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Distinction and Temporal Stability of Conformational Epitopes on Myelin Oligodendrocyte Glycoprotein Recognized by Patients with Different Inflammatory Central Nervous System Diseases

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Autoantibodies targeting conformationally intact myelin oligodendrocyte glycoprotein (MOG) are found in different inflammatory diseases of the CNS, but their antigenic epitopes have not been mapped. We expressed mutants of MOG on human HeLa cells and analyzed sera from 111 patients (104 children, 7 adults) who recognized cell-bound human MOG, but had different diseases, including acute disseminated encephalomyelitis (ADEM), one episode of transverse myelitis or optic neuritis, multiple sclerosis (MS), anti-aquaporin-4 (AQP4)–negative neuromyelitis optica (NMO), and chronic relapsing inflammatory optic neuritis (CRION). We obtained insight into the recognition of epitopes in 98 patients. All epitopes identified were located at loops connecting the β strands of MOG. The most frequently recognized MOG epitope was revealed by the P42 mutation positioned in the CC‘-loop. Overall, we distinguished seven epitope patterns, including the one mainly recognized by mouse mAbs. In half of the patients, the anti-MOG response was directed to a single epitope. The epitope specificity was not linked to certain disease entities. Longitudinal analysis of 11 patients for up to 5 y indicated constant epitope recognition without evidence for intramolecular epitope spreading. Patients who rapidly lost their anti-MOG IgG still generated a long-lasting IgG response to vaccines, indicating that their loss of anti-MOG reactivity did not reflect a general lack of capacity for long-standing IgG responses. The majority of human anti-MOG Abs did not recognize rodent MOG, which has implications for potential mimotopes and pave the way to Ag-specific depletion.  

Only Abs recognizing MOG in its correctly folded form, as on the cell surface, can be demyelinating and thus pathogenic (12, 13). Only such conformationally intact MOG—for example, as an in vitro translated streptavidin-linked tetramer or expressed on the surface of transfected cells—is suitable to identify proportions of patients with autoantibodies to MOG. Such autoantibodies to MOG are found in a substantial proportion (∼20–40%) of children with ADEM, chronic relapsing inflammatory optic neuritis (CRION), or MS, but they are rarely found in adult MS (14–23). Recently anti-MOG Abs were also found in a few anti–aquaporin-4 (AQP4) negative pediatric and acquired demyelinating event (including transverse myelitis, longitudinally extended transverse myelitis; NMO, neuromyelitis optica; mono ADS, patients experiencing only one disseminated encephalomyelitis; AQP4, aquaporin-4). We also analyzed sera from 111 patients (104 children, 7 adults) who recognized cell-bound human MOG, but had different diseases, including acute disseminated encephalomyelitis (ADEM), one episode of transverse myelitis or optic neuritis, multiple sclerosis (MS), anti-aquaporin-4 (AQP4)–negative neuromyelitis optica (NMO), and chronic relapsing inflammatory optic neuritis (CRION). We obtained insight into the recognition of epitopes in 98 patients. All epitopes identified were located at loops connecting the β strands of MOG. The most frequently recognized MOG epitope was revealed by the P42 mutation positioned in the CC‘-loop. Overall, we distinguished seven epitope patterns, including the one mainly recognized by mouse mAbs. In half of the patients, the anti-MOG response was directed to a single epitope. The epitope specificity was not linked to certain disease entities. Longitudinal analysis of 11 patients for up to 5 y indicated constant epitope recognition without evidence for intramolecular epitope spreading. Patients who rapidly lost their anti-MOG IgG still generated a long-lasting IgG response to vaccines, indicating that their loss of anti-MOG reactivity did not reflect a general lack of capacity for long-standing IgG responses. The majority of human anti-MOG Abs did not recognize rodent MOG, which has implications for potential mimotopes and pave the way to Ag-specific depletion.

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Abbreviations used in this article: AChR, acetylcholine receptor; ADEM, acute disseminated encephalomyelitis; AQP4, aquaporin-4; CRION, chronic relapsing inflammatory optic neuritis; hMOG, human MOG; MCF, mean channel fluorescence; mMOG, mouse MOG; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMO, neuromyelitis optica; mono ADS, patients experiencing only one acquired demyelinating event (including transverse myelitis, longitudinally extended transverse myelitis and optic neuritis).  

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adult patients with neuromyelitis optica (NMO) (16, 18, 21) and in patients at high risk of developing NMO (18).

These Abs to MOG are expected to be not only a biomarker; they also contribute to tissue destruction for the following reasons: human anti-MOG Abs recognized MOG in its native conformation, they are mostly of the complement-activating isotype IgG1 (18–20), and the blood-brain barrier is breached in CNS inflammation, allowing anti-MOG IgG to gain access to the CNS.

