immunostaining of OSC

OSC were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) dissolved in PBS (Life-Technologies) for 1 h at room temperature, permeabilized with 1% Triton X-100 (Thermo-Scientific) in PBS for 30–45 min and blocked with 10% normal goat serum (Sigma-Aldrich) in 0.2% Triton X-100 in PBS for 1 h after 3 washes with PBS. Primary antibodies (anti-NF200, N52, Millipore; anti-CD68, FA-II, BioLegend, San Diego, CA) were diluted in PBS supplemented with 1% goat serum and 0.2% Triton X-100, according to the manufacturer's instructions and were incubated for 2–3 days at 4°C. After three consecutive washes in PBS, OSC were incubated overnight with secondary antibodies (goat α mouse-Cy5 and goat α rabbit-Cy3, both Millipore), diluted in PBS, supplemented with 1% goat serum and 0–2% Triton X-100 according to the manufacturer's instructions at 4°C in the dark. Images were acquired using an LSM500 confocal laser-scanning microscope (Zeiss, Oberkochen, Germany) and analyzed with Zen black software (Zeiss), ImageJ, and Photoshop. Some images were contrast enhanced to facilitate visibility in composite figures.

Quantitative RT-PCR

RNA was isolated from a single OSC following a standard TRIZOL protocol. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT (Invitrogen). Quantitative PCR was performed according to the manufacturer's instructions, using the SensiFASTTM Probe Lo-ROX Kit from Bioline (London, UK). Primer-Probe sets specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1), myelin basic protein (MPB, Mm01266402_m1), CNPase (Cnp, Mm01306640_m1), Iba1 (Mm00479862_g1), NG2 (Mm00507257) and NF200 (Mm01191456_m1) were purchased from Life Technologies. PCR was performed in an ABI Prism 7500 (Applied Biosystems, Darmstadt, Germany) with 45 cycles. Gene expression was normalized to GAPDH and the untreated control.

Results

IVIG inhibits immune-mediated demyelination in a dose-dependent manner

We induced demyelination in OSC with 5 $\mu\text{g}/\text{mL}$ recombinant humanized MOG-specific antibody and 6% complement as described previously.³³ This treatment induces rapid and pronounced demyelination, which can be monitored by the quantification of the GFP fluorescence emitted by intact myelin/oligodendrocytes (as described in

Material and Methods) before and during treatment. In untreated OSC, GFP-fluorescence remained stable (or slightly increased due to still ongoing myelination) during the observation period of several days, while demyelinated OSC showed a progressive decrease in GFP-fluorescence over time. IVIG itself, with or without complement, had no effect on myelin integrity (Fig. S2), whereas the addition of IVIG significantly reduced the decrease of GFP-fluorescence in OSC treated with anti-MOG antibody and complement (Fig. 1B) in a dose-dependent fashion. The addition of BSA (12 mg/mL) as a control protein had no effect on demyelination (Fig. 1A and B). Likewise, HSA (12 mg/mL) did not inhibit demyelination (Fig. S1). In Figure 1 A representative images of the GFP-expression are depicted. For confocal microscopy, fixed OSC were counterstained with an antibody specific for the axonal protein NF200. As we have shown previously,³³ anti-MOG

antibody and complement efficiently caused demyelination, while axons remained largely intact (Fig. 1C). Whereas axons in the anti-MOG/complement-treated group were demyelinated, myelin was well preserved at the two highest concentrations of IVIG (6 and 12 mg/mL). Additionally, we analyzed gene expression in OSC one and 2 days after the beginning of demyelination, when antibody and complement were still on the slices (Fig. 2A). The kinetics of mRNA-expression of the myelin genes MBP and CNPase paralleled the degradation of GFP in demyelinating OSC (Fig. 2B and C), supporting that the time course and extent of demyelination is adequately represented by the quantification of GFP-fluorescence. On the level of gene transcription, IVIG at a concentration of 6 mg/mL ameliorated the reduction of myelin-associated gene-expression. Interestingly, the expression of Iba1, a marker for microglia, was also reduced in demyelinating

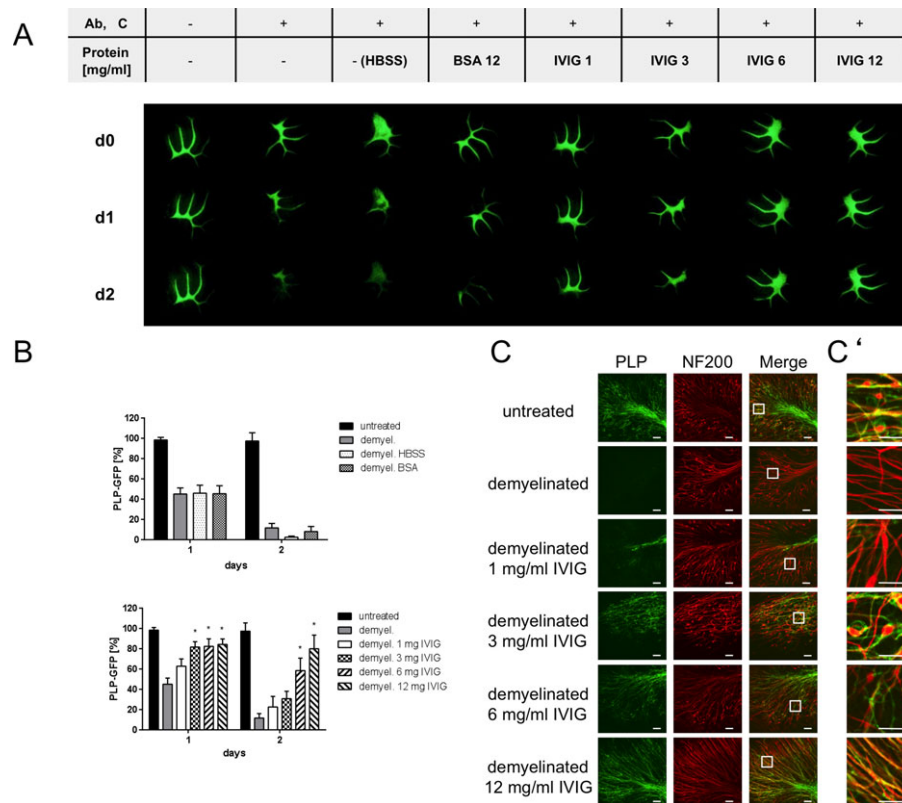


Figure 1. Intravenous immunoglobulin (IVIG) inhibits immune-mediated demyelination in organotypic cerebellar slice culture (OSC). OSC were treated as indicated and green fluorescent protein (GFP)-expression was assessed in living OSC using a fluorescence microscope 1 and 2 days after the induction of demyelination. Quantification was performed with Image J Software. After 2 days, OSC were fixed, stained with anti-NF200 antibody and analyzed via confocal microscopy. (A) Representative images (40 \times magnification) of PLP-GFP-expression: IVIG suppresses immune-mediated demyelination in a dose-dependent fashion. (B) Quantification of the GFP⁺ area relative to day 0 ($n = 6$ OSC per group); Upper graph: Addition of irrelevant protein bovine serum albumin (12 mg/mL) or solvent (HBSS) does not inhibit demyelination. Lower graph: IVIG inhibits demyelination in a concentration-dependent manner. (C) Representative confocal images: IVIG preserves the myelin sheath around axons. (C') Magnification of boxed area. Scale bars (C) 50 μ m (C') 25 μ m. Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

