More Efficient Complement Activation by Anti–Aquaporin-4 Compared With Anti–Myelin Oligodendrocyte Glycoprotein Antibodies

Magdalena Lerch, MSc, Kathrin Schanda, MSc, Elliott Lafon, MSc, Reinhard Würzner, MD PhD, Sara Mariotto, MD PhD, Alessandro Dinoto, MD, Eva Maria Wendel, MD, Christian Lechner, MD, Harald Hegen, MD PhD, Kevin Rostásy, MD, Thomas Berger, MD, MSc, Doris Wilflingseder, PhD, Romana Höftberger, MD, and Markus Reindl, PhD

Neurol Neuroimmunol Neuroinflamm 2023;10:e200059. doi:10.1212/NXI.0000000000200059

Abstract

Background and Objectives
The objective was to study complement-mediated cytotoxicity induced by immunoglobulin G (IgG) anti–aquaporin-4 antibodies (AQP4-IgG) and anti–myelin oligodendrocyte glycoprotein antibodies (MOG-IgG) in human serum samples from patients suffering from the rare demyelinating diseases of the CNS neuromyelitis optica spectrum disorder (NMOSD) and MOG-IgG–associated disease (MOGAD).

Methods
A cell-based assay with HEK293A cells expressing different MOG isoforms (MOGα1-3β1-3) or AQP4-M23 was used. Cells were incubated with human MOG-IgG or AQP4-IgG–positive serum samples together with active or heat-inactivated human complement, and complement-dependent cytotoxicity (CDC) was measured with a lactate dehydrogenase assay. To further quantify antibody-mediated cell damage, formation of the terminal complement complex (TCC) was analyzed by flow cytometry. In addition, immunocytochemistry of the TCC and complement component 3 (C3) was performed.

Results
AQP4-IgG–positive serum samples induced higher CDC and TCC levels than MOG-IgG–positive sera. Notably, both showed a correlation between antibody titers and CDC and also between titers and TCC levels. In addition, all 6 MOG isoforms tested (MOGα1,3β1-3) could induce at least some CDC; however, the strongest MOG-IgG–induced CDC levels were found on MOGα1, MOGα3, and MOGβ1. Different MOG-IgG binding patterns regarding recognition of different MOG isoforms were investigated, and it was found that MOG-IgG recognizing all 6 isoforms again induced highest CDC levels on MOGα1 and MOGβ1. Furthermore, surface staining of TCC and C3 revealed positive staining on all 6 MOG isoforms tested, as well as on AQP4-M23.

Discussion
Both MOG-IgG and AQP4-IgG are able to induce CDC in a titer-dependent manner. However, AQP4-IgG showed markedly higher levels of CDC compared with MOG in vitro on target cells. This further highlights the role of complement in AQP4-IgG–mediated disease and diminishes the importance of complement activation in MOG-IgG–mediated autoimmune disease.
Autoantibody-mediated autoimmune diseases comprise a broad spectrum that is rapidly evolving. Immunoglobulin G (IgG) antibodies against aquaporin-4 (AQP4-IgG) are found in most patients with neuromyelitis optica spectrum disorder (NMOSD), whereas antibodies against myelin oligodendrocyte glycoprotein (MOG-IgG) are associated with MOG-IgG–associated disease (MOGAD). Both conditions are rare demyelinating disorders of the CNS, which have distinct clinical, neuroradiologic, neuropathologic, and pathophysiologic features than multiple sclerosis (MS).

Autoantibodies can mediate their pathogenic potential through different immunologic pathways, some of which are activation of the complement system leading to complement-dependent cytotoxicity (CDC) through the formation of the terminal complement complex (TCC), internalization of antigen-antibody complexes, direct agonism/antagonism on a receptor, or induction of protein interactions, and antibody-dependent cellular cytotoxicity (ADCC). Most AQP4-IgG and MOG-IgG access to the CNS parenchyma through a disrupted blood-brain barrier.

The pathogenic role of AQP4-IgG has been well studied and is characterized by activation of the complement pathway, astrocytopathy with loss of AQP4, and damage of oligodendrocytes and neurons. Activation of the classical terminal complement pathway (CP) is a result from the interaction of C1q with orthogonal arrays of particles formed by the shorter AQP4-M23 isoform, which ultimately leads to TCC formation and consequent cell damage. In 2019, eculizumab (anti-C5 antibody) was approved by the FDA and EMA as a treatment option for AQP4-IgG–positive NMOSD, highlighting the importance of CDC in the disease.

By contrast, the role of complement activation in MOGAD is yet to be fully determined. Studies have shown the variable ability of MOG-IgG to activate complement in different experimental approaches. However, 2 neuropathologic studies observed complement deposition only in a subset of patients. Other mechanisms for the pathogenicity of MOG-IgG were also discussed. In addition, it has been shown that most patient-derived MOG-IgG require bivalent binding to their antigen, which disfavors C1q binding.

In a recent study, we have shown that there are distinct binding patterns of human MOG-IgG to 6 major MOG isoforms (MOGA1,3β1,3), suggesting that the role of MOG-IgG in disease pathogenesis is even more complex. Here, we aimed to compare the ability of human serum samples with MOG-IgG or AQP4-IgG to induce complement-mediated cell damage in vitro.