Although human autoantibodies to MOG are associated with inflammatory demyelinating CNS diseases and are presumably pathogenic, their target epitopes have not been mapped. In this study, we analyzed 111 sera with anti-MOG Abs obtained from patients with inflammatory CNS diseases, including MS, patients experiencing only one acquired demyelinating event (including transverse myelitis, longitudinally extended transverse myelitis and optic neuritis) (mono ADS), ADEM, anti-AQP4 negative NMO, CRION, and other relapsing ADS cases.

We addressed the following questions: 1) Can we define distinct epitopes on conformationally intact MOG recognized by human autoantibodies? 2) Is the response of an individual patient focused on a single, individually dominant epitope or is it broadly distributed? 3) Are certain epitopes preferentially recognized in certain CNS diseases? 4) In patients with long-term persistence of anti-MOG Abs, do we find evidence for intramolecular epitope spreading, or is the epitope pattern in a given individual stable over time? 5) In patients, with rapid decline of anti-MOG IgG, is there evidence for an inability to mount long-lived plasma cells?

Materials and Methods

Patient samples

This study included sera of 111 patients with different inflammatory CNS diseases and Abs to cell-bound MOG: mono ADS, ADEM, MS, NMO, CRION, and other relapsing ADS cases (Table 1). Of these, 7 patients were adults (18 or older); 54 of these patients had been recognized as anti-MOG diseases and Abs to cell-bound MOG: mono ADS, ADEM, MS, NMO, CRION, and other relapsing ADS cases.

Variant and mutants of MOG

Responses to human MOG (hMOG), mouse MOG (mMOG), and seven mutants of hMOG (N31D, S104E, H103A/S104E, P42S, P42S/H103A/S104E, R9G/H10Y, and R86Q) were analyzed. The mutations are shown in Fig. 1. The tip of the FG-loop of MOG (Fig. 1) is recognized by the mAb 8-18C5 (24). Therefore, we mutated the two amino acids H103 and S104 to obtain H103A/S104E. We also created the single amino acid mutant S104E, because this mutant already reduced binding of rMOG to mAb 8-18C5 (24). For visualization of the substitutions, a homology model of the Ig-V-like domain of hMOG (rmsd to mMOG: 0.07 Å, 116 aligned Cα atoms) was described in (15). In this case, binding percentage to a mutant was calculated as % binding = (MCF(mutant)−MCF(GFP only)) / MCF(GFP only) × 100%.

Western blot

HeLa cells transfected with anti-MOG-EGFP constructs were lysed at 4°C for 1 h in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM Tris pH 8.0, 1% SDS) containing complete protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). The lysate was then pelleted, and the supernatant was analyzed. For deglycosylation, the supernatant was digested with PNGaseF (New England Biolabs, Ipswich, MA) in Glycoprotein Denaturing Buffer (New England Biolabs), G7 Reaction Buffer (New England Biolabs) and 1% NP-40 (New England Biolabs) at 37°C overnight. The protein digested or undigested was loaded onto an SDS gel and separated by gel electrophoresis. The proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked on PBS containing 3% BSA overnight. The membrane was
incubated with a rabbit anti-GFP mAb (Research Diagnostics, Flanders NJ) at a dilution of 1:5000 for 1 h at room temperature, washed three times, and then incubated with a peroxidase-labeled goat anti-rabbit Ab (Dianova, Hamburg, Germany) at a dilution of 1:10,000 for 1 h at room temperature. The blots were developed with ECL.

IgG response to vaccines

The IgG responses to measles and rubella virus were measured by routine ELISA in the department of clinical chemistry using the Enzygnost Anti-Rubella Measles Virus/IgG and Anti-Measles Virus/IgG assays (Siemens Healthcare, Erlangen, Germany).

Results

Validation of transiently transfected MOG variants and reproducibility of binding ratios

hMOG, mMOG, and seven mutants of hMOG (Fig. 1) were analyzed for recognition by human IgG and mAbs. Similar extents of expression were obtained with the different constructs, (supplementary Supplemental Fig. 1). Cells with the FL1 fluorescence intensity of $10^3$–$10^6$ (see Supplemental Fig. 1) were gated to evaluate the binding to the respective transfected cell. This range was selected to ensure a high expression level and good comparability between the transfectants.

Surface expression of each MOG variant was evaluated with two anti-MOG mAbs, 8-18C5 and Y11. We chose these two mAbs because they recognize different epitopes on MOG (24). 8-18C5 did not recognize the three MOG variants containing the S104E mutation, but bound well to all other mutants we used. All MOG variants were recognized by Y11 (supplementary Supplemental Fig. 1). This shows that the mutations we introduced did not interfere with MOG surface expression. Furthermore, it was shown previously that the introduction of the H103A/S104E mutation did not disturb the overall structure of rat MOG (24). The P42S mu-
tation is also unlikely to change the overall structure of MOG, as both proline and serine are found in different species at this position.