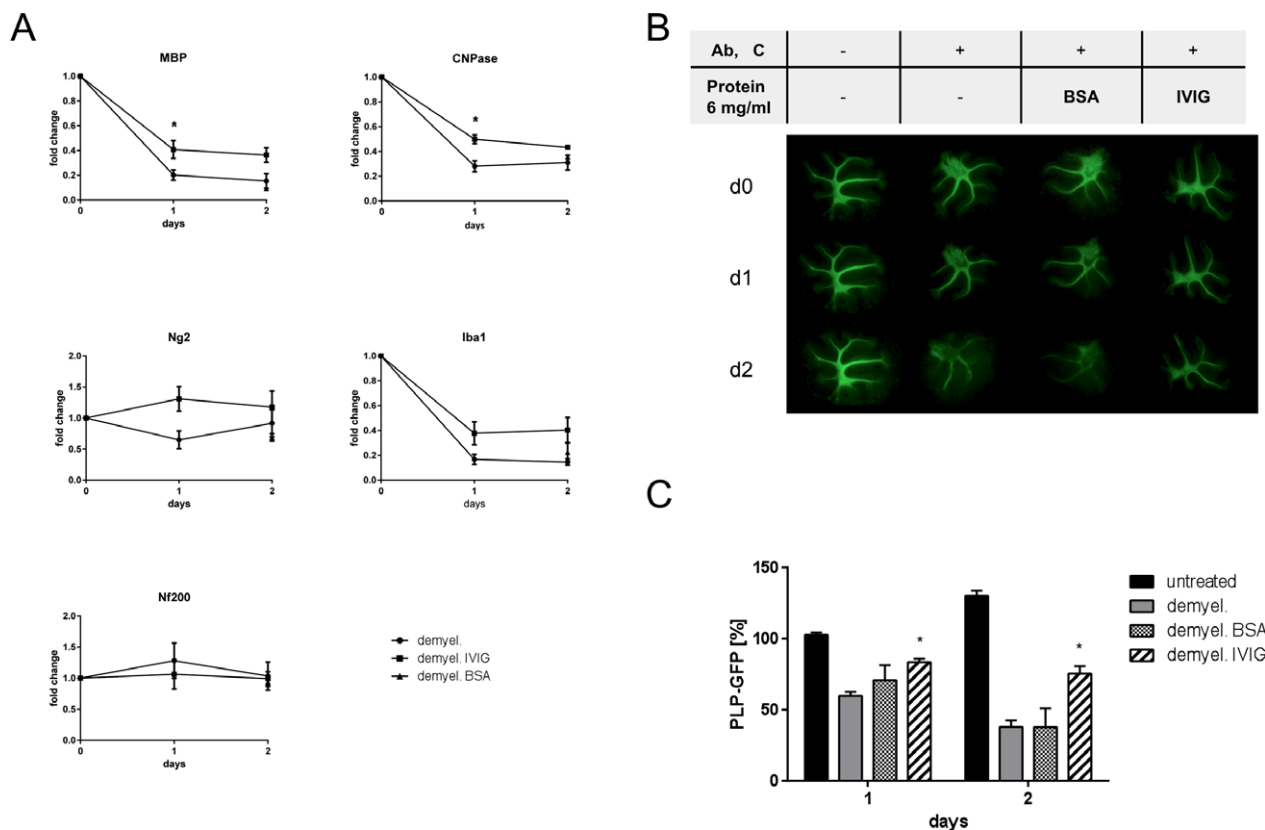


Figure 2. Intravenous immunoglobulin (IVIG)-mediated protection of oligodendrocytes in demyelinated organotypic cerebellar slice culture (OSC) is reflected on the level of gene expression. OSC were demyelinated with 5 μ g/mL anti-MOG antibody and 6% complement alone (circles), with additional 6 mg/mL IVIG (squares) or with additional 6 mg/mL bovine serum albumin (BSA) (triangles). The expression of indicated genes was assessed 1 and 2 days after the onset of demyelination via quantitative rtPCR. Values for BSA treated controls (triangles) were determined for day two only. (A) Expression of indicated genes normalized to GAPDH, relative to the untreated control: While gene expression of axonal (neurofilament N200) and oligodendrocyte progenitor cell (NG2) markers is hardly affected by demyelination/IVIG treatment, IVIG treatment protects against demyelination-induced reduction in gene expression of myelin (MBP, CNPase) and microglial (Iba1) gene expression. Significances were calculated with the student's *t*-test. (B) Representative images of PLP- green fluorescent protein (GFP) expression of OSC used for RT-PCR on days 1 and 2. (C) Quantification of the GFP⁺ area (*n* = 3–6 OSC per group): IVIG but not BSA inhibits demyelination of OSC. Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. **P* \leq 0.05. Values are depicted as mean \pm SEM.

OSC. This effect was partially inhibited by IVIG (Fig. 2A). Corresponding to our results from confocal microscopy, mRNA expression of the axonal marker NF200 was not affected by either demyelination or IVIG treatment. Similarly, NG2 expression remained relatively stable, indicating that neither the demyelination-treatment, nor IVIG had a strong influence on the population of oligodendrocyte precursor cells (Fig. 2A).

IVIG prevents immune-mediated oligodendrocyte death in anti-MOG antibody and complement-treated OSC

For a better quantification of this protective effect, we additionally analyzed oligodendrocyte and microglia survival by flow cytometry: Single cell suspensions, prepared from

untreated and treated OSC following enzymatic dissociation, were stained with Fixable Viability Dye eFluor 450 and CD11b (both eBioscience) before flow cytometry. GFP⁺ cells not stained by eFluor 450 were quantified as “living oligodendrocytes” and expressed in percent of all gated living cells (Fig. 3). As expected, the percentage of living oligodendrocytes was significantly decreased in demyelinated OSC with or without the presence of BSA, while IVIG clearly promoted oligodendrocyte survival (Fig. 3). No significant effects were observed in the microglia population.

IVIG does not neutralize the binding of anti-MOG antibody

To further characterize the mechanisms underlying the IVIG-mediated inhibition of demyelination and cell

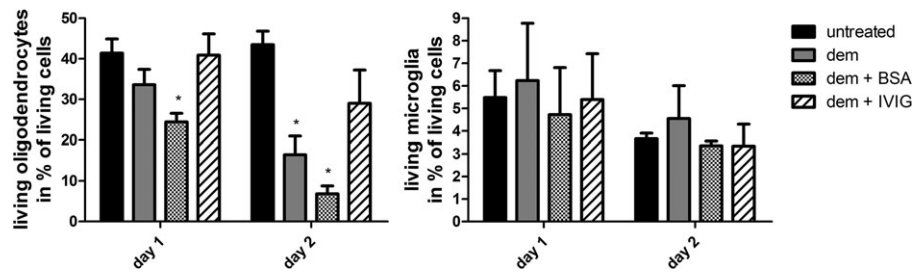


Figure 3. Intravenous immunoglobulin (IVIG) inhibits oligodendrocyte death in antibody-mediated demyelination. Organotypic cerebellar slice cultures (OSC) were demyelinated with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and 5% complement with or without 6 mg/mL IVIG or 6 mg/mL bovine serum albumin (BSA). One and two days after the onset of demyelination, single cell suspensions of OSC were stained for viability and microglia (CD11b), fixed and analyzed by flow cytometry (4 OSC per condition, oligodendrocytes $n = 4$, microglia $n = 3$). Green fluorescent protein (GFP) positive cells not stained by eFluor 450 were quantified as “living oligodendrocytes”. CD11b positive cells not stained by eFluor 450 were quantified as “living microglia”. Living oligodendrocytes and living microglia were expressed in percent of all living and gated cells. Demyelination in the absence or presence of BSA significantly reduced the percentage of viable oligodendrocytes, while the addition of IVIG prevented a significant relative reduction of viable oligodendrocytes. No significant effects on the viability of microglia cells were detected. Significances were calculated in respect to the untreated control using one-way analysis of variance and Dunnett’s post hoc test. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

death, we analyzed whether IVIG interferes with the binding of the anti-MOG antibody to its cognate targets, e.g. by anti-idiotypic neutralization of anti-MOG antibodies. If that were the case, we would expect that increasing concentrations of pathogenic antibody counteract the protective effect of IVIG. To test this hypothesis, we stepwise raised anti-MOG antibody concentrations from 5 to 40 $\mu\text{g}/\text{mL}$. Yet, IVIG-mediated inhibition of demyelination was unaffected by increasing antibody concentrations as assessed by the quantification of GFP expression (Fig. 4A and B). Confocal micrographs confirmed that even at the highest anti-MOG antibody concentration (40 $\mu\text{g}/\text{mL}$), the integrity of myelin and axons were maintained by IVIG (Fig. 4B and B’).

