

Frontline:**Epitope recognition on the myelin/oligodendrocyte glycoprotein differentially influences disease phenotype and antibody effector functions in autoimmune demyelination****Hans-Christian von Büdingen^{1,2}, Stephen L. Hauser¹, Jean-Christophe Ouallet^{1,3}, Naoyuki Tanuma^{1,4}, Til Menge¹ and Claude P. Genain¹**¹ Department of Neurology, University of California, San Francisco, USA² Department of Neurology, Universitätsspital Zürich, Zürich, Switzerland³ Service de Neurologie-Federation des Neurosciences Cliniques, Hôpital Pellegrin-Tripode, Bordeaux, France⁴ Department of Pediatrics, Metropolitan Fuchu Medical Center for Severe Motor and Intellectual Disabilities, Fuchu, Tokyo, Japan

Preliminary observations of humoral immunity against the myelin oligodendrocyte glycoprotein (MOG) in experimental allergic encephalomyelitis (EAE) and human multiple sclerosis (MS) suggest that a subset of anti-MOG autoantibodies directed against conformational epitopes is of pathogenic predominance. Here, we provide proof that in marmoset EAE, autoantibodies reactive against conformational epitopes of MOG are not only responsible for aggravating demyelination, but also an essential factor for disease dissemination in space within the central nervous system, a hallmark for typical forms of human MS. In terms of effector mechanisms, IgG deposition and complement activation occur exclusively in association with presence of these conformational antibodies, while microglial/macrophage activation appears to be a common immunopathological finding regardless of the fine determinant specificity of anti-MOG antibodies. These findings highlight for the first time the complex heterogeneity of function and pathogenicity in the polyclonal anti-MOG antibody repertoire of outbred species. Because the linear and conformational antibody determinants of MOG are shared between marmosets and humans, these results are directly relevant to understanding effector mechanisms of organ damage in MS.

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1 Introduction

Myelin oligodendrocyte glycoprotein (MOG)-induced experimental allergic encephalomyelitis (EAE) in the common marmoset (*Callithrix jacchus*) is a multifocal disease of central nervous system (CNS) white matter that closely approximates human multiple sclerosis (MS) [1, 2]. Myelin-directed T cell reactivity is obligatory for disease development in marmosets as in all EAE models;

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Abbreviations: **MOG:** Myelin oligodendrocyte glycoprotein **CNS:** Central nervous system **MS:** Multiple sclerosis **rMOG₁₋₁₂₅:** Recombinant extracellular domain of MOG **pep-MOG:** Mixture of 20mer peptides spanning the extracellular domain of MOG **Anti-MOG-P:** Antibodies against peptide epitopes of MOG **Anti-MOG-C:** Antibodies against conformational epitopes of MOG **aa:** Amino acids **MPB:** Myelin basic protein

however, involvement of anti-MOG antibodies is necessary for development of the typical MS-like neuropathological phenotype [3]. Not unexpectedly, a broader heterogeneity of epitopes within MOG antibody responses is found in primates compared to rodents (reviewed in [4]). Although limited preliminary observations suggest that epitope recognition may underlie differences in antibody effector functions in humans [5], these relationships remain largely unexplored.

Structurally, antibodies against MOG can be differentiated on the basis of their ability to recognize either linear or conformational, tertiary structure-dependent epitopes [6]. Standard ELISA methods cannot separately detect antibody reactivity against conformational and linear epitopes of MOG, thus pathogenic properties and effector functions of these different antibody populations cannot be understood unless they are isolated and separately

studied. Such information is needed in order to properly interpret anti-MOG antibody reactivity in MS, which has been reported with varying frequencies depending on the study, the method of detection and the protein used [7–14].

Cloning of MOG-reactive antibodies present in the marmoset immune repertoire has revealed monoclonal antibody specificities that define several distinct conformational, surface-exposed epitopes of MOG, which are also present in human antibody repertoires [6]. Here, we have taken advantage of this model system to characterize the immunopathogenicity of anti-MOG antibodies according to their epitope recognition. Using combinations of active immunizations and passive antibody transfers of affinity-purified antibody fractions in combination with immunohistochemical analysis, we found evidence that both linear and conformation-dependent anti-MOG antibodies are potentially pathogenic by contributing to macrophage recruitment and activation in EAE lesions. However, conformation-dependent antibodies appear to be required for dissemination of CNS pathology and complement deposition in lesions. These results have important implications for interpretation of anti-MOG antibody reactivity in health and disease.

2 Results

2.1 Clinical and neuropathological characteristics of EAE induced with MOG peptides or rMOG_{1–125}

Table 1 recapitulates the clinical and neuropathological phenotypes of EAE induced with either the recombinant extracellular domain of MOG (rMOG_{1–125}; group I) or linear MOG peptides (group II). The course of MOG peptide-induced EAE tended to be progressive, with more rapid progression in the two animals immunized with a mixture of 20mer peptides spanning the extracellular domain of MOG (pepMOG; animals 39–95 and 65–92). rMOG_{1–125}-induced EAE was either rapidly progressive or relapsing-remitting (not shown), as previously described in animals observed chronically. It is noteworthy that overall severity of disease was not associated with a particular immunization regimen: both animals J2–97 (rMOG_{1–125}-immune) and 39–95 (pepMOG-immune) developed hyperacute EAE symptoms requiring immediate euthanasia.