The similar expression levels of the mutants, the gating on cells with a defined expression level \((10^5-10^7)\), and the demonstration of binding of at least one MOG-specific mAb allowed the comparison of binding to the different mutants.

We assessed the reproducibility of our system. Binding of the 111 sera (Table I) to our mutants was analyzed up to three times in independent experiments, yielding a good reproducibility of the binding percentage. For 36 sera, we compared recognition of the three most important constructs P42S, mMOG, and H103A/S104E to hMOG (i.e., 108 measurements and each in triplicates) and found the following. First, in 33 of 108 measurements, the mutation reduced the binding to less than 10%, and we found an absolute SD of 7.8%. Second, in the other 75 measurements, binding was either strongly reduced (<65%), comparable to hMOG (65–200%) or strongly increased (>200%); here, the SD of the binding was 20% of the binding percentage. Sera representing each of the seven epitope patterns (see below) were analyzed three times (Fig. 2).

Two sera were serially diluted, and Ab binding to the MOG variants was assessed by FACS. The binding patterns were comparable at different serum dilutions (Supplemental Fig. 2).

A subset of 16 sera was independently analyzed for binding to P42S and mMOG by titration in an immunofluorescence assay: the resulting 32 binding percentage values were highly comparable between the independent assays (Spearman \( r = 0.7136; p < 0.0001 \)).

**Recognition of MOG-epitopes analyzed with single and multiple amino acid mutants**

Anti-MOG positive sera (98/111) showed a reduced binding to at least one of our variants of MOG. In 52 of 111 samples, the immune response was clearly reduced by mutations of amino acids positioned in only one loop. In 39 of 52 patients, binding to single mutated loops was decreased to less than one third, indicating that the IgG response is focused on one epitope. In 32 of 111 samples, we saw a reduction in binding to multiple mutants. In 14 of 111 samples, we saw reduced or no binding to mMOG, but were not able to assign a specific responsible amino acid. The responses to our mutants allowed us to distinguish seven different patterns of MOG recognition (Figs. 2, 3). The intensity of the recognition of hMOG did not differ significantly between sera recognizing the seven patterns, as summarized in Fig. 3. The structure of MOG with the strands and loops referred to in this work is shown in Fig. 1. An overview of these patterns is shown in Fig. 3, and examples of the seven patterns are shown in Fig. 2.

**Patients with anti-MOG IgG directed to one epitope.** The most frequently recognized MOG-epitope was revealed by the P42S mutation positioned in the CC α1-loop (Fig. 1); 66/111 patients (59%) showed reduced or no binding to this mutant (patterns 1, 5, and 7). Moreover, in 41 of 111 patients (37%), the MOG-specific Ab response was focused on the CC α1-loop (pattern 1, Fig. 2A). Thirty-six of these 41 sera also showed reduced or no binding to mMOG, which was expected because mMOG contains S42. In 21 of these 36 cases, binding to mMOG was lower than binding to P42S, indicating that other differences between human and murine MOG further decreased binding to mMOG. The CC α1-loop is hence identified as the most frequently recognized epitope on hMOG and as the dominant epitope in patient sera that recognize a single epitope.

**Patients with anti-MOG IgG directed to multiple epitopes.** Reduced binding to multiple mutants was detected in 32 of 111 (29%) patient samples. The most common combination, present in 19 of 32 samples, was reduced binding to P42S and to H103A/S104E (pattern 5, Fig. 2E). These mutations are located in loops at opposite ends of the A’GFCC’ C’ β-sheet of MOG (distance of Cα atoms: \( d_{\text{average}} = 23 \AA \)) and the observed maximum dimensions of Ab epitopes account for 21 A × 28 A (32).

In the MOG IgD (8-18C5) Fab complex, the FG-loop forms the center of the epitope, whereas S42 is located too far away to interact with Ab amino acids (27); thus, P42 and H103A/S104E cannot simultaneously bind to the center of this Ab paratope. In our study, 64 of 111 (58%) patient Abs recognized either P42 (pattern 1, 7) or H103A/S104E (pattern 2, 6). For this reason, sera showing pattern 5 reactivity (reduced binding to H103A/S104E and to P42S) can be assumed to contain at least two different Ab populations rec-