Furthermore, we investigated whether IVIG could possibly supplant anti-MOG antibodies from binding to their epitope on the myelin sheath. To this end, OSC were preincubated with 6 mg/mL IVIG for 3 days prior to fixation and IHC with the biotinylated anti-MOG antibody. Confocal colocalization of PLP-GFP-fluorescence (green) and anti-MOG staining (red) (Fig. 5) clearly indicates that binding of the anti-MOG antibody to its target structures was not affected by IVIG pretreatment. It therefore seems unlikely that IVIG exerts “anti-idiotypic” effects by IgG specific for the MOG epitope of the hu8-18c5 antibody or for the antibody used in the experiments itself.

IVIG-mediated protection is overruled by rising complement concentrations

To determine whether IVIG exerts its effects on demyelination by interfering with complement deposition and/or

activation, we induced demyelination with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and varied the concentration of complement from 3% to 12%. As shown in Figure 6, at a complement concentration of 3%, only very mild demyelination occurred. Yet, raising the concentration of complement to 6%, 9% and 12% clearly led to increasing demyelination (Fig. 6A). While IVIG (6 mg/mL) significantly protected from demyelination at a complement concentration of 6%, this protection was overruled at complement concentrations of 9% and 12% (Fig. 6A and B), and demyelination occurred irrespectively of the presence of IVIG. These findings clearly support that IVIG interferes with complement deposition and/or activation in a dose-dependent fashion.

Interestingly, these effects were paralleled by morphological changes of tissue resident microglia cells: Confocal images revealed that in untreated slices and slices treated with anti-MOG antibody and 3% complement, CD68⁺ microglia cells were small, ramified and evenly distributed within the tissue, resembling an inactivated state, irrespectively of the presence of IVIG. In contrast, in slices treated with anti-MOG antibody and 6%, 9% or 12% complement, microglia cells displayed a typical activated phenotype and appeared rounded up, swollen, mainly being located at the sites of myelin destruction. In OSC demyelinated with anti-MOG antibody and 6% complement, IVIG prevented these morphological changes typical for microglia activation. Similar to demyelination, IVIG failed to prevent microglia activation when OSC were demyelinated with 9% and 12% complement, indicating that IVIG inhibits the activation of microglia cells by either direct or indirect interaction but can be overruled by excess concentrations of complement.

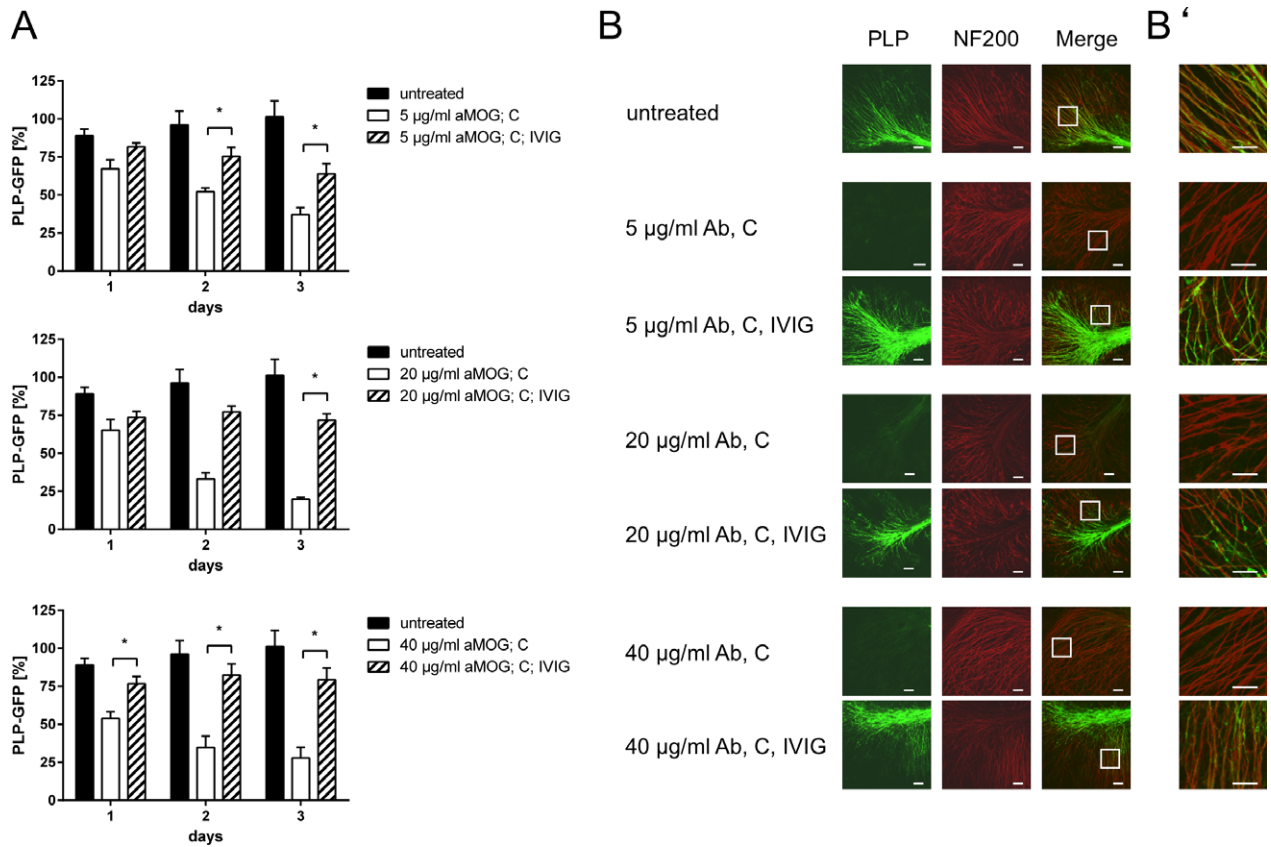


Figure 4. Intravenous immunoglobulin (IVIG)-mediated inhibition of demyelination is independent of the anti-MOG antibody concentration. Organotypic cerebellar slice cultures (OSC) were demyelinated with varying concentrations of anti-MOG antibody and 6% complement with or without 6 mg/mL IVIG. Green fluorescent protein (GFP)-expression in living OSC was assessed with a fluorescence microscope at days 1, 2 and 3. Quantification of GFP-fluorescence was performed with Image J Software. After 3 days, OSC were fixed, stained with anti-NF200 antibody and analyzed by confocal microscopy. (A) Quantification of the GFP⁺ area ($n = 4-6$ OSC per group) showed that IVIG-mediated inhibition of demyelination was not overruled by increasing concentrations of the anti-MOG antibody. (B) Representative confocal images illustrate that 6 mg/mL IVIG preserved the integrity of myelin sheaths around axons at all concentrations of the anti-MOG antibody. (B') Magnification of boxed area. Scale bars (B) 50 μm , (B') 25 μm . Significances between the demyelinated condition with and without IVIG were calculated with the student's *t*-test. Control bars were included for better comparability. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