Neuropathologically, the first remarkable difference between animals in the two immunization groups was a tremendously reduced white matter lesion burden in group II (group I: 163±56.3 lesions; group II:

Table 1. Characteristics of EAE in groups I (rMOG_{1–125}-immune, gray shading) and II (MOG peptide-immune) marmosets^{a)}

Animal ID	Immunogen	Max. clinical score	No. of lesions	Inflammation	Demyelination
U004–99	rMOG _{1–125}	14	326	+++	+++
U009–99	rMOG _{1–125}	8	135	+++	+++
Cj72–88	rMOG _{1–125}	5.5	67	+++	+++
J2–97	rMOG _{1–125}	29	124	+++	+++
199–94	aa 21–40	8	4	+++	+
368–94	aa 21–40	10	4	++	+
39–95	pepMOG	19	4	+++	+
65–92	pepMOG	9	33	++	+ ^{b)}
252–93	aa 1–40	5	6	++	+
TX245–90	aa 1–40	10	8	+++	+
14–91	aa 21–40, 51–90	12	8	+	+
TX75–92	aa 51–90	10	13	++	+
Tx256–93	aa 81–120	9	3	+	+

^{a)} pepMOG denotes a mixture of 11 20mer peptides overlapping by ten aa and spanning the sequence of MOG aa 1–120.

^{b)} Demyelination was found with the grade indicated in all lesions except in animal 65–92, in which only 18 of 33 (55%) lesions were demyelinated.

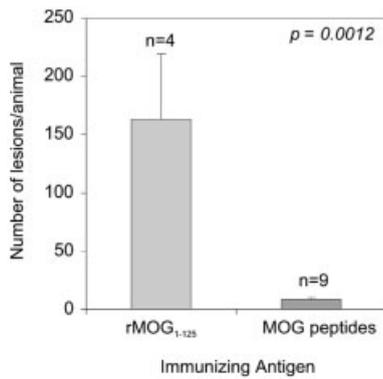


Fig. 1. Average CNS lesion load in rMOG₁₋₁₂₅- and MOG peptide-immunized marmosets; means \pm SEM.

9.2 \pm 3.1 lesions; mean \pm SEM, $p=0.0012$; Table 1, Fig. 1). Second, in contrast with the multifocal disease that we and others have consistently observed in many rMOG₁₋₁₂₅-immune marmosets (typically involving optic nerves, spinal cord, brain hemispheres, and brainstem with perivascular intraparenchymal distribution [1]), the distribution of lesions in MOG peptide-immune animals was mainly restricted to brainstem and spinal cord with a pattern of subpial space infiltration reminiscent of MOG peptide-induced EAE in mice [15, 16] (Fig. 2). Animal 39–95 developed a large hemorrhagic lesion in the left optic tract and nerve. Only one of the pepMOG-immunized animals developed inflammatory lesions within the cerebral white matter (animal 65–92), none of which showed evidence of demyelination (not shown). The third difference was that the extent of demyelinated areas was reduced in lesions of MOG peptide-induced EAE compared with those of rMOG₁₋₁₂₅-induced EAE, in the presence of roughly similar degrees of inflammation (Table 1). The demyelination in MOG peptide-induced EAE did not extend beyond the margin of inflammatory infiltrates, in contrast to the protracted and expanding lesions of rMOG₁₋₁₂₅-induced EAE (Fig. 2). The most abundant pattern of demyelination in lesions of MOG peptide-induced EAE was myelin vacuolation (Fig. 2A), a feature that is present at the periphery of expanding lesions in rMOG₁₋₁₂₅-induced marmoset EAE [17] (Fig. 2B).

2.2 Epitope specificities of antibody responses

All monkeys developed serum antibodies that reacted to both rMOG₁₋₁₂₅ and linear peptides as shown by standard ELISA of unfractionated serum (Fig. 3, left panels). To separately characterize conformational and linear specificities, the following selected sera were depleted from pepMOG-reactive antibodies: group I (rMOG₁₋₁₂₅-immune): J2–97, 72–88, U004–99, U009–99; group II:

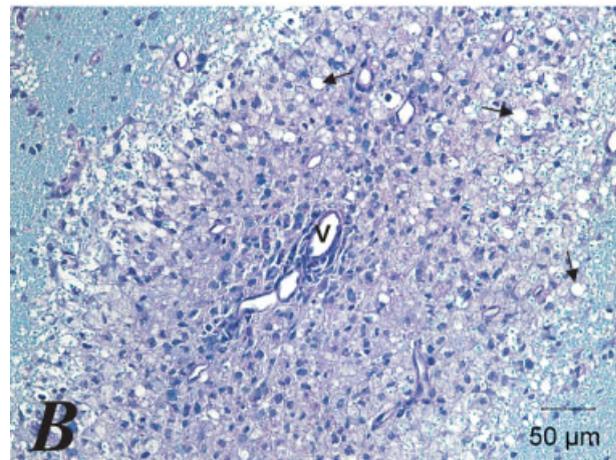
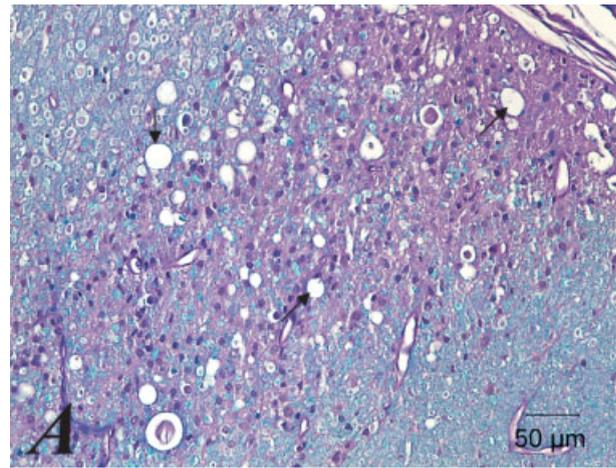


Fig. 2. Neuropathology of rMOG₁₋₁₂₅- and MOG peptide-induced EAE. High-power views ($\times 200$) of cervical spinal cord sections stained with LFB. (A) Typical inflammatory infiltrate in a marmoset immunized with MOG aa 21–40 (368–94). Note contiguity with the subpial space (upper right corner) and the limited amount of demyelination. (B) Perivascular, inflammatory infiltrate in deep periventricular white matter (V = blood vessel) with pronounced concentric demyelination, characteristic of rMOG₁₋₁₂₅-immunized animals (J2–97). Such lesions were never found in MOG peptide-immune *C. jacchus*. Note myelin vacuolation (arrows) in both MOG peptide-induced EAE (A) and at the lesion edges in rMOG₁₋₁₂₅-induced EAE (B).

pepMOG-immune: 39–95, 65–92; MOG amino acids (aa) 21–40-immune: 199–94, 368–94; a pool of equal amounts of sera from animals 14–91, 75–92, 252–93, 245–90, 256–93. The results from representative animals and the pooled sera from group II are shown in the right panels of Fig. 3.