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**Table 1. Patient data**

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients with Abs to hMOG (Adults)</th>
<th>No. of Females</th>
<th>Mean Age in Years (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono ADS other than ADEM</td>
<td>45 (2)</td>
<td>25</td>
<td>11.0 (1.5–51.1)</td>
</tr>
<tr>
<td>ADEM</td>
<td>40 (1)</td>
<td>19</td>
<td>7.0 (1.4–47.1)</td>
</tr>
<tr>
<td>MS</td>
<td>10 (1)</td>
<td>7</td>
<td>11.3 (3.4–34)</td>
</tr>
<tr>
<td>NMO like</td>
<td>2 (1)</td>
<td>1</td>
<td>34.7 (13.5–55.9)</td>
</tr>
<tr>
<td>CRION</td>
<td>10 (2)</td>
<td>6</td>
<td>15.3 (7.4–32)</td>
</tr>
<tr>
<td>Other relapsing ADS</td>
<td>4</td>
<td>1</td>
<td>7.9 (3.2–15.7)</td>
</tr>
</tbody>
</table>

Other relapsing ADS cases: three patients had one ADEM attack plus one non-ADEM attack, and one patient had a monolesional transverse myelitis.
ognizing distinct epitopes. Four of these 19 samples, however, showed strongly reduced binding (<10%) to both H103A/S104E and to P42S, which indicates that in these sera both loops influence Ab binding simultaneously. Regarding the observed maximum dimensions of Ab epitopes and the distance between the two MOG loops, the epitopes recognized by these samples have to be highly extended with hot spots of binding in the two loops at the edges of the epitope.

In 7 of 111 patient samples, binding to H103A/S104E and mMOG was reduced or abolished, but binding to P42S, R9G/H10Y, and R86Q was good (pattern 6, Fig. 2F). In 6 of 111 samples, binding was reduced to the P42S mutant and to the R9G/H10Y or to the R86Q mutant (pattern 7, Fig. 2G). We noted that sera showing pattern 7 reactivity differed in reactivity to mMOG (binding to mMOG was reduced in 4/6), indicating that within this pattern additional epitopes are recognized to different extents.

A subset of 32 of 111 sera showed reduced reactivity to various MOG constructs carrying distant mutations. As discussed earlier, the most frequently recognized epitopes are found at the CC-loop and at the FG-loop of hMOG. In addition, the reactivity to both of these two epitopes is the most common combination among sera recognizing multiple epitopes.

The triple mutant P42S/H103A/S104E. Binding to the triple mutant P42S/H103A/S104E, which combined the two most important epitopes of human anti-MOG IgG, was reduced in 64% of all sera. As expected, all 19 sera that showed a reduced binding to both P42S and H103A/S104E showed strongly decreased binding to the triple mutant (less than 35% binding; pattern 5, Fig. 2E).
Within the group of 41 sera with pattern 1 recognition, 13 showed increased (i.e., more than 200%) binding to H103A/S104E. Nine of these 13 sera also showed reduced binding to the triple mutant P42S/H103A/S104, whereas the remaining 4 of 13 bound this triple mutant comparably strong as hMOG (also see later). Thus, our triple mutant revealed a further heterogeneity of sera with pattern 1 recognition.

The triple mutant also allowed further differentiation of sera that recognized the second most important epitope, the FG-loop. Within the subgroup of 17 sera with decreased binding to H103A/S104E (patterns 2 and 6), four sera showed increased binding to P42S. The one serum with pattern 6 recognition and a strongly increased P42S recognition showed poor recognition of mMOG. This result could be explained by other more subtle structural differences between human and mMOG. In only 1 of these 4 sera, binding to the triple mutant was decreased. The remaining 3 sera bound well to the triple mutant.

The triple mutant also allowed further differentiation of sera that recognized the second most important epitope, the FG-loop. Within the subgroup of 17 sera with decreased binding to H103A/S104E (patterns 2 and 6), four sera showed increased binding to P42S. The one serum with pattern 6 recognition and a strongly increased P42S recognition showed poor recognition of mMOG. This result could be explained by other more subtle structural differences between human and mMOG. In only 1 of these 4 sera, binding to the triple mutant was decreased. The remaining 3 sera bound well to the triple mutant.

One example is the optic neuritis serum AEB048 that showed only 12% binding to H103A/S104E (pattern 2). This serum showed strongly increased binding to the P42S mutant (2057% binding to P42S and 1518% binding to mMOG, compared with hMOG). In this case, binding to the triple mutant was not reduced, but was also stronger than to hMOG (610%).

Sera with decreased reactivity to both P42S and to H103A/S104E also showed decreased reactivity to the triple mutant P42S/H103A/S104E. Thirteen sera showed increased reactivity to H103A/S104E, but did not recognize P42S. In 9 of 13 cases, these autoantibodies did not recognize the triple mutant. Four sera showed increased reactivity to the P42S mutant. In 3 of 4 sera, these autoantibodies also bound the triple mutant well, although they did not recognize H103A/S104E.