Myelin protection by IVIG depends on the Fc terminus, not the variable region

In order to elucidate which part of the immunoglobulin molecules present in IVIG is responsible for the observed effects, we cleaved IVIG to obtain pure Fab fragments via papain digestion. Figure 7A shows the purified denatured and native Fab fragments in lanes 2 and 4, respectively. OSC were demyelinated with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and 6% complement in the presence or absence of either 6 mg/mL IVIG or equimolar IVIG-derived Fab fragments. As control protein, 6 mg/mL BSA was added to demyelinating OSC. In contrast to intact IVIG, neither the Fab fragments nor BSA effectively suppressed demyelination (Fig. 7B and C). Confocal images revealed that only OSC, which were treated with intact IVIG, were

still myelinated after 3 days. We additionally stained phagocytizing microglia with an anti-CD68 antibody. In untreated OSC, microglia appeared small, ramified and evenly distributed within the tissue, while microglia in demyelinated OSC had an activated phenotype and strongly expressed CD68. IVIG reduced microglial activation to control levels.

Additionally, we studied the effect of Fc fragments derived from a polyclonal preparation of human IgG. Interestingly, Fc fragments protected axons from demyelination even more efficiently than equimolar concentrations of IVIG: Both 1 and 2 mg/mL huFc protected myelin to about 75-90%, while 6 mg/mL IVIG inhibited myelin degradation only to about 60% (Fig. 8A). Confocal imaging confirmed that the addition of IVIG or equimolar amounts of huFc both inhibited microglia

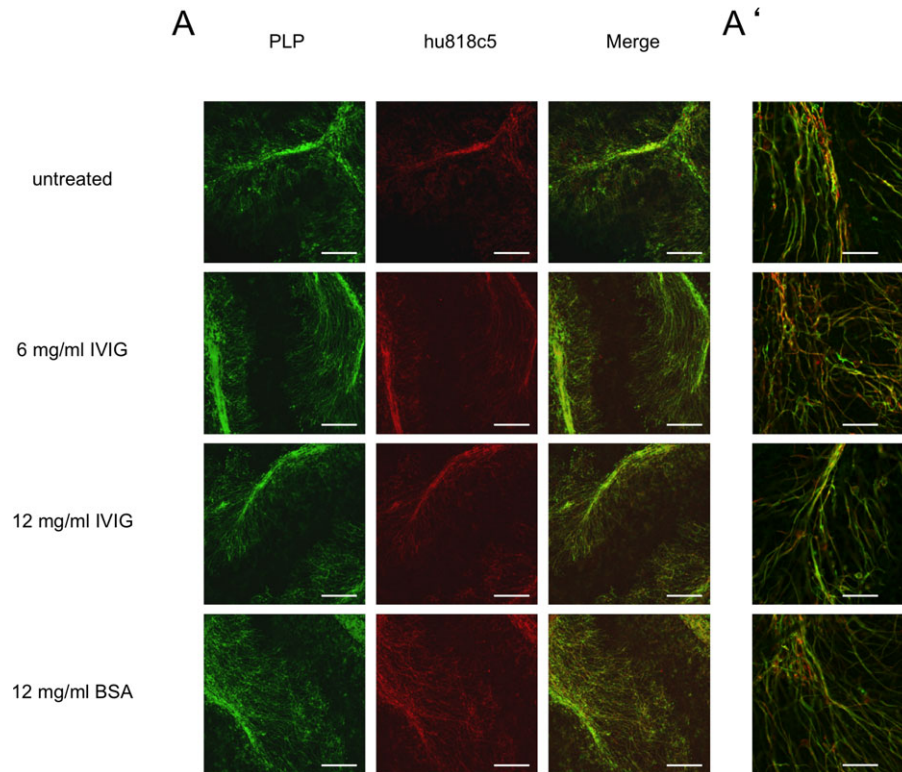


Figure 5. Intravenous immunoglobulin (IVIG) does not compete with the binding of anti-MOG antibody during demyelination. Organotypic cerebellar slice cultures were incubated with or without IVIG or bovine serum albumin (BSA) as indicated for 3 days and subsequently fixed and stained with a biotinylated hu8-18C5 anti-MOG antibody: The anti-MOG antibody used for demyelination in our experiments bound strongly to myelin irrespective of pretreatment with IVIG or BSA. Scale bars (A) 100 μm , (A') 50 μm .

activation and severe demyelination in anti-MOG antibody and complement-treated OSC (Fig. 8B).

IVIG-mediated protection seems to depend on specific modifications of the Fc terminus or the composition of isotypes

Since the great variety of IgG-specificities within the IVIG preparation apparently was dispensable for the observed protective effects, the question remained open, whether equal concentrations of a monoclonal IgG would suffice to inhibit demyelination in our model system. We therefore assessed whether the monoclonal antibody Rituximab[®] also inhibits immune-mediated demyelination in OSC. While 6 and 12 mg/mL IVIG clearly protected OSC from myelin degradation, Rituximab had no significant protective effect at either concentration (Fig. 9A and B). Likewise, while microglia activation was inhibited in IVIG-treated demyelinating OSC, microglia was highly activated in demyelinated OSC, which was not altered by the addition of Rituximab (Fig. 9B), arguing for the necessity of specific modifications of the Fc terminus of IgG antibodies.

Discussion

In this study, we aimed to assess direct protective effects of IVIG on CNS tissue during demyelination, a damage characteristic for multiple sclerosis.⁴¹ As compared to animal models on the one hand and primary cell cultures on the other, the use of OSC as an *ex vivo* model of the CNS-immune interface has the advantage that effector mechanisms of CNS damage can be clearly defined while the complex spatial microarchitecture of the CNS is maintained.

To mimic immune-mediated demyelination, OSC were incubated with a humanized recombinant anti-MOG antibody together with complement for 2–3 days. At the concentrations of anti-MOG antibody and complement tested, this treatment resulted in rapid and severe demyelination and damage of oligodendrocytes, while axons remained largely intact for the duration of the experiment as shown before.³³ The addition of IVIG blocked this immune-mediated demyelination in a concentration-dependent manner, arguing for IVIG-dependent actions within the CNS that exceed the modulation of peripheral