Complete depletion of sera from the MOG peptide-reactive antibody fraction (anti-MOG-P) was achieved

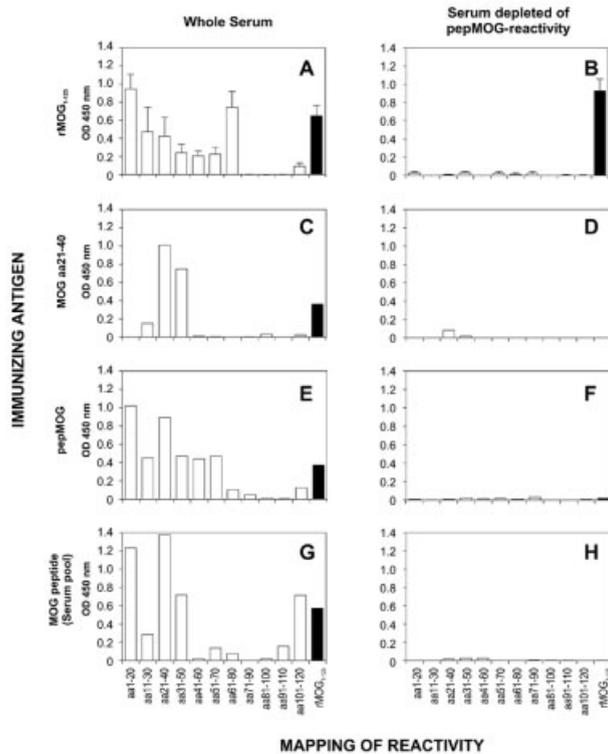


Fig. 3. Fine specificities of unfractionated sera and anti-MOG-P-depleted sera from representative animals of groups I and II. The left panels show reactivity of whole sera at a dilution of 1:200. The right panels show residual reactivity after removal of anti-MOG-P antibodies by affinity chromatography. (A, B) Antibody specificities in rMOG_{1–125}-immunized monkeys ($n=4$, means \pm SEM), demonstrating that strong reactivity against rMOG_{1–125} is retained after removal of all MOG peptide-specific antibodies. (C–F) Representative experiments for individual animals immunized with individual or all MOG-derived peptides (aa 21–40, 199–94; pepMOG, 39–95). (G, H) Reactivity of a pool of MOG peptide-immune sera (animals 252–93, Tx245–90, 14–91, Tx75–92, Tx256–93). The MOG-reactivity is completely removed in all animals immunized with MOG-derived peptides by passage on pepMOG columns, indicating that this immunization regimen does not induce conformation-dependent antibodies. Compare to (A, B), rMOG_{1–125}-immune animals.

after three to five passes over the pepMOG columns, as shown by the lack of binding to individual peptides (Fig. 3, right panels). Depletion from anti-MOG-P resulted in complete loss of reactivity to rMOG_{1–125} in each of the animals immunized with MOG peptides (Fig. 3D, F, H), regardless of the sequence of the immunizing peptides. By contrast, sera from rMOG_{1–125}-immune animals always retained reactivity against whole

rMOG_{1–125} after being depleted from anti-MOG-P antibodies (Fig. 3B).

Anti-MOG-P and/or the fraction of antibodies reactive against conformation-dependent epitopes of MOG (anti-MOG-C) from animals of both groups were eluted from the respective affinity columns. Only anti-MOG-P displayed binding to MOG peptides, as did the respective sera from which they were purified. These antibody fractions were also capable of binding to rMOG_{1–125} *in vitro* in the ELISA system (not shown). Incubation of normal marmoset CNS sections with anti-MOG-P antibodies showed that these antibodies strongly stained white matter, as did anti-MOG-C antibodies. This was observed regardless of the immunization regimen used to produce anti-MOG-P antibodies (e.g. rMOG_{1–125} or MOG peptides) (Fig. 4). No significant reactivity to either recombinant glutathione S-transferase (GST) expressed in *Escherichia coli* or the (His)₆ C-terminal peptide was detected in any of the antibody fractions (not shown). Together, these findings demonstrated that (1) both linearly defined (anti-MOG-P) and conformational (anti-MOG-C) antibodies are capable of binding to MOG *in situ*, thus epitope recognition *per se* does not appear to be the determining factor for antibody binding to MOG embedded in intact myelin sheaths; (2) anti-MOG-C antibodies that were isolated after depletion of pepMOG-specific antibodies were not directed against the C-terminal peptide of rMOG_{1–125} or against bacterial contaminants in the rMOG_{1–125} preparation used to synthesize the chromatography columns.

2.3 MOG-specific T cell proliferative responses

Circulating T cell proliferative responses to rMOG_{1–125} were observed in peripheral blood mononuclear cells (PBMC) of all animals at euthanasia. The magnitude of these responses was similar in MOG peptide-immune animals and rMOG_{1–125}-immune animals (10 ± 3.1 vs. 12.7 ± 5.8 ; not significant; Fig. 5). T cell proliferative responses mapped to 20mer peptides corresponding either to the immunodominant T cell epitopes in rMOG_{1–125}-immune marmosets [4] or to the immunizing peptide(s) in MOG peptide-immune animals (not shown).