Higher reactivity to mutated variants of MOG. Thirty-two of 111 patients showed a clearly elevated (more than 2-fold) reactivity to mutated variants of MOG as compared with hMOG, and some bound multiple mutants better than hMOG. These 32 patients included 8 who recognized MOG better in the absence of glycosylation (N31D mutant, details are discussed later). Twelve patients recognized mMOG better than hMOG, six of these also bound better to P42S than to hMOG. This increase in binding to P42S might be explained by the rigidity of proline and the flexibility of serine, which will allow an induced fit of the protein to the Ab. For 6 of 12 patients showing higher reactivity to mMOG, we were not able to assign an epitope recognition pattern; 3 of 12 showed pattern 2 recognition and 3 of 12 recognized other patterns.

Seventeen of 111 patient sera bound H103A/S104E better than hMOG, 4 of which also bound better to mMOG. 13 of these 17 sera recognized pattern 1 (reduced binding to P42S). Nine of 13 sera with increased binding to H103A/S104E and pattern 1 recognition (reduced binding to P42S) also showed reduced binding to the triple mutant P42S/H103A/S104E, as described in the previous paragraph. As explained earlier, these sera could contain at least two different kinds of anti-MOG Abs—one recognizing the CC'-loop and another with improved binding to the artificial mutant H103A/ S104E. However, we cannot exclude the possibility that an Ab recognizes both loops simultaneously at the edge of the epitope.
Another possible explanation for increased recognition of MOG variants could be that high-affinity anti-MOG Abs do not recognize a MOG variant allowing multiple other lower affinity anti-MOG Abs to bind to this variant. Theoretically, the sum of binding of these low-affinity Abs could result in an increased FACS ratio.

A subset of 32 of 111 sera showed increased reactivity to mutated variants of MOG. The most common mutants to elevate autoantibody binding were H103A/S104E, mMOG and N31D.

**Comparison of MOG epitope analysis with mutated variants versus competition with defined mAbs**

We compared our single amino acid mutation assay to blocking assays with defined mAbs. The mAbs 8-18C5 and Y11 recognize different epitopes on MOG (24). We analyzed IgG binding of 15 sera in competition with either 8-18C5 or Y11; we found both mAbs to compete with the human IgG in 15 of 15 sera. The tested sera recognized different epitopes; 13 of them recognized patterns 1, 2, 4, 5, 6 and 7; and two bound well to all mutants. Fig. 4 shows two sera that were blocked by the mAbs 8-18-C5 and Y11 to a similar extent, although they recognized different epitopes as revealed by our mutants; the mutation H103A/S104E abrogated the binding of one, but did not interfere with the binding of the other serum. This result shows that mAbs can block binding of human anti-MOG IgG, even when a different epitope is recognized by the human Abs. Therefore, systematic mutation of amino acids gives more detailed information about the epitopes recognized by human anti-MOG Abs than competition assays do with defined mAbs.

**The role of MOG-glycosylation in autoantibody binding**

Deglycosylation with PNGaseF yielded MOG proteins with the same size as the N31D mutant (Fig. 5), indicating that N31D is the only N-glycosylation site used in our constructs. The “no glycosylation” mutant N31D did not significantly lower binding in any of the sera. Examples of recognition of N31D are shown in Figs. 2 and 6 and in Supplemental Fig. 4. We conclude that the glycosylated part of MOG is not recognized by autoantibodies. Instead, 8 sera recognized the unglycosylated MOG better than the hMOG. Five of these patients showed low recognition of hMOG, with a FACS ratio less than 2.0. Enhanced recognition of the unglycosylated mutant was not linked to recognition of a certain epitope pattern (3/8 recognized pattern 4, 2/8 recognized pattern 1; 1/8 recognized pattern 2; 1/8 recognized pattern 7, and 1/8 bound well to all mutants.)

**MOG epitopes recognized by patients with different disease entities**

Distinct epitope patterns were recognized by anti-MOG Abs in the serum of patients with six different clinical entities: ADEM, mono ADS, MS, CRION, NMO, and other relapsing ADS cases. Each epitope pattern was found in several clinical presentations (Table II). No statistically significant association between epitope recognition and diagnosis was found ($\chi^2$ test, $p = 0.27$). Nevertheless, a larger sample size might potentially reveal such an association. It is interesting to note that in the CRION group, 4 of 10 and only 4 of 101 other patients showed more than 200% binding to the N31D mutant.

We analyzed whether patients with chronic inflammatory diseases had an anti-MOG response directed against more epitopes than did patients with a monophasic disease. Of 26 cases of chronic inflammatory diseases—namely MS, NMO, CRION, and other relapsing ADS cases—35% recognized multiple epitopes (patterns 5, 6, and 7) and 31% recognized a single epitope (patterns 1, 2, and 3). Of 85 patients with a monophasic disease (ADEM and mono ADS), 27% recognized multiple epitopes (patterns 5–7) and 44% recognized a single epitope (patterns 1–3). These results are summarized in Table II, and they indicate that recognition of a single epitope of multiple epitopes is not linked to monophasic or chronic disease.