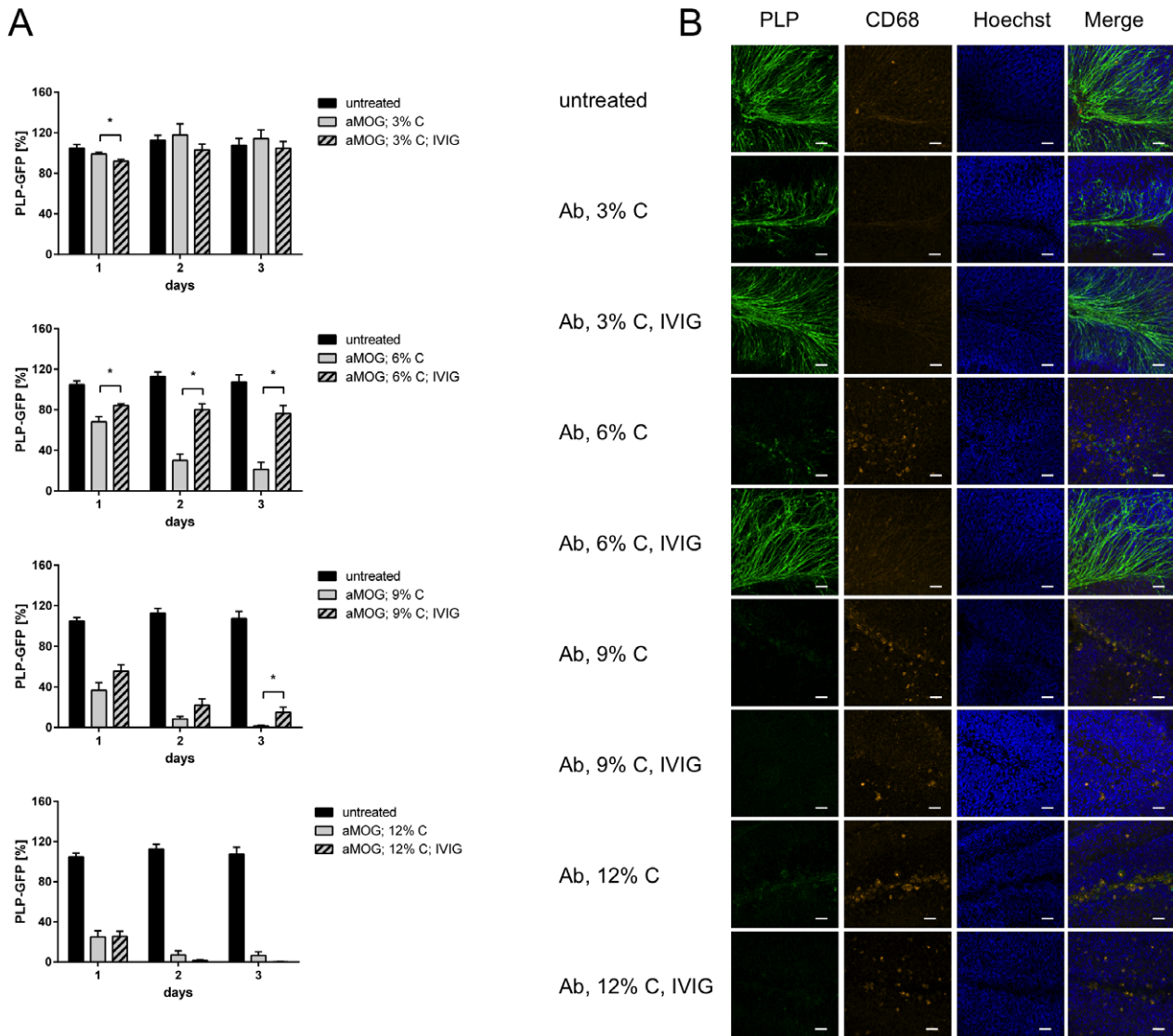


Figure 6. Intravenous immunoglobulin (IVIG)-mediated inhibition of demyelination is overruled by increasing concentrations of complement. Organotypic cerebellar slice cultures (OSC) were demyelinated with 5 μ g/mL anti-MOG antibody and varying concentrations of complement with or without 6 mg/mL IVIG. Green fluorescent protein (GFP)-expression in living OSC was assessed with a fluorescence microscope at days 1, 2 and 3. Quantification was performed with Image J Software. After 3 days, OSC were fixed, stained with anti-CD68 antibody and Hoechst dye and analyzed by confocal microscopy. (A) Quantification of the GFP⁺ area ($n = 7-8$ OSC per group): At the higher concentrations of complement, the protective effect of IVIG is abrogated. (B) Representative confocal images: At complement concentrations above 6%, IVIG does not preserve the myelin sheaths or prevent microglial activation. (B') Magnification of boxed area. Scale bars (B) 50 μ m, (B') 25 μ m. Significances between the demyelinated condition with and without IVIG were calculated with the student's *t*-test. Control bars were included for better comparability. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

immune mechanisms. IVIG clearly reduced demyelination of axons as depicted in Figure 1, while albumin at the same concentrations did not affect demyelination, excluding effects merely due to a higher protein-concentration in the IVIG-treated cultures. This protective effect of IVIG was reproducible both by flow cytometry and on the level of gene expression: flow cytometry of single cell suspensions prepared from OSC confirmed a significant

reduction of living oligodendrocytes induced by anti-MOG antibody and complement, irrespective of the presence of BSA, while in IVIG-treated OSC, the fraction of viable oligodendrocytes was not significantly reduced (Fig. 3). No significant effect on the viability of microglia cells was observed. Correspondingly, mRNA levels of the myelin genes MBP and CNPase were both lowered in demyelinating OSC, an effect which was reduced by IVIG.