2.4 Immunohistochemical characterization of lesions

Results of immunostaining are summarized in Fig. 6, 7. Macrophage infiltration was a consistent feature of inflammatory infiltrates in all animals, as indicated by staining for HAM56 (Fig. 6A, B). Pronounced IgG deposition was found in rMOG_{1–125}-immune animals, either in

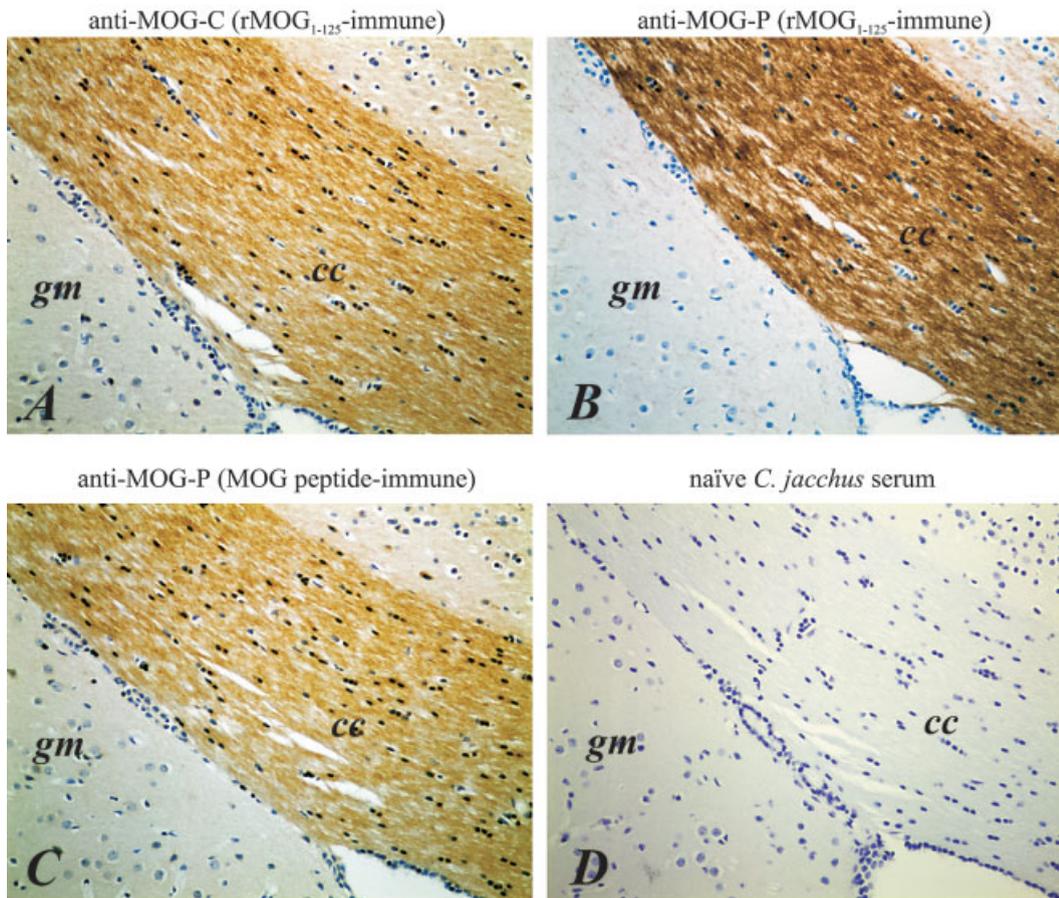


Fig. 4. Reactivity of affinity-purified anti-MOG antibody fractions with native MOG. Immunohistochemical staining of normal corpus callosum (cc) from an unimmunized *C. jacchus*. (A, B) Anti-MOG-C and anti-MOG-P from an rMOG₁₋₁₂₅-immune serum pool; (C) anti-MOG-P from a MOG peptide-immune serum pool; (D) naïve *C. jacchus* serum; gm, gray matter.

the immediate perivascular vicinity or deeper within the white matter parenchyma (Fig. 6C). In sharp contrast, lesions that showed IgG deposition were observed in

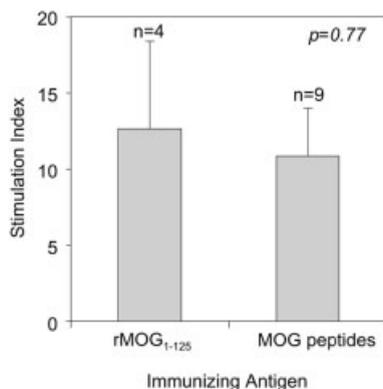


Fig. 5. T cell proliferation against rMOG₁₋₁₂₅ in rMOG₁₋₁₂₅- and MOG peptide-immune animals; means \pm SEM.

only two animals immunized with MOG peptides (39–95 and one in 252–93). This involved a single hemorrhagic lesion in both cases (not shown), which raises the possibility that this was the result of exudation of blood into the lesion. In addition to parenchymal deposition, IgG could also be detected in cells present in close vicinity of blood vessels in rMOG₁₋₁₂₅-immune animals (B cells or plasmocytes; Fig. 6C). Some of the lesions found in MOG peptide-immune animals also showed IgG-positive cells, though much less frequently (not shown). Quantitatively, the differences in IgG deposition and IgG-positive cells between rMOG₁₋₁₂₅- and MOG peptide-immune animals were significant ($p=0.003$ and $p=0.038$, respectively; Fig. 7). Highly significant differences were also observed for C9neo deposition, which was prominently observed in rMOG₁₋₁₂₅-immune animals (56% positive of 204 lesions analyzed), but was uniformly absent from any of the 83 lesions analyzed in MOG peptide-immune animals (0%; $p<0.0001$; Fig. 6E, F; 7).