**Methods**

**Patient Serum Samples**

In this cross-sectional study, we included 107 serum samples from patients with antibody-associated demyelinating diseases, of whom 68 were positive for MOG-IgG and 39 patients were positive for AQP4-IgG. Samples were obtained from 3 laboratories (Innsbruck and Vienna, Austria; Verona, Italy). All samples were analyzed for antibody positivity with either full-length MOGα1 or AQP4-M23 in 2 immunofluorescence live cell-based assays as described previously, where the cutoff titer value for MOG-IgG positivity was ≥1:160 and for AQP4-IgG positivity was ≥1:20. Further dilutions of sera were made to determine the antibody titer of each sample. Furthermore, all MOG-IgG–positive samples were tested for their binding to the 6 major MOG isoforms (MOGA1,3 and MOGβ1,3) to determine their respective binding pattern, as described recently. Demographic, serologic, and clinical data of patients are shown in Table and eTables 1, 2, and 3 (links.lww.com/NXI/A767).

**Standard Protocol Approvals, Registrations, and Patient Consents**

This study was approved by the ethical committees of the Medical University of Innsbruck, Austria (AM3041A and AM4059) and Medical University of Vienna (EK 1636/2019 and 1123/2015). Forty samples were obtained from the Neuropathology-Verona biobank. All patients or their caregivers gave written informed consent. All samples from participating centers were anonymized before sending them to Innsbruck, Austria.

**Lactate Dehydrogenase Cytotoxicity Assay**

For the determination of CDC, a lactate dehydrogenase (LDH) assay was used (CytoTox 96 Nonradioactive Cytotoxicity Assay, Promega, Madison, WI) to measure the activity of LDH that was released into the cell culture medium after cell lysis. According to the manufacturer’s instructions. Briefly, HEK293A (ATCC; LGC Standards GmbH, Wesel, Germany) cells were transfected with different MOG isoform constructs (MOGA1,3 and MOGβ1,3) either fused to enhanced green fluorescent protein (EGFP) or without tag, referred to as STOP; sequence variants of MOGα1: N30Q, P42S, E64K, A75S, R86Q, and H103A + S104E) in the pEGFP-N1 vector (Takara Bio USA, San Jose, CA) or with AQP4-M23 fused to...
Table Demographic, Clinical, and Immunologic Characteristics of Included Patients

|                  | MOG-IgG positive (n = 68) | AQP4-IgG positive (n = 39) |
|------------------|---------------------------|---------------------------|
| MOG-IgG titer (1:), median with range | 1,280 (160–102400) | 1,280 (20–163840) |
| MOG-IgG isoform binding pattern | | |
| α1,β1 | 17 (25%) | 20 (29%) |
| α1,β1 | 20 (29%) | 31 (46%) |
| AQP4-IgG titer (1:), median with range | 1,280 (20–163840) | |
| Sex | | |
| Females | 35 (51%) | 36 (92%) |
| Males | 33 (49%) | 3 (8%) |
| Age (y, median with range) | 20 (0–76) | 50 (3–98) |
| Age groups | | |
| Children (<18 years) | 32 (47%) | 4 (10%) |
| Adults (≥18 years) | 36 (53%) | 34 (87%) |
| Disease course | | |
| Relapsing | 24 (62%) | 18 (69%) |
| Monophasic | 15 (38%) | 8 (31%) |
| Acute relapse at sampling | | |
| Relapse | 9 (30%) | 6 (50%) |
| Remission | 21 (70%) | 6 (50%) |

Abbreviations: AQP4 = aquaporin-4; MOGAD = MOG-IgG-associated disorder; MOG = myelin oligodendrocyte glycoprotein.

We also used monoclonal humanized anti-MOG 8-18-C5 (rh8-18-C5) and mouse anti-AQP4 E5415A (E5415A; produced in-house using E5415A-1H6-68 Hybridoma cells according to the manufacturer’s instructions; #RBC4883, Riken, Tsukuba-shi, Ibaraki, Japan) as positive controls on MOG- and AQP4-transfected cells, respectively. Furthermore, Fab and F(ab’)2 fragments of rh8-18-C5 were produced using the Pierce F(ab’)2 and the Pierce Fab Micro Preparation Kits according to the manufacturer’s instructions (Thermo Fisher).

Analysis of Complement Products by Flow Cytometry and Immunocytochemistry

Complement activation product TCC was quantified by flow cytometry using an anti-C9neo antibody (clone WU13-15, Hycult Biotech, Uden, The Netherlands; 5 μg/mL) for both AQP4- and MOG-expressing cells. Furthermore, the cell surface deposition of TCC and C3 (clone 10C7, Cedarlane, Burlington, ON, Canada; 7.5 μg/mL) was assessed by immunocytochemistry. A detailed description of the methods is given in the Supplementary information (links.lww.com/NXI/A767).

Statistics

Statistical analyses were performed using GraphPad Prism 9.1 (GraphPad Software Inc., La Jolla, CA) and SPSS 26 (IBM SPSS Statistics, Armonk, NY: IBM Corp.). Comparison of complement activation between MOGAD and AQP4 was examined with the Mann-Whitney test, and correlations were analyzed with the Spearman test. A Fisher exact test was used for calculating the OR for above cutoff positive sera. A Friedman test with post hoc Dunn was used for the comparison of CDC between MOG isoforms. For calculating the difference between expression vectors and MOG and AQP4 constructs, a Wilcoxon test was applied, and two-way ANOVA with the post hoc Sidak test was used for the analysis of CDC on MOG-EGFP vs MOG-STOP constructs. A four-parameter nonlinear least-squares fit regression was applied to calculate EC50 values.
of the concentration-dependent CDC response of rh8-18-C5 and E5415A. EC_{50} values were compared using Brown-Forsythe and Welch ANOVA with post hoc Dunnett. Two-way ANOVA with post hoc Tukey was used for the analysis of rh8-18-C5 Fab and F(ab')_2 fragments and for MOG_{β1} sequence variant comparisons. To compare the CDC of the 3 MOG-IgG binding patterns on different MOG isoforms, we used the Kruskal-Wallis test with post hoc Dunn, and resulting p values were adjusted for multiple comparisons. Predictive factors for CDC were analyzed using multivariate regression analysis. Hodges-Lehmann median differences and Woolf’s logit OR were used to assess the influence of disease course and acute relapses on CDC.