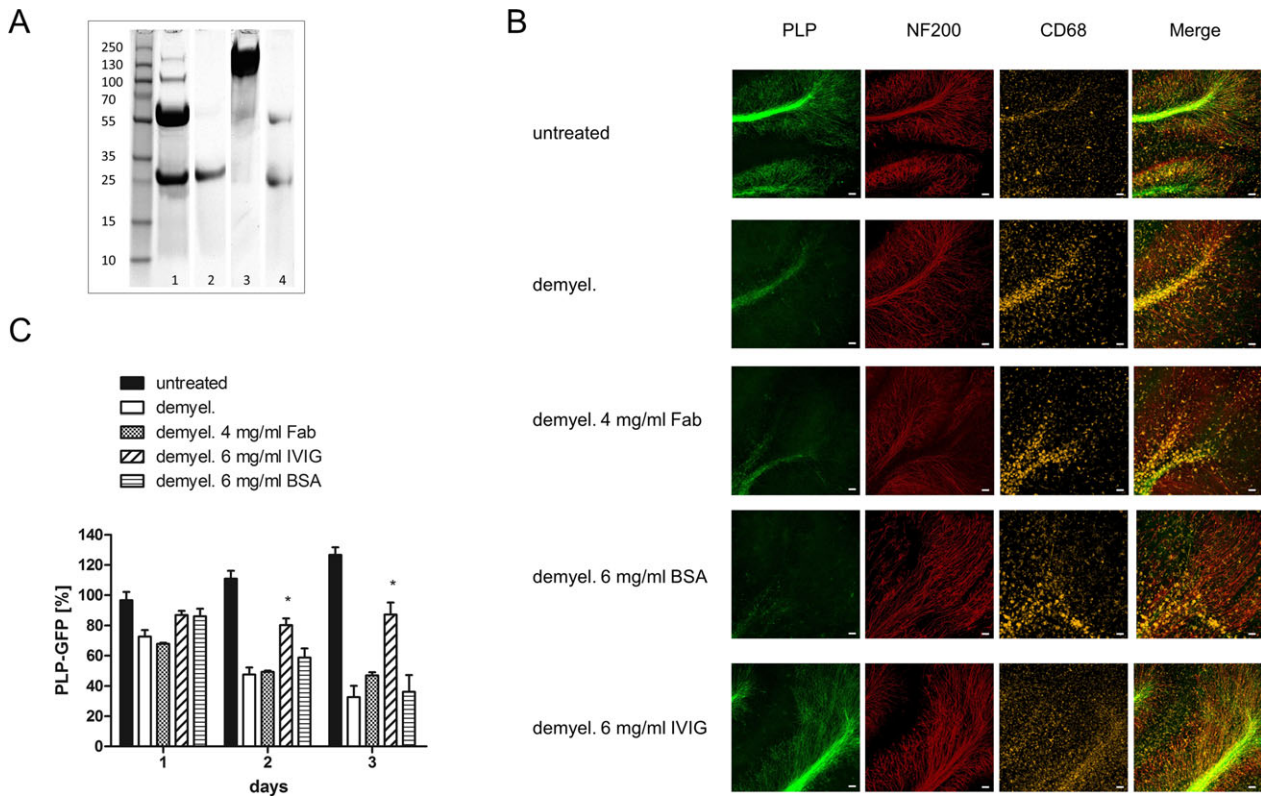


Figure 7. Ig-variable regions do not mediate intravenous immunoglobulin (IVIG)-mediated inhibition of demyelination. Polyclonal Fab fragments were generated from whole IVIG by papain cleavage (see material and methods). (A) To verify the purity of the preparation, Fab fragments were separated on a 12% SDS-gel under denaturing (lanes 1 and 2) or native (lanes 3 and 4) conditions. Lanes 1 and 3 show whole IgG, while lanes 2 and 4 show purified Fab fragments. (B, C) organotypic cerebellar slice culture (OSC) was demyelinated with 5 μ g/mL anti-MOG antibody and 6% complement with or without 6 mg/mL IVIG, 4 mg/mL Fab fragment or 6 mg/mL bovine serum albumin (BSA) or were left untreated. Green fluorescent protein (GFP) expression in living OSC was assessed by fluorescence microscopy at days 1, 2 and 3. Quantification was performed with Image J Software. After 3 days, OSC was fixed, stained with anti-NF200 and anti-CD68 antibody and analyzed via confocal microscopy. (B) Representative confocal images: IVIG but not Fab fragments or BSA inhibit demyelination of axons and microglia activation. (C) Quantification of the GFP⁺ area ($n = 3$ OSC per group): IVIG but not Fab fragments or BSA inhibit immune-mediated demyelination in OSC. Scale bar 50 μ m. Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

Paralleling microglia activation, mRNA expression of the microglia marker *Iba1* was reduced in demyelinated OSC (Fig. 2A), in line with findings by Silverman and colleagues in a different experimental model.⁴² Microglia cells in demyelinated OSC, when compared to the untreated controls, appeared swollen and rounded, resembling the morphology of highly activated, phagocytizing microglia. In untreated OSC, microglia appeared ramified and small, reflecting the phenotype of resting microglia (Figs. 6-9). As visualized by immunohistochemistry, IVIG strongly reduced microglia activation (Figs. 6-9) in demyelinating OSC, whereas BSA added instead of IVIG at the same concentration did not visibly modulate microglia (Fig. 7). Previously, it was demonstrated that IVIG influences microglia activation by different mechanisms, involving both Fc- and Fab-dependent pathways: Stangel and

colleagues showed, that IVIG activates matrix-metalloproteinase (MMP-9) in cultured microglia *in vitro*,⁴³ while earlier, the same group demonstrated that IVIG down regulates endocytosis via F(ab')₂-, and receptor-mediated phagocytosis by Fc-dependent mechanisms.⁴⁴ Additionally, IVIG was described to activate TNF α -secretion and NO-production in isolated microglia cells via Fc receptors.^{45,46} These findings suggest that IVIG may influence both activating and silencing mechanisms in microglia, fine-tuning the activation status of these cells. Our data implicate that IVIG modulates microglia activation within the CNS tissue, which is likely to contribute to the preservation of myelin in OSC. Nevertheless, the question remains open whether modulation of microglia activation is a primary, direct effect of IVIG treatment or a result of IVIG-mediated reduction of oligodendrocyte/myelin damage in

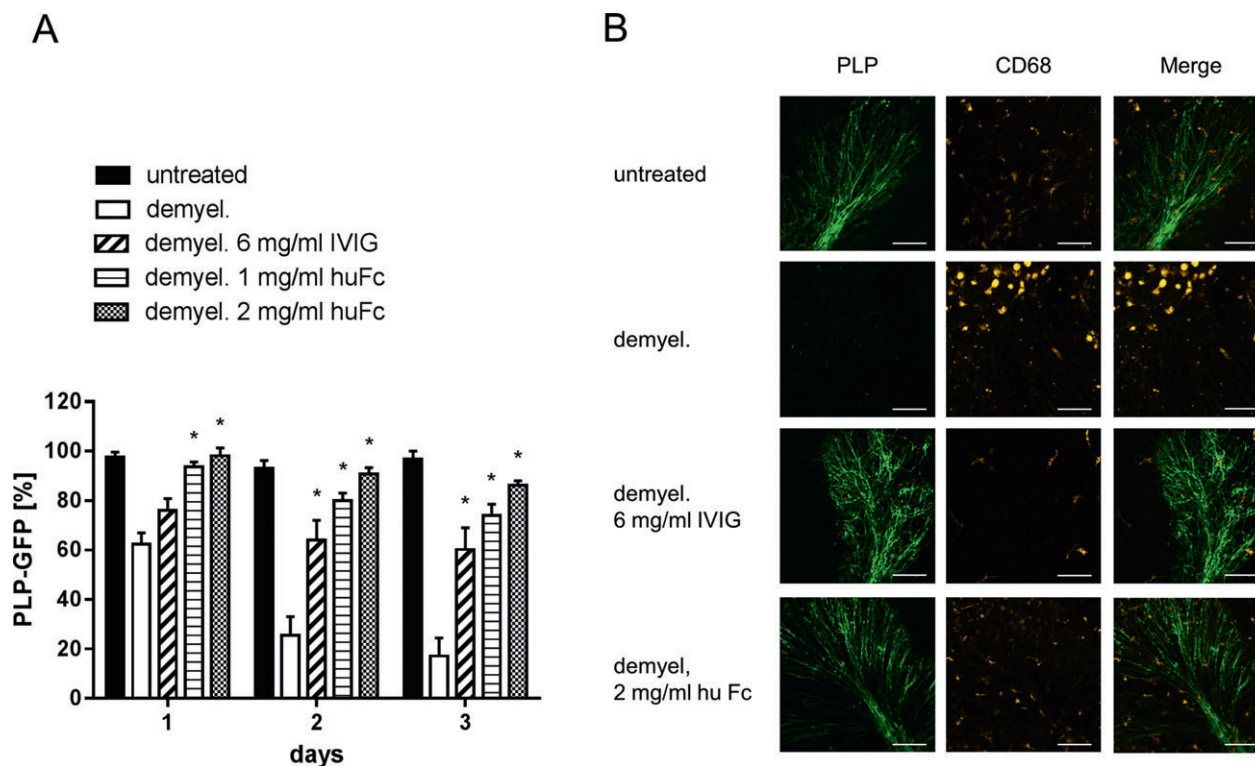


Figure 8. Intravenous immunoglobulin (IVIG) induced protection from demyelination is Fc-mediated. Organotypic cerebellar slice cultures (OSC) were demyelinated with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and 6% complement with or without 6 mg/mL IVIG or 2 mg/mL human polyclonal Fc fragment or were left untreated. Green fluorescent protein (GFP)-expression was assessed in living OSC under a fluorescence microscope 1, 2 and 3 days after the induction of demyelination. Quantification was performed with Image J Software. After 3 days, OSC were fixed, stained with an anti-CD68 antibody and analyzed via confocal microscopy. (A) Quantification of the GFP⁺ area ($n = 3\text{--}12$ OSC per group): Fc fragments suppress demyelination even more potently than whole IVIG at an equimolar concentration. (B) Representative confocal images. Scale bars 100 μm . Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. $*P \leq 0.05$. Values are depicted as mean \pm SEM.

demyelinating OSC, leading to a secondary decrease of activation stimuli in the microenvironment of the inflamed brain tissue.