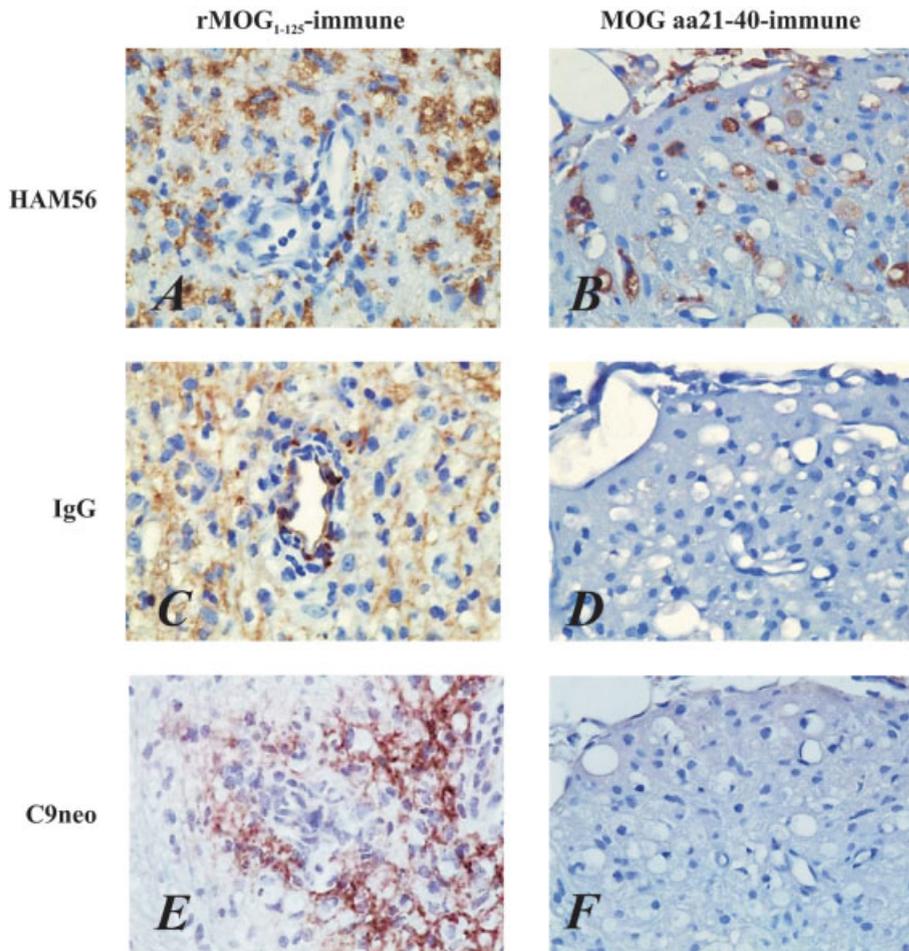


Fig. 6. Immunohistochemical characterization of CNS lesions. Representative lesions from an rMOG₁₋₁₂₅-immunized animal (J2-97, left) and an animal immunized with MOG aa 21-40 (199-94, right). From top to bottom: staining for macrophages (HAM56; A, B); IgG (C, D); C9neo (E, F). Activation of complement (C9neo) was a characteristic of rMOG₁₋₁₂₅-induced EAE and was not found in MOG peptide-immune animals. Original magnification 600x.

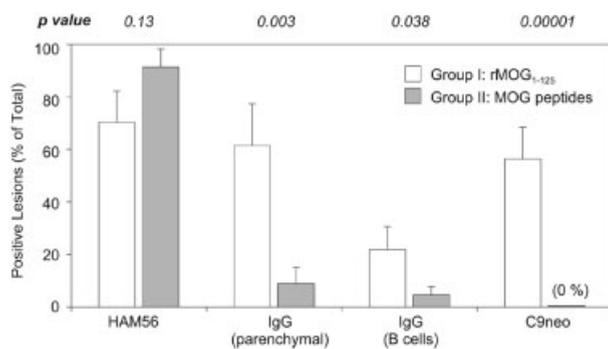


Fig. 7. Quantitative analysis of HAM56, IgG and C9neo in CNS lesions. Values on y axis represent the percentages of positive lesions for each marker (rMOG₁₋₁₂₅-immune animals: 204 lesions, open bars; MOG peptide-immune animals: all visible lesions (n=83), closed bars; means ± SEM).

2.5 Anti-MOG antibody transfer experiments in myelin basic protein-immunized animals

All animals developed an immune response against myelin basic protein (MBP) as assayed by ELISA (not shown); however, none of the animals showed obvious signs of clinical EAE during the observation period. Neuropathologically, the three groups markedly differed: animals that received anti-MOG-P/C (U015-99 and 437-99) developed 135 and 35 inflammatory lesions, respectively (Fig. 8A, B; Table 2), mostly located in the cerebral white matter, with pronounced demyelination, highly similar to lesions found in rMOG₁₋₁₂₅-immune animals; animals that received anti-MOG-P (U026-00 and U029-00) developed 35 and 1 inflammatory lesions, respectively. All lesions in the latter animals showed significantly less demyelination and were characterized mostly by perivascular cuffing with mononuclear cells (Fig. 8C, D;

Table 2. Passive antibody transfer of fractionated anti-MOG antibodies in MBP-immunized marmosets

Animal ID	Antibody transferred	No. of CNS lesions	Demyelination
437–99	Anti-MOG-P/C	135	+++
U015–99	Anti-MOG-P/C	35	++
U026–00	Anti-MOG-P	35	+
U029–00	Anti-MOG-P	1	+
118–99	Naive Ig κ	0	–
335–97	Naive Ig κ	0	–

Table 2). Most of the lesions in animal U026–00 were located in the cerebral hemispheres and were reminiscent of the lesions found in the pepMOG-immunized animal 65–92 (not shown), whereas the 1 lesion found in animal U029–00 was located in the brain stem and thus shared an important characteristic with most of the MOG peptide-immunized animals; no lesion was detectable in the CNS of either animal that received naive *C. jacchus* Ig κ (not shown).

3 Discussion

Studies of the murine anti-MOG antibody repertoire [18–22] and recent crystallization studies of MOG/anti-MOG antibody complexes [23, 24] indirectly suggest that recognition of conformational determinants of MOG may be an important requirement for pathogenicity. However, according to some [25] but not all [26] investigations, Lewis rats immunized with MOG aa 35–55 can develop multifocal demyelinating disease despite the demonstration that these animals do not develop conformation-dependent anti-MOG antibodies. This implies that MOG peptide-specific antibodies may be pathogenic in the rat, as seems to be the case in several mouse strains [27].