**Long-term analysis of individual epitope patterns on MOG**

Follow-up sera of 11 anti-MOG Ab positive patients were analyzed with the different MOG mutants. In 9 of 11 patients, we were able to assign one of the aforementioned epitope patterns. The patterns stayed constant in 9 of 9 analyzed cases for an observation period of up to 50 mo (MS) without evidence for intramolecular epitope spreading (Fig. 6 and Supplemental Fig. 4). Constant epitope patterns were found in MS, CRION, and ADEM; this was especially remarkable for patients with MS. In pediatric patients with MS, anti-MOG IgG persists with fluctuations (20). For example, the anti-MOG reactivity of the MS patient ACJ-162 (Supplemental Fig. 4H) decreased below detection level after 12 mo, but clear anti-MOG reactivity was seen after 24 mo (20). The anti-MOG Abs still recognized the same pattern (pattern 1) after a follow-up period of 36 mo. Different epitope patterns, 1, 2, 4, 5, 6 and 7, stayed constant over years. Additional details of this follow-up part of our study are presented in Supplemental Fig. 4.
Comparison of the dynamic of anti-MOG IgG with anti-measles virus and anti-rubella virus IgG

We selected three patients with a rapid decline of anti-MOG IgG (20) for a comparative analysis of the dynamic of IgG produced in response to vaccines. All three patients had been vaccinated against measles and rubella virus. IgGs against these vaccines typically persist. We found that these patients mounted a persevering IgG response against both measles and rubella virus, but they lost the anti-MOG IgG rapidly (Fig. 7).

Discussion
In this study, we define epitopes of conformationally intact MOG recognized by human autoantibodies. The mutants of MOG we applied allowed us to obtain insight into recognized epitopes in 98 of 111 patients. Based on the tested mutants, half of the patients showed an immune response directed against one single epitope, the other half recognized multiple epitopes.

Mutation of the single amino acid P42 in the CC’-loop abrogated or reduced recognition of MOG in the majority of anti-MOG–positive patients. The second most frequently recognized epitope is located at the tip of the FG-loop (H103; S104), which is bound by the mAb 8-18C5 (27). Overall, we distinguished seven patterns of Ab recognition. All epitopes identified in this work are located at loops that connect the β-stands of the IgV-like fold of MOG. This observation is in harmony with the concept that antigenicity correlates with solvent accessibility and flexibility in proteins (33). It is currently unknown whether the serum anti-MOG Ab response is polyclonal. Our data provide direct evidence for the polyclonal nature of at least a subgroup of anti-MOG sera, because we observed reduced binding to multiple mutants in about a third of all donors.

Most of the patients recognizing hMOG did not recognize mMOG, largely because the majority of sera did not bind to P42S, which is also found in mMOG. Other amino acid differences between the two species also contribute to the differential recognition of human and murine MOG (patterns 3, 4, and 7). This species-specific recognition pattern is different from the features of anti-AQP4 autoantibodies. Human anti-hAQP4 Abs cross-react with mAQP4; staining of mouse tissue was even used to identify NMO IgG (34). Our study shows that other human autoantigens might be missed when screening with rodent tissue.

The pathogenic potential of human autoantibodies is best demonstrated in transfer studies into experimental animals as done with anti-AQP4 Abs (35–38). The human Abs to MOG have all the characteristics of pathogenic autoantibodies: they recognize MOG in its correct conformation, they are mostly of the complement-fixing isotype IgG1 (18–20), and they activate Ab-dependent cellular cytotoxicity (14, 18). Their pathogenic activity, however, has not yet been shown with affinity-purified Abs. Transfer experiments with concentrated human sera (39) are difficult to interpret, because human sera could have pathogenic compounds beyond anti-MOG IgG. Our study shows that only a minority of human sera with anti-MOG IgG are suitable for transfer experiments in mice. The recognized proline at position 42 is not present in mice and rats, not even in the New World primate Callithrix jacchus, but appears in the rhesus monkey (Macaca mulatta).

The major autoantibody epitope found here is different from the immunodominant and pathogenic epitope in rodents (24). Human anti-MOG Abs mainly recognized the CC’-loop around P42 of hMOG, whereas most mouse mAbs to MOG recognized the FG-loop. Animal experiments have shown that not all Abs against MOG are pathogenic (12, 40, 41). Mouse mAbs, which recognize MOG on the cell surface and are pathogenic, can recognize different epitopes: both the mAb 8-18C5 and the mAb Y11 are pathogenic (31). Thus, one would expect that not only those human anti-MOG Abs recognizing the FG-loop (patterns 2, 5, and 6), but that also other Abs recognizing another part of the surface of MOG, e.g., the CC’-loop, are pathogenic. The CC’-loop is closer to the membrane than the FG-loop. Because this loop is recognized by Abs when displayed on the surface of transfected cells, we anticipate that it is also recognized on the surface of myelin. It is evident from features of the anti-MOG mAb Y11 that the same Ab can recognize both a linear peptide and the cell-bound conformationally intact MOG protein (12). Thus, it is likely that some of the anti-MOG Abs in patients recognizing cell-bound MOG also recognize linear peptides.