In line with our previously published data³³ axons were not affected by demyelination or by IVIG treatment, as shown by immunohistochemical staining and RT-PCR analysis of the axonal marker NF-200. At least within the time frame observed, oligodendrogenesis also did not seem to be influenced by IVIG, since IVIG did not alter NG2-expression in demyelinating OSC. BSA again had no effect on the expression of any gene assessed, excluding artifacts due to a higher concentration of protein in the culture medium.

Others and we have demonstrated that IVIG itself together with complement does not result in demyelination (Fig. S2,⁴⁷), indicating that IVIG does not contain proportions of naturally occurring oligodendrocyte/myelin-specific antibodies sufficient to cause demyelination in the presence of complement. Alternatively,

oligodendrocyte/myelin-specific antibodies contained in IVIG preparations may be of isotypes unsuited for complement-mediated lysis, e.g. IgG4, but sufficient to compete with "pathogenic" autoantibodies in patients with demyelinating diseases. To further address this point, we assessed, whether IVIG competes with the anti-MOG antibody for binding to its target epitope within the tissue, a prerequisite for efficient assembly of anti-MOG-C3b complexes at the oligodendrocyte surface. Confocal images show that preincubation of OSC with IVIG for 3 days does not reduce staining of myelinated structures with the anti-MOG antibody by immunohistochemistry (Fig. 5). Therefore, it seems unlikely that IVIG reduces demyelination by masking target epitopes of the anti-MOG antibody used for demyelination.

To further elucidate the mode of action of IVIG in our model, we varied concentrations of anti-MOG antibody or complement in separate experiments to dissect, whether IVIG interferes with either component. The

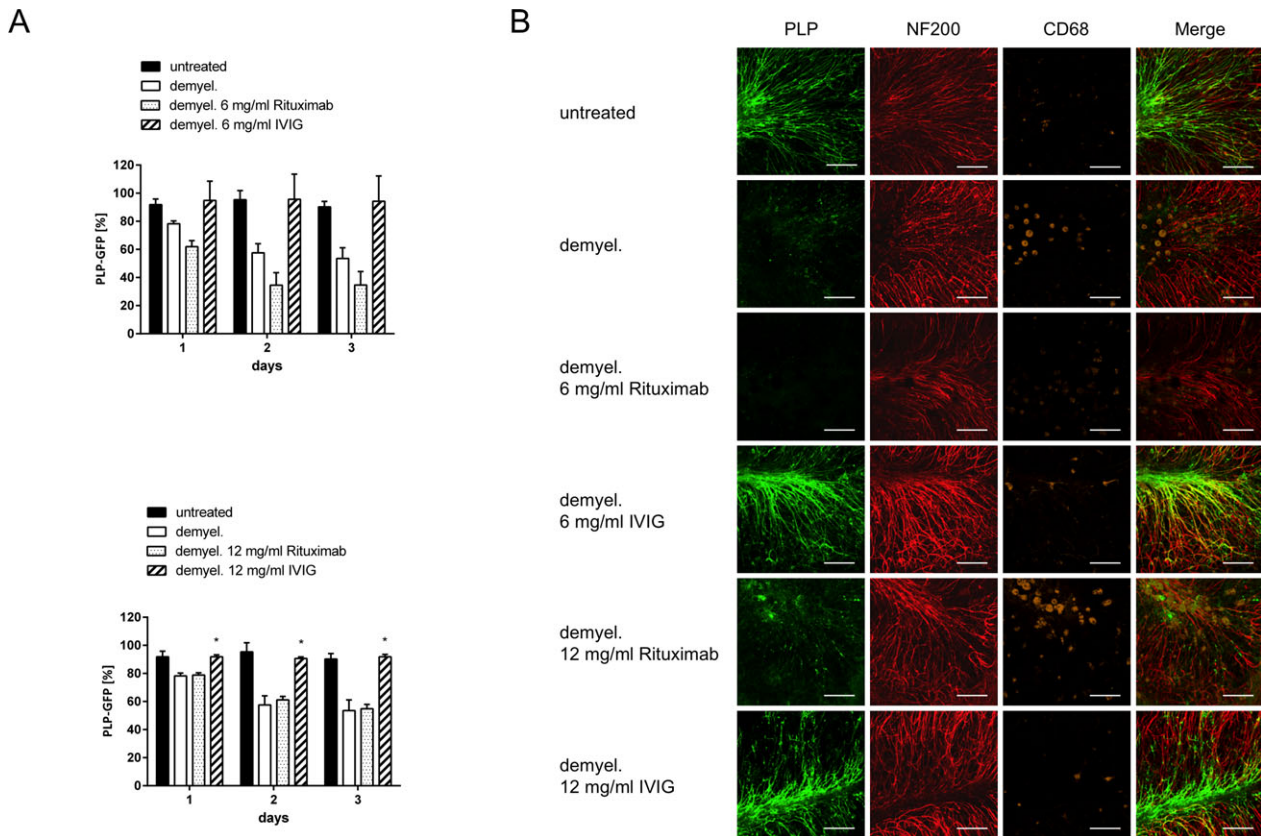


Figure 9. Monoclonal IgG does not protect organotypic cerebellar slice cultures (OSC) from immune-mediated demyelination. OSC were demyelinated with 5 μ g/mL anti-MOG antibody and 6% complement with or without intravenous immunoglobulin (IVIG) or Rituximab or were left untreated as indicated. Green fluorescent protein (GFP)-expression in living OSC was assessed by fluorescence microscopy at day 1, 2 and 3. (A) Quantification of the GFP⁺ area ($n = 4-5$ OSC per group) was performed with Image J Software: IVIG but not Rituximab suppresses demyelination in OSC. (B) Representative confocal images: IVIG but not the recombinant monoclonal antibody Rituximab prevents demyelination of axons and microglial activation. Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. * $P \leq 0.05$. Values are depicted as mean \pm SEM.

results were remarkably clear: While elevating the concentration of the anti-MOG antibody had no influence on IVIG-mediated protection of myelin, increasing the concentration of complement gradually overruled IVIG protection. This finding strongly argues for an interference of IVIG with the complement cascade, rather than anti-idiotypic blocking of anti-MOG-antibodies. This is partially congruent with the literature. Using FcR γ ^{-/-} and C1q^{-/-} mice in a model of anti-MOG antibody enhanced experimental autoimmune encephalomyelitis (EAE), Becher and colleagues demonstrated that - while the functional expression of FcR γ on systemic accessory cells appeared to be vital for the *development* of CNS inflammation - the *demyelinating* capacity of such autoAb *in vivo* relies on complement activation and is FcR-independent.⁴⁸ Since the 1990s, it is known that IVIG acts as acceptor for complement components like C3b and C4b, thereby scavenging them from cell-bound immune complexes.⁴⁹

However, the formation of soluble C3b₂-IgG complexes results in amplification of complement activation, rather than in attenuation, since these soluble complexes are even more effective in cleaving C3 and thereby amplifying the complement cascade than cell-bound complexes. IVIG however was shown to additionally lower the half-life of soluble C3b_n-IgG complexes both *in vitro* and *in vivo*, which reduces the concentration of C3-convertase and by that the nascence of reactive complement-factors and the proceeding of the complement-cascade.^{50,51}

Since IVIG presumably interferes with the complement reaction, we asked the question, whether autoantibodies against complement components may scavenge complement factors from the medium, preventing the formation of membrane-bound C3b₂-IgG complexes and subsequent lysis of oligodendrocytes. To this end we tested the effects of IVIG-derived Fab fragments versus human Fc fragments from a polyclonal preparation of IgG on

demyelination in our model. IVIG-derived Fab fragments could not provide protection from anti-MOG antibody and complement-mediated demyelination or microglia-activation, while Fc fragments were even more potent than complete IVIG in inhibiting demyelination (Figs. 7 and 8).