A role for humoral mechanisms of demyelination in human MS is even less clear than in EAE, although suggested by the presence of intrathecal Ig synthesis, clonal expansion of B cells, and complement activation (reviewed in [28]). Despite one *in vitro* study that provides clues to the nature of exposed determinants of MOG in humans [5], to what extent the recognition of conformational determinants of MOG by B cells and/or antibodies influences the expression of MS phenotypes in humans

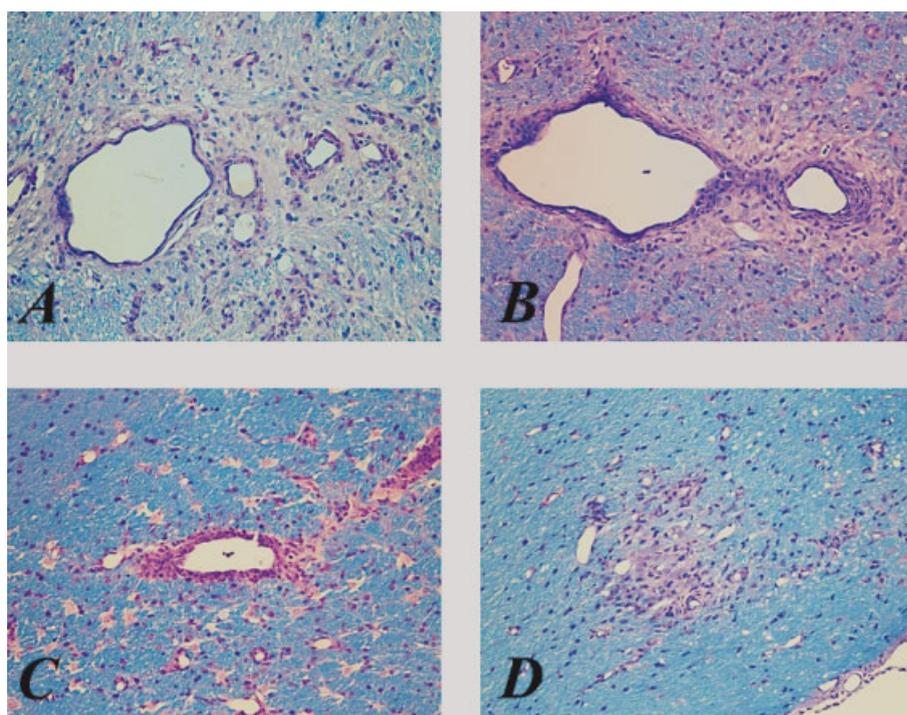


Fig. 8. Neuropathology of MBP-immunized animals after passive transfer of anti-MOG antibodies. Representative CNS lesions in animals that received anti-MOG antibodies reactive against both conformational and linear epitopes [anti-MOG-P/C; (A) 437–99, (B) U015–99], and in animals receiving MOG peptide-specific anti-MOG antibodies [anti-MOG-P; (C) U026–00, (D) U029–00]. All antibody fractions were derived from rMOG_{1–125}-immune serum. LFB stains, magnification 200 \times .

remains largely unknown. The concomitant presence in MS serum of pathogenic and non-pathogenic antibodies that cannot easily be distinguished one from another may be responsible for the lack of clear correlation between antibody presence and disease phenotype that has so far been reported. The current studies systematically investigated functional properties of anti-MOG antibodies according to epitope recognition in outbred marmosets, an EAE model where the diversity of humoral responses parallels that encountered in MS, and include determinants of MOG commonly found in humans [4, 6]. We show that marmosets develop radically different forms of EAE after immunization with either MOG peptides or rMOG_{1–125}, and that the EAE phenotype correlates with the presence or absence of a conformation dependent anti-MOG antibody response.

It is important to note that the differences between rMOG_{1–125}- and MOG peptide-induced EAE predominantly involved patterns of disease dissemination and demyelination, and not severity of EAE. Some animals in both groups developed either severe, rapidly progressive disease or mild to moderate forms, as can be expected in this outbred species. However, MOG peptide-immunized animals showed reduced, albeit significant demyelination compared to rMOG_{1–125}-immune animals. Demyelinating lesions in the former animals were mostly observed in spinal cord and brain stem, and not in cerebral hemispheres where they typically occur after rMOG_{1–125}-immunization [1]. This pattern of pathology was consistent in all MOG peptide-induced EAE cases regardless of the choice of immunizing peptide within the sequence of the extracellular domain of MOG, likely indicating that the observed differences were not a consequence of T cell epitope immunodominance. Rather, we propose that the recognition of conformational determinants of MOG was the basis for certain pathogenic properties of antibodies and/or B cells, which together with T cell responses resulted in MS-like multifocal disease and prominent demyelination.

A major finding of the current study is the clear demonstration of a link between antibody determinant recognition and density and distribution of CNS lesions. The presence of anti-MOG-C appears strictly associated with disease in a typical MS-like distribution (brain hemispheres, optic nerve and spinal cord), whereas linear-dependent antibodies are clearly associated with focal disease mostly restricted to brain stem and spinal cord in most animals. The subpial localization of demyelinating infiltrates in MOG peptide-immunized marmosets is also strikingly similar to CNS pathology observed in C57BL/6 mice immunized with MOG aa 35–55 [16, 29]. Possible biological explanations for these differences include differential binding affinity or, as discussed below, different

effector mechanisms activated by anti-MOG-P and anti-MOG-C antibodies. It is also possible that the density of expression of MOG molecules and/or presentation of its accessible epitopes on myelin sheaths differ within the different parts of the CNS, thus influencing lesion dissemination and location.