The identification of precise epitopes of autoantibodies can provide the basis for an Ag-specific depletion of relevant B cells. A proof of concept for such an Ag-specific therapy has recently been obtained in an animal model of diabetes (42). In a different approach, intracerebral injection of competing nonpathogenic anti-AQP4 Abs reduced AQP4 and myelin loss in a mouse model of NMO (43).

Our study shows that the application of mutant variants of MOG allows a more precise insight into epitope recognition than blocking with defined mAbs does. In agreement with previous studies, we found that the anti-MOG mAb 8-18C5 competes with binding of human Abs to cell-bound MOG (19, 20). We show that this mAb also inhibits sera that recognize different epitopes. This is not

Table II. Recognition of epitope patterns in different disease groups

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients</th>
<th>Pattern 1</th>
<th>Pattern 2</th>
<th>Pattern 3</th>
<th>Pattern 4</th>
<th>Pattern 5</th>
<th>Pattern 6</th>
<th>Pattern 7</th>
<th>No Epitope Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono ADS other than ADEM</td>
<td>45</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>ADEM</td>
<td>40</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>3</td>
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<td>MS</td>
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<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>NMO like</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>CRION</td>
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<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other relapsing ADS</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Other relapsing ADS cases: three patients had one ADEM attack plus one non-ADEM attack, and one patient had a monolesional transverse myelitis.
This study analyzes epitopes of human anti-MOG Abs with mutated cell-bound MOG. Earlier studies using peptide ELISA assays (47–49) would not give information on epitopes of conformationally intact MOG. In this study, we show that human anti-MOG Abs recognize the loops of structurally intact MOG, which should not be provided in a peptide ELISA, and indeed two studies failed to identify these epitopes in an ELISA assay (47, 48). Another study reported that linear epitopes aa 37–48 and aa 42–53 are immunodominant in a peptide ELISA assay, but these peptides were also recognized by controls at a lower frequency (49). The donors assessed in (49) were adult patients with MS, who rarely have IgG against conformationally intact MOG. In addition, because a secondary Ab recognizing IgG, IgA, and IgM was used in that study, it is likely that mainly low-affinity IgM was detected.

To study the potential relevance of MOG-glycosylation in our study, we applied the N31D mutant (23). This application completely abolished N-glycosylation of MOG, indicating that in our MOG-constructs N31 is the only used N-glycosylation site. A second potential glycosylation site N52, which lacks the consensus N-glycosylation sequence N-\(\text{X}\)-Ser/Thr, was found in mouse brain by tandem mass spectrometry (50). This site was not considered a high-confidence glycosylation site (50) and was not used to glycosylate MOG in our case. In our study, unglycosylated MOG was recognized well by all human anti-MOG Abs, in agreement with O’Connor et al. (23). We noted that 8 of 111 sera even showed increased reactivity to unglycosylated MOG, which might be due to the better accessibility of MOG lacking the polycarbohydrate chain at its upper, very exposed edge of its extracellular domain. This effect is reminiscent of observations made in HIV, in which deglycosylation of the HIV envelope glycoprotein gp120 led to increased recognition by neutralizing Abs (51).

In addition to unglycosylated MOG, other mutated variants of MOG were also recognized at least twice as good as hMOG: 17 of 111 sera recognized H103A/S104E better, and 6 of 111 recognized P42S better than hMOG. Mutation of serine to glutamic acid, as in the H103A/S104E mutant is used experimentally to mimic phosphorylation, a strategy called “pseudo-phosphorylation” (52). It is possible that these Abs are generated against phosphorylated MOG. We have used NetPhos 2.0 (53) to predict phosphorylation sites on hMOG and found S104 to be a likely site for phosphorylation, with a score of 0.994 (data not shown). Further experiments are required to confirm whether MOG in the human CNS is indeed phosphorylated at S104 or other positions.

Although patients, in particular with ADEM, only show a transient IgG response to MOG, others (in particular with MS) tend to have a persisting Ab response to MOG (15, 20). We have addressed two issues related to the dynamic of anti-MOG IgG. First, we analyzed whether there was epitope spreading. Second, we examined whether those patients with a rapid decline of anti-MOG IgG were still able to mount a persisting IgG response to other Ags.