**Data Availability**
The data used in this manuscript are available from the corresponding author after reasonable request or are included within this paper.

**Results**

**Human AQP4-IgG Activates the Complement System Stronger Than MOG-IgG**

We tested 39 serum samples positive for AQP4-IgG from patients with NMOSD and 68 serum samples positive for MOG-IgG from patients with MOGAD for their ability to activate the complement system. We used a cell-based LDH assay with HEK293A cells expressing different MOG isoforms fused with an EGFP tag or AQP4-M23 with an EmGFP tag and incubation of human sera together with active or inactive human complement alone (cutoff value compared with 34% for MOG_{α1} (Figure 1C) (Fisher exact test: p = 0.0044; OR = 3.5, 95% CI: 1.5–8.2). Using multivariate linear regression analysis, predictive values for CDC were significantly lower for MOG-IgG than for AQP4-IgG and were predicted by antibody titer (Figure 1D). In addition, CDC levels adjusted for antibody titers were significantly higher for AQP4-IgG (median 7.5, 25–75th percentile 4.7–9.8) compared with MOG-IgG (median 2.1, 25–75th percentile 1.6–3.0, Mann-Whitney test: p < 0.0001).

To show that the complement activation observed was specific for a certain antigen and not directed against the cells themselves, we compared complement activation on HEK293A cells transfected with the expression vectors (pEGFP-N1 for MOG and pcDNA 6.2-EmGFP for AQP4) and could not find significant LDH activities in the media of the treated cells (eFigure 2, A and B, links.lww.com/NXI/A767). Although we used HEK293A cells for all our assays, we studied the expression of 2 complement inhibitors (CD46 and CD59) after transfection with MOG_{α1} or AQP4-M23. We observed increased CD59 expression of the transfected cells compared with nontransfected cells. However, expression levels were comparable between MOG- and AQP4-expressing cells (eFigure 2D). We also investigated the monoclonal anti-MOG antibody rh8-18-C5 and anti-AQP4 antibody E5415A for their ability to activate the complement pathway as a positive control (eFigure 3, A and B). These data showed that the rh8-18-C5 was able to induce higher maximal CDC values to MOG, but starting at higher concentrations, than the E5415A antibody to AQP4-M23 (Welch test comparison of EC_{50} values: ns; eFigure 3C), but this could also be due to the fact that the E5415A is completely of mouse origin. However, after incubation with rh8-18-C5 Fab and F(ab')_2 fragments, there was no leakage of LDH into the media, showing the necessity of a Fc region for complement activation (eFigure 4A), and in addition, we studied sequence variants of the extracellular domain of MOG_{α1} to further underline the specificity of the assay. As expected, the H103A/S104E sequence variant (containing the rh8-18-C5 binding motif) was not able to induce CDC, as shown in eFigure 4B. Finally, we found no clinical associations of CDC and antibody titers with acute relapses and disease course (eTable 4, links.lww.com/NXI/A767).

**Comparison of Complement Activation on Different MOG Isoforms**

Like many other eukaryotic genes, the human MOG gene undergoes extensive splicing, leading to the formation of different isoforms. Recently, we have reported different binding patterns of human MOG-IgG to the major human MOG isoforms (MOG_{α1,3} and MOG_{β1,3}), namely the α_{1,3}β_{1,3} (only recognizing the longest α_{1,3} and β_{1,3} isoforms), α_{1,3}β_{1,3} (recognizing all 3 α and β isoforms), and α_{1,3}β_{1,3} (binding to all 6 isoforms) pattern. Therefore, we wanted to evaluate whether the major 6 MOG isoforms (MOG_{α1,3} and MOG_{β1,3}) differ in their ability to activate the complement system.
after antibody binding. We found that MOGα₁, MOGα₃, and MOGβ₁ could activate the complement system better compared with the other isoforms, which is shown in Figure 2A. As a next step, we again calculated which percentage of serum samples was above the cutoff value for each isoform and found 34% for MOGα₁, 15% for MOGα₂, 24% for MOGα₃, 28% for MOGβ₁, 4% for MOGβ₂, and 10% for MOGβ₃ (Figure 2B). These findings are as expected because MOGα₁ and MOGβ₁ isoforms were also those most recognized by human MOG-IgG as demonstrated in a previous study. eFigure 3A (links.lww.com/NXI/A767) shows the CDC rh8-18-C5 concentration curves to the 6 MOG isoforms tested, and a comparison of the EC₅₀ values revealed a lower EC₅₀ for MOGα₃ (EC₅₀: 0.9; 95% CI: 0.6–1.2) compared with MOGα₁ (EC₅₀: 3.2; 95% CI: 2.2–4.3), β₁ (EC₅₀: 2.3; 95% CI: 1.7–2.9), and β₂ (EC₅₀: 4.1; 95% CI: 2.0–6.1) (Brown-Forsythe and Welch ANOVA with post hoc Dunnett: p = 0.0017, 0.0017, and 0.47, respectively). To analyze differences in CDC induction of IgG belonging to the different binding patterns mentioned above, we sorted the sera based on their respective binding behavior. We found no difference in LDH activity on the MOGα₁ and MOGβ₁ isoforms between distinct binding patterns (eFigure 5A and 6A). However, for MOGα₃, there was a difference between the α₁β₁, 3 and α₁β₁, 1 patterns (eFigure 5C).