Our findings in actively induced EAE were fully corroborated by passive antibody transfer experiments in MBP-immunized marmosets, which develop non-demyelinating antibody responses [3]. MBP-immune animals that received only anti-MOG-P did not develop widespread demyelinating disease unlike those that received preparations containing the antibodies reactive against conformational MOG epitopes. We conclude that the latter fraction is responsible for the typical MS-like phenotype induced by immunization with rMOG_{1–125}, in partial agreement with studies of mice lacking B cells which fail to develop severe EAE after immunization with rMOG_{1–120}, but do so after passive transfer of whole serum from rMOG_{1–120}-immune wild-type mice [30].

However, the marmoset experiments demonstrate that anti-MOG-P antibodies may also be pathogenic, because MBP-immune animals that received these antibodies showed significantly more pathology than animals that received naive marmoset Igκ. Indeed, certain similarities were observed between MOG peptide- and rMOG_{1–125}-induced EAE. The lesion pattern observed in the former includes evidence of myelin vacuolation, which is also present at the edge of lesions in rMOG_{1–125}-induced EAE [2, 17]. This phenomenon has previously been shown to result, for example, from exposure of the intact myelin sheath to a variety of toxic soluble substances such as TNF-α [31], and could also be an effect of MOG-specific antibodies. Antibodies specific for MOG aa 21–40 have been detected in close association with disintegrating myelin membranes in lesions of rMOG_{1–125}-induced marmoset EAE, and in those of human MS [2]. Thus, it is possible that anti-MOG-P antibodies play a pathogenic role in sustaining myelin destruction by binding to epitopes newly exposed during active demyelination, as could be the case when naturally occurring MOG dimers are being disrupted [24].

The recognition of structural epitopes of MOG could not only influence antibody binding *in vivo* but also result in different effector mechanisms for antibody pathogenicity. While it was noteworthy that macrophage infiltration and activation was present to a similar extent in both MOG peptide- and rMOG_{1–125}-induced EAE, consistent deposition of IgG and the C9 component of the complement lytic complex was only detected in rMOG_{1–125}-immunized animals, and was absent from lesions of MOG peptide-induced EAE. This suggests that anti-

bodies against conformation-dependent MOG epitopes, and not those against the linear epitopes are capable of activating lytic complement pathways, thus potentially augmenting the destructive potential of these antibodies. Studies of EAE [18, 32] and MS [33–36] support a role for complement in lesion pathogenesis; however, there is no practical marker to detect this type of pathology. The knowledge of what antibodies within the MOG repertoire have specific pathogenic properties in primates is thus of essential importance.

In summary, we present here a comprehensive analysis of functional heterogeneity of the MOG-specific antibody repertoire in an outbred species that shares complexity and similar structural epitopes with humans. We formally demonstrate for the first time that antibody responses against a single target antigen of myelin can be sharply dichotomized in terms of pathogenic and functional properties, and that antibody epitope recognition is a critical factor for phenotypic expression of CNS autoimmunity. Our observations bear important implications for the interpretation of anti-MOG antibody serotypes in humans and will be essential to guide the choice of future therapies antagonizing pathogenic antibody responses in MS.

4 Materials and methods

4.1 Antigens

A recombinant protein corresponding to the sequence of the extracellular domain of rat MOG (rMOG_{1–125}) was expressed and purified to homogeneity as fusion protein with a His₆-Tag in *E. coli* following published procedures [37]. Eleven synthetic overlapping linear 20mer peptides corresponding to the sequence of rat MOG aa 1–120, and the C-terminal peptide of rMOG_{1–125} (WINPGRSRSHHHHHH) were synthesized using standard solid phase chemistry (Research Genetics, Huntsville, AL) and purified >95% by HPLC. Purity was confirmed by mass spectrometry. Human MBP was purified from brain as previously described [38].

4.2 Animals, immunization and characterization of EAE

C. jacchus marmosets used in this study were cared for in accordance with all guidelines of the Institutional Animal Care and Usage Committee. Marmosets were actively immunized with either 50 µg of rMOG_{1–125} (group I) or 100 µg of MOG-derived 20mer peptides (group II, individual peptides or combinations, please also refer to Table 1) dissolved in phosphate-buffered saline (PBS) and emulsified with complete Freund's adjuvant (CFA) as previously described [3]. The peptides or combinations of peptides were selected according to previous mapping studies that have character-

ized the immunodominant T cell and antibody epitopes of rMOG_{1–125} in marmosets [4, 39].

EAE was assessed by daily clinical examination and animals were observed for a total of 12–140 days (marmoset expanded scale, score 0 to 45 [40]). At the end of the observation period, euthanasia was performed under deep pentobarbital anesthesia by intracardial perfusion with 4% paraformaldehyde, and the entire neuraxis obtained and examined in serial consecutive sections (2 mm each). Five-micrometer, paraffin-embedded sections were stained with luxol fast blue /periodic acid-Schiff (LFB/PAS) or used for immunohistochemical analysis. Pathological findings were graded according to separate inflammation and demyelination scores. Inflammation score: 0, no inflammation present; +, rare (1–3) inflammatory infiltrates/average whole section; ++, moderate numbers (3–10) of inflammatory infiltrates/section; +++, widespread parenchymal infiltration by inflammatory cells, with numerous large confluent lesions. Demyelination score: 0, no demyelination; +, rare (1–3 lesions/section) foci of demyelination; ++, moderate (3–10 lesions/section) demyelination; +++, extensive demyelination with large confluent lesions.

4.3 Fractionation and purification of antibodies from immune *C. jacchus* sera

Sera were collected from each animal at euthanasia, and stored at –20°C until use. The respective fractions of serum antibodies with binding specificities for linear peptide or conformational epitopes were separated by affinity chromatography. Sera or pools of sera from animals in groups I and II were repeatedly passed over columns containing a mixture of the 11 20mer overlapping peptides spanning MOG aa 1–120 (pepMOG) covalently linked to Sepharose. Bound material containing the MOG peptide-reactive fraction (anti-MOG-P) was eluted with glycine buffer pH 2.5, immediately brought to neutral pH with 1 M Tris buffer (pH 8.0) and extensively dialyzed against PBS. Thus, in these experiments antibody reactivity found in follow-through fractions (if present) could not represent any epitope of MOG directed against a linear feature, and was considered to represent anti-MOG-C.