We found that the recognized epitopes remained constant. This was seen not only in patients with ADEM who rapidly lose their anti-MOG Abs, but also in childhood patients with MS and CRION who have anti-MOG Abs persisting for years. Therefore, we find that for MOG-Abs there is neither intramolecular epitope spreading nor epitope loss. Autoantibody epitope spreading has been reported for a number of autoimmune targets, among them anti-AChR Abs in myasthenia gravis (54), anti-mitochondrial Abs in primary biliary cirrhosis (55), and anti-citrullinated protein Abs in rheumatoid arthritis (56).

The maintenance of serum IgG is crucial for our ability to fight pathogens (57). The persistence of serum IgG is based on long-lived plasma cells that find a survival niche in the bone marrow or inflamed tissue (58). We found that those patients, who rapidly
lost their anti-MOG IgG, were still able to mount a persisting IgG response against two pathogens: measles and rubella virus. This finding shows that these patients do not have a general inability to generate long-lived plasma cells. Instead, it suggests that the IgG-secreting cells generated during their anti-MOG response are less competent to seed the survival niches than are plasma cells generated after vaccination.

This study shows that mutation of the single amino acid P42 in the membrane proximal CC′-loop disrupts MOG recognition in the majority of patient sera. We dissect seven patterns that describe the epitopes of 98 of 111 patients with anti-MOG Abs. Notably, the individual epitope patterns remained constant over time without evidence for intramolecular epitope spreading. This study has implications for the design of transfer studies in animals, anti-MOG detection assays, and future therapies aiming at Ag-specific depletion of MOG-reactive Abs.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary figures

Figure S1: Validation of transiently transfected MOG variants and surface expression of wild type and mutated variants of MOG. A) Processing of FACS data to calculate specific binding. Three different transfectants were stained with the same serum AEB085. Left: HeLa cells transfected with EGFP only (MCF FL-4= 4.47). Middle: HeLa cells transfected with hMOG-EGFP (MCF FL-4=79.7). Right: HeLa cells transfected with mMOG-EGFP (MCF FL-4=3.34). For binding calculation gates were set on the cells with an EGFP expression level between of 102 and 103. Calculation of the binding percentage for the serum AEB085 shown above:

\[
\text{AEB085 \% binding of mMOG compared to hMOG} = \frac{3.34 - 4.47}{79.7 - 4.47} \times 100\% = -1.5\% \approx 0\%.
\]

B) hMOG and all mutants were recognized equally by the anti-MOG mAb Y11 proving that they are correctly folded and expressed on the cell surface. In accordance with published data (Breithaupt et al., 2008) the mAb 8-18C5 does not recognize MOG anymore when the mutation S104E is introduced. C) Expression intensity of the applied MOG variants. HeLa cells were transiently transfected with the indicated MOG-variant fused to EGFP. Depicted is the fluorescence intensity of the FL-1 channel (EGFP) of the 8 different MOG mutants, measured 24h after transfection. Frequency and fluorescence intensity were comparable, allowing for comparison of binding to different mutants.

Figure S2: Serial dilution of two sera with differential recognition of MOG variants. A) The adult ADEM serum showed enhanced recognition of the H103A/S104E mutant and did not bind to mMOG and the P42S mutant. Recognition of the R9G/H10Y mutant was decreased. This pattern was seen at a dilution of 1/50 and 1/200. At a dilution of 1/800, only binding to the H103A/S104E mutant was above the cut-off. B) The serum AEB-123 did not recognize
Distinction of MOG epitopes

P42S/H103A/S104E, mMOG or P42S. This pattern was seen up to a dilution of 1/3200. At a dilution of 1/12800, recognition of hMOG was slightly below the cut-off.

Figure S3: Binding to the double mutant H103A/S104E in comparison to binding to S104E. Binding to H103A/S104E was decreased (to less than 65%) in 34 of all sera. For these 34 and additional 42 sera binding well to H103A/S104E (76 in total) we also tested binding to the single mutant S104E. Depicted are the binding percentages for the 76 sera; sera with low reactivity to hMOG (FACS ratio below 2.0) are depicted as open circles. The correlation was highly significant (Spearman r= 0.85, p<0.0001). One optic neuritis patient recognized S104E with 1506% binding, but did not recognize the double mutant H103A/S104E.

Figure S4: Temporal stability of recognition of MOG-epitopes. The longitudinal analysis of epitope recognition of 10 sera (additional to figure 6) is shown. These patients were followed for an observation period of up to 37 months (I) and had different diseases, namely ADEM (A-F), CRION (G) and MS (H-J). Anti-MOG reactivity showed a second increase in all examined MS sera and the time point of the increase is labeled with a star (*). In the MS serum ACJ-162, the anti-MOG IgG fell below our detection limit at 12 months (n.d., not detectable), but reappeared later. Despite these fluctuations in the intensity of anti-MOG reactivity, the epitope recognition patterns stayed the same. The CRION patient experienced her 11th and 12th episode of optic neuritis.