Both AQP4 and MOG Antibodies Lead to Deposition of the TCC and C3 on the Cell Surface

To further establish cytotoxicity of human MOG-IgG and AQP4-IgG in our cell-based system, we urged to study the formation and deposition of complement products on the surface of MOG- or AQP4-expressing cells treated with MOG-IgG– or AQP4-IgG–positive serum samples.

For a confirmation of the higher values of AQP4-IgG–induced cell lysis compared with MOG-IgG, we quantified the formation of the TCC by flow cytometry. Therefore, HEK293A cells transfected with either MOGα₁-EGFP or AQP4-M23-EmGFP-transfected cells is plotted against the antibody titers. Semi-log linear regression lines are shown. Spearman correlation: MOG: r = 0.58, p < 0.0001; AQP4: r = 0.71, p < 0.0001. (B) Violin plot (dashed line: median, dotted lines: quartiles) of all MOG-IgG (blue; n = 68) and AQP4-IgG (red; n = 39) positive serum samples tested is shown. Asterisks indicate the statistical difference of a Mann-Whitney test: ****p < 0.0001. The solid lines indicate the cutoff levels (mean + 2SD of cells treated with complement alone). (C) Percentage of MOG-IgG (blue) or AQP4-IgG (red) positive samples above the cutoff level (mean + 2SD of cells treated with complement alone) is demonstrated as bar graph. (D) The predictive role of MOG-IgG and AQP4-IgG antibody titers and status, age, and sex on complement-dependent cell lysis were analyzed by multivariate regression (R = 0.827, R² = 0.684, F = 53.1, p < 0.001). Standardized regression coefficient beta is shown with 95% CI. ***p < 0.001. AQP4 = aquaporin-4; CDC = complement-dependent cytotoxicity; LB = lysis buffer; MOG = myelin oligodendrocyte glycoprotein.
AQP4-M23-EmGFP were incubated with human heat-inactivated MOG-IgG (n = 19) or AQP4-IgG (n = 19) positive serum samples together with human complement. We found a correlation between antibody titers and TCC formation for both groups (Spearman r for MOGα1: 0.56, p = 0.012; Spearman r for AQP4-M23: 0.77, p < 0.0001, Figure 3A). Importantly, incubation with AQP4-IgG again lead to a stronger activation of the complement cascade compared with MOG-IgG as shown in Figure 3B (TCC values of AQP4-M23: median 37,405, 25–75th percentile 20,122–59,174; MOGα1: median 15,303, 25–75th percentile 6,207–26,145, p = 0.002). We also studied the correlation between TCC formation and cell lysis (LDH values) of the respective serum samples and found a correlation in the AQP4 group (Spearman r: 0.57, p = 0.011) and a trend toward correlation for MOG (Spearman r: 0.48, p = 0.04, Figure 3C).

In our immunocytochemistry experiments, human MOG-IgG and AQP4-IgG showed positive TCC staining together with active human complement but not after incubation with heat-inactivated complement on MOGα1- and AQP4-M23-expressing cells (eFigure 7, links.lww.com/NXI/A767). Cell morphology was clearly altered after complement activation, and many dead cells were present. Furthermore, TCC staining was present on cells expressing other MOG isoforms (α2,3, β1,3) after incubation with a human serum belonging to the α1,β1,3 binding pattern (eFigure 9A) but was visible only on MOGα1 and MOGβ1 after incubation with a serum that shows an α1β1 binding pattern (eFigure 10A). However, treatment with a serum negative for MOG-IgG and AQP4-IgG did not result in TCC deposition (TCC; eFigure 13, A and C), and incubation of MOG-IgG or AQP4-IgG harboring sera on the transfection control cells showed no signs of complement activation (TCC; eFigure 13, B and D). As expected, the monoclonal antibodies rh8-18-C5 and ES415A were also able to activate the complement cascade, leading to TCC formation and deposition on cell surfaces (MOG isoforms, eFigure 11A; AQP4, eFigure 11B). As C3 is an important protein in the CP as well as in the alternative pathway (AP), we further wanted to investigate the extend of C3 cleavage after complement activation. Here, we found C3/C3b/iC3b staining for both MOG-IgG and AQP4-IgG after incubation with active complement but, interestingly, also with inactivated complement (eFigure 8). In addition, we observed positive staining after incubation with a representative α1,β1,3 pattern MOG-IgG-positive serum on all MOG isoforms tested (eFigure 9B) and on MOGα1 and MOGβ1 after treatment with an α1β1 pattern serum but, to a much lesser extent, on the other isoforms (eFigure 10B). A small amount of C3b (and iC3) on cells treated with active complement was expected because the AP is always slightly active or because of failure of the CP to move forward to terminal activation.38 This was also observed after incubation with a representative double-negative serum (C3; eFigure 13, A and C) and after treatment with a MOG-IgG or AQP4-IgG-positive serum on the transfection controls (C3; eFigure 13, B and D) in combination with active complement. However, incubation with MOG-IgG or AQP4-IgG-positive serum samples without complement showed no positive staining (data not shown). Furthermore, as with TCC, the rh8-18-C5 and ES415A antibodies showed cleavage of C3 after incubation with active complement (MOG, eFigure 12A; AQP4, eFigure 12B). Finally, we used factor B-depleted serum to study the impact of the AP in our LDH assay and observed reduced cytotoxicity for MOGα1, and for AQP4-M23–transfected cells compared with normal human serum (eFigure 14).
Discussion