The binding characteristics of all eluted and follow-through fractions were analyzed by ELISA. Anti-MOG-C, if present, were further affinity-purified by passing pepMOG column follow-through fractions over Sepharose columns containing covalently linked rMOG_{1–125}, followed by elution, neutralization and dialysis as described above. In addition to characterization of fine specificity by ELISA, the ability of purified anti-MOG-P and anti-MOG-C to bind to native marmoset MOG *in situ* was determined by immunohistochemistry as described below using antibody fractions biotinylated with a sulfo-NHS biotinylation reagent following the manufacturer's instruction (Pierce). Unreacted sulfo-NHS biotin was removed by extensive dialysis against PBS.

4.4 Epitope specificity

Epitope specificities of whole unfractionated sera, fractionated sera or affinity-purified antibodies were determined by ELISA. Plastic wells (maleic anhydride plates; Pierce) were coated with rMOG_{1–125} or MOG-derived 20mer peptides. Control wells contained no antigen, the recombinant GST from *E. coli*, and the (His)₆ C-terminal peptide of rMOG_{1–125}. Wells were blocked with PBS containing 0.05% Tween-20 (PBS-T) and 3% bovine serum albumin, and the following samples were added in blocking buffer and incubated for 1 h at 37°C: (1) whole immune serum, 1:200; (2) 3 µg/ml of affinity-purified anti-MOG-P antibodies; (3) group I (rMOG_{1–125}-), or group II (MOG peptide-immune) sera depleted of anti-MOG-P antibodies, 1:200. Next, a horseradish peroxidase-labeled anti-monkey IgG (A0170; Sigma, St. Louis, MO) was added, and after incubation for 1 h, wells were developed with tetramethylbenzidine (Pierce) and read at 450 nm.

4.5 Immunohistochemistry

Sections of *C. jacchus* brain were deparaffinized, hydrated and treated with a citrate-based antigen-unmasking solution (Vector Labs, Burlingame, CA) at high temperature for 20 min. Endogenous peroxidase activity was blocked by incubation of sections in 0.3% H₂O₂ in methanol for 30 min. Sections were blocked with 5% normal goat serum (Sigma) in PBS-T for 1 h at 37°C, washed with PBS-T, and incubated with the following primary antibodies in blocking buffer: (1) mouse anti-human C9neo (IgG1, Novocastra; 1:25) for staining of the terminal membrane attack complex; (2) mouse anti-human HAM56 (IgM, Accurate Chemicals; 1:20), pan-macrophage/microglia marker; (3) mouse anti-human IgG (IgM, DAKO; 1:25). After incubation for 1 h at 37°C and washes with PBS-T, the appropriate biotinylated secondary antibodies were applied and incubated for another hour at 37°C [rabbit anti-mouse IgG1 (Zymed); goat anti-mouse IgM (Vector)]. Slides were rinsed again, incubated with the Vectastain Elite ABC Kit (Vector) and stained with 3,3'-diaminobenzidine (Vector). All slides were counterstained with hematoxylin and permanently mounted. Biotinylated anti-MOG-P (from rMOG_{1–125}- and MOG peptide-immune animals, 7 µg/ml and 20 µg/ml, respectively) and anti-MOG-C (10 µg/ml) were used to measure their ability to bind to native, full-length MOG expressed *in situ* by oligodendrocytes in marmoset CNS.

4.6 T cell proliferative responses

Antigen-specific T cell proliferative responses were measured in PBMC obtained at euthanasia by centrifugation, using a standard 3-day [³H]thymidine incorporation assay with 10 µg/ml of rMOG_{1–125} or MOG-derived peptides. Stimulation indices were calculated as the ratio of counts in stimulated/control (no antigen) wells.

4.7 Passive antibody transfers

A total of six animals were immunized with 2 mg MBP emulsified in CFA as described previously^[3], and after the development of MBP-specific antibody responses (37–43 days post-immunization, determined by ELISA) received by i.v. injection the following antibody preparations: (1) the whole rMOG-reactive antibody fraction from rMOG-immune *C. jacchus* sera obtained by immuno-affinity chromatography over rMOG columns as described above (anti-rMOG-P/C, 250 µg/animal, *n*=2); (2) the peptide-reactive fraction from rMOG-immune sera obtained by affinity purification over MOG peptide columns (anti-MOG-P, 125 µg/animal, *n*=2); (3) Igκ from naive marmoset sera obtained by affinity purification over protein L columns (protein L binds to κ light chains of all antibody isotypes) (naive antibodies, 153–180 µg/animal, *n*=2). All animals were euthanized 2–7 days after the passive antibody transfer and their tissue processed for histology as described above. Persistence of anti-MOG antibodies in peripheral blood was confirmed by ELISA (data not shown).

4.8 Statistics

The following parameters were analyzed for each animal of groups I (*n*=4) and II (*n*=9): (1) total lesion load, by counting the number of lesions in 20–24 sections covering the entire neuraxis; (2) immunohistochemical patterns of staining, by examining 2–4 sections of brain and spinal cord from group I (*n*=4, 24–82 lesions per animal) and all the sections containing lesions from animals in group II. We compared the percentages of lesions positive for each marker, with positivity defined as follows: HAM56, > ten stained cells/lesion; IgG (cellular distribution, B cells), > two stained cells/lesion in the immediate perivascular vicinity; IgG (parenchymal distribution), clearly positive staining above background along fibers within lesions and not associated with cells; C9neo, clearly positive staining above background. Comparisons between the two groups were performed using a two-tailed, unpaired Student's *t*-test.

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