Several autoantibodies against complement factors have been described and in other studies anti-idiotypic effects of IVIG were found to be responsible for the scavenging of complement in various model systems.^{52,53} We clearly show that blocking of complement by IVIG does not involve specific antigen binding via the variable antigen-binding regions (Fab fragments) of the IgG molecules contained in IVIG. Instead, our findings confirm that the Fc part of IVIG can interfere with the complement cascade, in line with Yuki *et al.*⁵⁴

Both animal experiments and clinical evidence suggest that the modification pattern of the Fc terminus by sugar residues is crucial for the Fc-mediated therapeutic effects of IVIG. Ravetch and colleagues showed that sialyl-residues bound to the Fc region seem to be indispensable for certain effects.^{55,56} Sialylated IgG preferably interacts with C-type lectins like the human DC-Sign (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) or the murine homolog SignR1.⁵⁷ Park *et al.*⁵⁸ demonstrated mSign-R1 expressed on microglial cells in the cerebellum, identifying microglia as a likely target for IVIG-interference. The lack of sialyl residues in the monoclonal antibody Rituximab⁵⁹ may also be an explanation for its inability to protect from demyelination in our experimental system. In a recent study however, Campbell *et al.* showed that the therapeutic effects of IVIG in two antibody and complement-dependent models of arthritis are dependent on the Fc part but are independent of the sialylation pattern, putting the importance of Fc modifications up for debate.⁶⁰ We propose that in the complex microenvironment of the CNS, Fc-mediated effector-mechanisms of IVIG may play a pivotal role, possibly acting on both microglia and complement factors in parallel by distinct or cumulative mechanisms.

In summary we demonstrate that IVIG modulates immune-CNS interactions in a multimodal fashion: In our model of immune (antibody/complement)-mediated demyelination, the preservation of myelin seems to largely depend on the inhibition of the complement cascade by IVIG. Additionally, IVIG-mediated suppression of microglia activation may be an important effect of IVIG, even though this hypothesis is yet to be addressed. Our data clearly shows that in this model system the observed effects entirely depend on Fc-dependent mechanisms, while the variable Fab fragment is not needed to convey protection.

Taken together our findings raise a number of potential clinical considerations:

- On the one hand, the applied dose of IVIG needs to be sufficient to counteract complement factors. While in most “autoimmune” conditions an empirical standard dose of 2 g IVIG/kg bodyweight is applied, clinical trials in the past have used a variety of dosing regimens, possibly offering an explanation for controversial effects. Since complement factors seem to be a major target of IVIG at least in diseases with clear “antibody/complement” mediated effector mechanisms (such as myasthenia gravis and many others), measurements of serum complement activity or derivatives thereof may be helpful for “personalized” treatment regimens both with regard to dosage and frequency of application.
 - While this is a relatively obvious conclusion from this study, it is also tempting to speculate on the potential beneficial direct effects of IVIG on a variety of CNS diseases. At first glance, the lack of a BBB in our experimental model seems to limit the interpretation of our findings with regards to a therapeutic *in vivo* situation. We found few data on the actual amount of systemically administered IVIG reaching the CNS compartment: van Engelen detected a mean increase of 44% in cerebrospinal fluid (CSF) IgG concentration in epilepsy patients after systemic IVIG delivery, with the extent of increase correlating with Q albumin, a measure of blood CSF permeability.⁶¹ Wurster and Haas measured an intrathecal Ig concentration reaching close to 1% of the serum Ig concentration (0.317 g/L vs. 32.1 g/L) in a patient with polyradiculitis after infusion of 30 g IVIG.⁶² More recent studies in rodents by Sha Mi and colleagues established that systemically applied therapeutic antibodies could reach the CSF in therapeutic concentrations.⁶³ Likewise, IVIG was detected “throughout the brain of injected mice, in capillaries, brain parenchyma and brain cells”.⁶⁴ While these studies support that immunoglobulin may reach the mammalian CNS in spite of an intact BBB, this is expected to be even more the case in inflammatory CNS conditions characterized by (an at least partial) BBB breakdown: Hawkins and colleagues described a partial enhancement of CNS lesions in chronic relapsing experimental encephalomyelitis by immunoglobulin added to gadolinium-DTPA (diethylenetriaminepentaacetic acid).⁶⁵
- In spite of these promising reports, lacking systematic studies, we can currently not answer which proportion of systemically applied IVIG reaches the CNS compartment, nor can we estimate which local IVIG concentrations would be needed to achieve therapeutic effects. Nevertheless, in debilitating immune-mediated diseases namely MS, also an intrathecal application suitable to

deliver high local IVIG concentrations to the CNS may be worth considering.

- Since our study confirms that solely the Fc part of IVIG conveys protection from immune-mediated demyelination, future trials with recombinant forms of Fc fragments may be considered, for which Ravetch and colleagues elegantly demonstrated anti-inflammatory activity if adequately sialylated.⁵⁶
- In our study, we did not touch on the issue whether IVIG has an influence on remyelination in the OSC model of immune-induced demyelination, but it will be interesting to assess direct effects of IVIG within the CNS on remyelination processes in OSC in the future.⁶⁶

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Author Contributions

M. W., C. B., N. G. conceived and designed the experiments. M. W., C. B., J. S., S. B., M. M., M. H.-K. performed the experiments. M. W., C. B., J. S., N. G. analyzed the data and M. W., C.B., H.-P. H., N. G. wrote the paper.

Conflict of Interest

NG received, with approval by the Rector of the Heinrich-Heine-University, travel support to scientific conferences from Bayer, Biogen, Genzyme, Novartis; research support (unrelated to this manuscript) from Novartis; honoraries for lectures (unrelated to this manuscript) from Biogen. HPH received fees for consulting and serving on steering committees from CSL Behring, Baxter, Novartis, Octapharma, with approval by the Rector of Heinrich-Heine-University.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Human serum albumin does not protect organotypic cerebellar slice cultures (OSC) from immune-mediated demyelination. OSC were demyelinated with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and 4% complement with or without 6 mg/mL IVIG (6 mg/mL) or HSA (12 mg/mL) or were left untreated. GFP expression in living OSC was assessed by fluorescence microscopy at days 1–3 and quantified relative to day 0 with Image J Software ($n = 4$ OSC per group). Treatment with the irrelevant protein (HSA, 12 mg/mL) does not inhibit demyelination. Significances were calculated in respect to the demyelinated control using one-way analysis of variance and Dunnett's post hoc test. $*P \leq 0.05$. Values are depicted as mean \pm SEM.

Figure S2. IVIG \pm complement does not initiate demyelination in organotypic cerebellar slice culture (OSC). OSC were treated as indicated. For demyelination the slices were incubated with 5 $\mu\text{g}/\text{mL}$ anti-MOG antibody and 6% complement. GFP expression in living OSC was assessed by fluorescence microscopy at days 1–3 and quantified relative to day 0 with Image J Software ($n = 6$ –8 OSC per group). Neither IVIG alone nor IVIG with complement lead to demyelination in OSC. Significances were calculated in respect to the untreated control using one-way analysis of variance and Dunnett's post hoc test. $*P \leq 0.05$. Values are depicted as mean \pm SEM.