We used a cell-based cytotoxicity assay to assess the ability of human serum samples positive for MOG-IgG or AQP4-IgG to activate the complement system on cells expressing different MOG isoforms or AQP4-M23, respectively. A cell-based assay was chosen because they are state of the art for the detection of MOG-IgG and AQP4-IgG in serum samples as it enables the binding to natively folded MOG or AQP4.32,33 Furthermore, cell-based assays were also used in former studies investigating CDC in AQP4-IgG– or MOG-IgG–mediated disease in vitro.18,21,39-41

We found increased LDH activity, released from damaged cells into the surrounding media, after incubation of serum samples together with active complement and could show that, in both cases, CDC was dependent on the MOG-IgG or AQP4-IgG titers but was absent on HEK293A cells lacking the respective antigen. A recently published article also found a correlation between AQP4-IgG titers and CDC; thus, we could confirm this association, but both studies showed no correlation with the clinical activity of patients.41 The role of complement activation in NMOSD is well established.3,9-18

Complement depositions have been observed in human AQP4-IgG–induced lesions in Lewis rats14 and in mice after injection of AQP4-IgG together with human complement into the brain.15 Furthermore, in studies investigating the neuropathology in patients with NMOSD, activated complement deposits were observed in human brain material.11,12 By contrast, in MOGAD, the role of complement is less clear, and studies made different observations.21,22,24-26 One study injected human MOG-IgG together with human complement into mouse brains and found only little complement activation,26 and furthermore, another investigation showed that only a portion of mouse monoclonal antibodies against MOG is able to induce demyelination in vivo.42 In an ex vivo mouse study, complement activation was only observed in 1 of 10 samples after treatment with purified human MOG-IgG, and this sample did not induce pathogenic lesions in vivo.22 Investigation of human MOG-IgG in vivo is hampered by the fact that a portion of human MOG-IgG does not cross-react with rodent MOG, or show reduced binding compared with human MOG.22,23,43 Of interest, one study showed that on transfer of cross-reactive human MOG-IgG into a rat model with MOG-specific T cells, T-cell infiltration was enhanced, but only traces of TCC were observed, whereas together with
MBP-specific T cells MOG-IgG induced complement activation, leading to TCC deposition.23 Of note, recent neuropathologic studies investigating human brain material found either prominent24 or only sparse activated complement products.25 However, TCC deposition in MOGAD lesions does not necessarily mean that it is caused by MOG-IgG alone, and a synergistic action of humoral and cellular immune mechanisms may have an important impact on the formation of TCC deposits in a subset of patients.23

Here, we could confirm the importance of complement activation in human AQP4-IgG-provoked pathogenicity compared with human MOG-IgG because higher CDC levels were observed after incubation of sera harboring AQP4-IgG in the presence of active complement on AQP4-M23–expressing cells. Furthermore, after subtraction of serum samples that showed CDC levels lower than the control cutoff, we found that only 34% of MOG-IgG–positive serum samples were able to induce CDC compared with 64% of AQP4-IgG–positive serum samples. A recently published study found significantly higher levels of soluble activated complement products in patients with MOGAD compared with patients with NMOSD and MS.46 However, the study failed to detect significant levels of complement products in patients with NMOSD, where the role of complement is well established, and the high levels of complement products in MOGAD could also be due to a preceding systemic inflammation.45 In our study, we did not find a correlation between CDC and disease course or relapse. One study found such a correlation after investigating the CSF soluble C5a levels in patients with NMOSD,46 whereas another investigation showed that AQP4-IgG and CDC lack predictive and prognostic utility in patients with NMOSD.47

We also conducted additional experiments to further evaluate the ability of human MOG-IgG to activate the complement cascade on 6 different MOG isoforms (MOGα1-3 and MOGβ1-3). A recent study observed that the hydrophobic intracellular domain (which is only present in MOGα1 and MOGβ1) is necessary for antigen binding for most MOG-IgG and argued that this domain keeps MOG proteins separated at a certain distance to enable bivalent antibody binding,30 which is known to poorly interact with C1.44 Here, we also observed that the highest CDC responses were found against the 2 longest isoforms MOGα1 and MOGβ1. However, all 6 isoforms tested were able to trigger at least some complement activation after incubation with human MOG-IgG–positive serum samples and active complement. Moreover, MOG-IgG recognizing all 6 MOG isoforms31 showed the highest LDH activity on MOGα1 and MOGβ1. Of interest, we observed a difference between CDC of the 3 binding patterns (α1β1, α1, β1, and α1β1) only on MOGα3, and furthermore, the EC50 value of the rh8-18-C5 monoclonal antibody on MOGα3 was lower when compared with MOGα1 and MOGβ1, which suggests an even more complex dynamic of complement activation on different MOG isoforms. To further investigate complement activation at a cellular level, we analyzed the presence of TCC and C3 on MOG- or AQP4-M23–expressing cells after initialization of the complement system through MOG-IgG or AQP4-IgG. IgG (more specifically IgG1 and 3 and relatively poorly IgG2) activate the complement system through the CP, which is conveyed by binding of C1q to a hexamer of surface bound IgG followed by formation of the CP C3 convertase that cleaves C3 into C3a and C3b. The latter binds to the surface of the target cell and mediates further activation, which ultimately leads to TCC formation. However, the AP serves as an amplification loop by generating the AP C3 convertase and is activated through a tick-over that constantly takes place and is tightly regulated.7,38,49 In our flow cytometry experiments, we found a correlation between TCC levels and antibody titers for both MOG and AQP4. The overall TCC level was higher for AQP4 compared with MOG, again demonstrating the more efficient complement activation through human AQP4-IgG.

We also observed positive C9neo staining on the surface of HEK293 cells expressing MOG isoforms or AQP4-M23 after treatment with human sera harboring the respective IgG together with active complement, but after incubation with heat-inactivated complement no staining could be observed visually. Again, we found visible TCC deposition on all 6 MOG isoforms tested. Of interest, C3 positive staining was visible after incubation with active and with heat-inactivated complement but only if human MOG-IgG or AQP4-IgG containing serum samples was present. A possible explanation would be the detection of iC3b, which is a degradation product of C3b, because the anti-C3 antibody used also binds to iC3b. Furthermore, other degradation products of C3b have been detected also in heat-inactivated sera.50 Moreover, C3b is also able to bind directly on IgG heavy chains, which could also lead to a positive staining in our setting.7 However, in all experiments conducted with active complement, we found a slight positive staining for C3, even without any IgG, which is explained by the constant activation of the AP. This is also supported by the reduced cytotoxicity observed after incubation with factor B-depleted serum in both AQP4- and MOG-expressing cells.

A recent study used a triple knockout cell line (CD46, CD55, and CD59 deficient) to study complement activation in patients with myasthenia gravis.36 We observed higher CD59 levels in the transfected cells compared with nontransfected cells; however, levels were comparable between MOG and AQP4 transfections. Despite the possible interference of CD59 with complement activation, we were able to measure CDC in our cell-based system and, importantly, used the same cell lines for AQP4 and MOG; we decided that this influence is negligible in our experimental setting.

Our study has several limitations. First, the sample size is small and limited by the amount of MOG-IgG– or AQP4-IgG–positive serum samples available, and the retrospective nature of the study is a restriction, too. Second, immunocytochemistry and flow cytometry experiments were only...
performed for representative serum probes. Third, whether human MOG-IgG induces CDC to MOG sequence variants would have also been interesting to study but was again limited by the available human material. In addition, a direct comparison of complement activation with both antigens in a system using primary oligodendrocytes and astrocytes or organoid-based experiments could lead to further insights into this important topic. Finally, purification of MOG- and AQP4-specific antibodies from patient sera would have been an interesting tool to normalize the antibody concentration for complement activation, but again this was limited by the amount of available patient serum samples.

To summarize, we demonstrated that human AQP4-IgG serum samples showed stronger activation of the complement system on AQP4-M23–expressing cells compared with MOG-IgG on MOG-expressing cells, but in both cases, this was correlated with the IgG titers. Furthermore, CDC was directed to all 6 MOG isoforms tested, but the strongest responses were detected to MOGα1, MOGα3, and MOGβ1. Together, these findings further underline the more pronounced role of complement activation in autoimmune diseases directed against AQP4 compared with MOG. Moreover, this study emphasizes the need for further studies to fully establish the role of complement activation in MOGAD including the potential use of complement inhibition as a therapeutic strategy.

**Study Funding**

This study was funded by a research grant from the Austrian Science Fund (FWF project P32699, Markus Reindl and Romana Höffberger).

**Disclosure**

M. Lerch, K. Schanda, E. Lafon, and R. Würzner have no disclosures to report; S. Mariotto received support for attending scientific meetings by Merck and Euroimmun and received speaker honoraria from Biogen; A. Dinoto and E.M. Wendel have no disclosures to report; C. Lechner has served as a consultant for Roche; H. Hegen has participated in meetings sponsored by and received speaker honoraria or travel funding from Bayer, Biogen, Celgene, Merck, Novartis, Sanofi-Genzyme, Siemens, and Teva and received honoraria for acting as a consultant for Biogen, Celgene, Novartis, and Teva; Kevin Rostasy has acted as a consultant for the Ocrevus study/Roche and has received speaker honoraria from Merck; Thomas Berger has participated in meetings sponsored by and received honoraria (lectures, advisory boards, and consultations) from pharmaceutical companies marketing treatments for MS: Allergan, Bayer, Biogen, Bionorica, BMS/Celgene, GSK, GW/Jazz Pharma, Horizon, Janssen-Cilag, MedDay, Merck, Novartis, Octapharma, Roche, Sandoz, Sanofi-Genzyme, Teva, and UCB. His institution has received financial support in the past 12 months by unrestricted research grants (Biogen, Bayer, BMS/Celgene, Merck, Novartis, Roche, Sanofi-Genzyme, and Teva) and for participation in clinical trials in MS sponsored by Alexion, Bayer, Biogen, Merck, Novartis, Octapharma, Roche, Sanofi-Genzyme, and Teva; D. Willflingseder has no disclosures to report; R. Höffberger received speaker’s honoraria from Novartis and Biogen. The Medical University of Vienna (Austria; employer of Dr. Höffberger) receives payment for antibody assays (MOG, AQP4, and other autoantibodies) and for MOG and AQP4 antibody validation experiments organized by Euroimmun (Lübeck, Germany); M. Reindl was supported by a research support from Euroimmun and Roche. The University Hospital and Medical University of Innsbruck (Austria; employer of Dr. Reindl) receives payments for antibody assays (MOG, AQP4, and other autoantibodies) and for MOG and AQP4 antibody validation experiments organized by Euroimmun (Lübeck, Germany). Go to Neurology.org/NN for full disclosures.

**Publication History**

Received by *Neurology: Neuroimmunology & Neuroinflammation* May 24, 2022. Accepted in final form September 19, 2022. Submitted and externally peer reviewed. The handling editor was Josep O. Dalmau, MD, PhD, FAAN.

**Appendix Authors**

| Name                 | Location                          | Contribution                                                                 |
|----------------------|-----------------------------------|------------------------------------------------------------------------------|
| Magdalena Lerch, MSc | Medical University of Innsbruck, Austria | Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data |
| Kathrin Schanda, MSc | Medical University of Innsbruck, Austria | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Elliott Lafon, MSc   | Medical University of Innsbruck, Austria | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Reinhard Würzner, MD, PhD | Medical University of Innsbruck, Austria | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Sara Mariotto, MD, PhD | University of Verona, Verona, Italy | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Alessandro Dinoto, MD | University of Verona, Verona, Italy | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Eva Maria Wendel, MD | Olghospital Stuttgart, Stuttgart, Germany | Drafting/revision of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
### References
1. Prüts H. Autoantibodies in neurological disease. *Nat Rev Immunol*. 2021;21(12): 798-813. doi: 10.1038/s41577-021-00543-x
2. Wingerdchuk DM, Bansell B, Bennett JL, et al. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. *Neurology*. 2015;85(2):177-189. doi: 10.1212/WNL.000000000001729
3. Jarius S, Paul F, Weisshenker BG, Levy M, Kim HJ, Wildemann B. Neuromyelitis optica. *Nat Rev Dis Primers*. 2020;6(1):85. doi: 10.1038/s41572-020-0214-9
4. Reindl M, Waters P. Myelin oligodendrocyte glycoprotein antibodies in neurological disease. *Nat Rev Neurol*. 2019;15(2):89-102. doi: 10.1038/s41582-018-0112-x
5. Marignier R, Hacohen Y, Cobo-Calvo A, et al. Myelin-oligodendrocyte glycoprotein can cause complement-dependent demyelination. *J Neuroinflammation*. 2011;8(1):184. doi: 10.1186/1742-204X-8-184
6. Peschl P, Schanda K, Zeka B, et al. Human antibodies against the myelin oligodendrocyte glycoprotein can cause complement-dependent demyelination. *J Neuroinflammation*. 2017;14(1):208. doi: 10.1186/s12974-017-0984-5
7. Spadaro M, Winklmeier S, Beltran E, et al. Pathogenicity of human antibodies against myelin oligodendrocyte glycoprotein. *Ann Neurol*. 2018;84(2):315-328. doi: 10.1002/ana.25291
8. Höftberger R, Guo Y, Flanagan EP, et al. The pathology of central nervous system inflammatory demyelinating disease accompanying myelin oligodendrocyte glycoprotein autoantibody. *Acta Neuropathol. 2020*.39(3):875-892. doi: 10.1002/an.22132-y
9. Takai Y, Misu T, Kaneko K, et al. Myelin oligodendrocyte glycoprotein antibody-associated disease: an immunopathological study. *Brain*. 2020;143(5):1443-1466. doi: 10.1093/brain/awaa102
10. Saadoun S, Waters P, Owens GP, Bennett JL, Vincent A, Papadopoulos MC. Neuromyelitis optica MOC-IgG causes reversible lesions in mouse brain. *Acta Neuropathol Commun.* 2014;2(1):35. doi: 10.1186/s12974-013-00012-2
11. Zelinski S, Lehmann-Horn K, Torke S, et al. Myelin-reactive antibodies initiate T cell-mediated CNS autoimmune disease by activation of endogenous antigen. *Acta Neuropathol. 2016*.132(1):43-58. doi: 10.1007/s00401-016-1559-8
12. Flach A-C, Litke T, Strauss J, et al. Autoantibody-boosted T-cell reactivation in the target organ triggers manifestation of autoimmune CNS disease. *Proc Natl Acad Sci USA*. 2016;113(12):3322-3328. doi: 10.1073/pnas.1519808113
13. Macnair C, Gerhardt R, Winklmeier S, et al. Features of MOC required for recognition by patients with MOC antibody-associated disorders. *Brain*. 2014;137(5):2373-2389. doi: 10.1093/brain/awaa105
14. Schanda K, Peschl P, Lorch M, et al. Differential binding of autoantibodies to MOG isoforms in inflammatory demyelinating diseases. *Neurrol Neuroimmunol Neuroinflamm*. 2021;8(3):e1027. doi: 10.1212/NXI.000000000001027
15. Reindl M, Schanda K, Woodhall M, et al. Multicenter evaluation of MOG antibody assays. *Neuro Immunol Neuroinflamm*. 2020;7(2):e674. doi: 10.1212/NXI.000000000000674
16. Waters P, Reindl M, Saiz A, et al. Multicentre comparison of a diagnostic assay: aquaporin-4 antibodies in neuromyelitis optica. *J Neurol Neurophysy. 2016*;87(9):1005-1015. doi: 10.1007/jnnp-2015-312601
17. Mader S, Lutterotti A, Reindl M, et al. Patterns of antibody binding to aquaporin-4 isoforms in neuromyelitis optica. *PLoS One*. 2010;5(5):e10455. doi: 10.1371/journal.pone.0010455
18. Kurusova K, Misu T, Takai Y, et al. Severe exacerbations of neuromyelitis optica are associated with extensive astrocytopathy by high affinity anti-aquaporin-4 monoclonal antibody. *Acta Neuropathol Commun*. 2015;3(1):182. doi: 10.1186/s40478-015-0259-2
19. Obaid AH, Zografou C, Vardaxis DD, et al. Heterogeneity of acetylcholine receptor autoantibody-mediated complement activity in patients with myasthenia gravis. *Neurrol Neuroimmunol Neuroinflamm*. 2022;9(4):e169. doi: 10.1212/NXI.0000000000010169
20. Peschl P, Bradl M, Höftberger R, Berger M, et al. Myelin oligodendrocyte glycoprotein: deciphering a target in inflammatory demyelinating diseases. *Front Immunol*. 2017;8:529. doi: 10.3389/fimmu.2017.00529

### Appendix (continued)

| Name                        | Location                      | Contribution                                                                 |
|-----------------------------|-------------------------------|------------------------------------------------------------------------------|
| Christian Lechner, MD       | Medical University of Innsbruck; Medical University of Vienna, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Harald Hegen, MD, PhD        | Medical University of Innsbruck, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Kevin Rostásy, MD           | Witten/Herdecke University, Germany | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Thomas Berger, MD, MSc      | Medical University of Vienna, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Doris Wilflingseder, PhD    | Medical University of Innsbruck, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Romana Höftberger, MD       | Medical University of Vienna, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
| Markus Reindl, PhD          | Medical University of Innsbruck, Austria | Drafting/review of the manuscript for content, including medical writing for content, and analysis or interpretation of data |
38. Bexborn F, Andersson PO, Chen H, Nilsson B, Ekdahl KN. The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3bBb). Mol Immunol. 2008;45(8):2370-2379. doi: 10.1016/j.molimm.2007.11.003

39. Duan T, Smith AJ, Verkman AS. Complement-independent bystander injury in AQP4-IgG seropositive neuromyelitis optica produced by antibody-dependent cellular cytotoxicity. Acta Neuropathol Commun. 2019;7(1):112. doi: 10.1186/s40478-019-0766-7

40. Tradtrantip L, Yeaman MR, Verkman AS. Cytoprotective IgG antibodies in sera from a subset of patients with AQP4-IgG seropositive neuromyelitis optica spectrum disorder. Sci Rep. 2021;11(1):21962. doi: 10.1038/s41598-021-01294-3

41. Jitprapaikulsan J, Fryer JP, Majed M, et al. Clinical utility of AQP4-IgG titers and measures of complement-mediated cell killing in NMOSD. Neurrol Neuroimmunol Neuroinflamm. 2020;7(4):e727. doi: 10.1212/NXNI.0000000000000727

42. Piddlesden SJ, Lassmann H, Zimprich F, Morgan BP, Linington C. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. Am J Pathol. 1993;143(2):555-564.

43. Mayer MC, Breithaupt C, Reindl M, et al. Distinction and temporal stability of conformational epitopes on myelin oligodendrocyte glycoprotein recognized by patients with different inflammatory central nervous system diseases. J Immunol. 2013;191(7):3594-3604. doi: 10.4049/jimmunol.1301296

44. Keller CW, Lopez JA, Wendel E-M, et al. Complement activation is a prominent feature of MOGAD. Ann Neurol. 2021;90(6):976-982. doi: 10.1002/ana.26226

45. McCombe JA, Flanagan EP, Chen JJ, Zekeridou A, Luccionetti CF, Pittcock SJ. Investigating the immunopathogenic mechanisms underlying MOGAD. Ann Neurol. 2022;91(2):399-500. doi: 10.1002/ana.26279

46. Kuroda H, Fujihara K, Takano R, et al. Increase of complement fragment C5a in cerebrospinal fluid during exacerbation of neuromyelitis optica. J Neuroimmunol. 2013;254(1-2):178-182. doi: 10.1016/j.jneuroim.2012.09.002

47. Jitprapaikulsan J, Chen JJ, Flanagan EP, et al. Aquaporin-4 and myelin oligodendrocyte glycoprotein autoantibody status predict outcome of recurrent optic neuritis. Ophthalmology. 2018;125(10):1628-1637. doi: 10.1016/j.phphoto.2018.03.041

48. Strasser J, de Jong RN, Beurskens FJ, et al. Unraveling the macromolecular pathways of IgG oligomerization and complement activation on antigenic surfaces. Nano Lett. 2019;19(7):4787-4796. doi: 10.1021/acs.nanolett.9b02220

49. Walport MJ. Complement. First of two parts. N Engl J Med. 2001;344(14):1058-1066. doi: 10.1056/NEJM200104053441406

50. Fante MA, Decking S-M, Bruss C, et al. Heat-inactivation of human serum destroys C1 inhibitor, promotes immune complex formation, and improves human T cell function. Int J Mol Sci. 2021;22(5):2646. doi: 10.3390/ijms22052646