Antibody cross-reactivity between casein and myelin-associated glycoprotein results in central nervous system demyelination

Rittika Chunder[a,b], Alicia Weier[a,b], Hannah Mäurer[b], Nicolas Luber[b], Michael Enders[a,b], Gabriele Luber[c], Thorsten Heider[d], Alfred Spitzer[e], Sabine Tacke[b], Janine Becker-Gotot[f], Christian Kurts[f], Radhika Iyer[g], Peggy P. Ho[h], William H. Robinson[g], Tobias V. Lanz[g,i], and Stefanie Kuerten[a,1]

[a]Institute of Neuroanatomy, Medical Faculty, University of Bonn, 53115 Bonn, Germany; [b]Institute of Anatomy and Cell Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany; [c]Practice for Neurology, Psychiatry and Psychotherapy, 90491 Nürnberg, Germany; [d]Clinic for Neurology, Klinikum St. Marien Amberg, 92224 Amberg, Germany; [e]Neurological Medical Practice Erlangen, 91054 Erlangen, Germany; [f]Institute of Molecular Medicine and Experimental Immunology, Rheinische-Friedrichs-Wilhelms University of Bonn, 53127 Bonn, Germany; [g]Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305; [h]Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305; and [i]Department of Neurology, Medical Faculty Mannheim, University of Heidelberg, 68167 Mannheim, Germany

Edited by Lawrence Steinman, Stanford University, Stanford, CA; received September 15, 2021; accepted January 26, 2022

Multiple sclerosis (MS) is a neuroinflammatory demyelinating disease of the central nervous system (CNS) with a high socioeconomic relevance. The pathophysiology of MS, which is both complex and incompletely understood, is believed to be influenced by various environmental determinants, including diet. Since the 1990s, a correlation between the consumption of bovine milk products and MS prevalence has been debated. Here, we show that C57BL/6 mice immunized with bovine casein developed severe spinal cord pathology, in particular, demyelination, which was associated with the deposition of immunoglobulin G. Furthermore, we observed binding of serum from casein-immunized mice to mouse oligodendrocytes in CNS tissue sections and in culture where casein-specific antibodies induced complement-dependent pathology. We subsequently identified myelin-associated glycoprotein (MAG) as a cross-reactive antigenic target. The results obtained from the mouse model were complemented by clinical data showing that serum samples from patients with MS contained significantly higher B cell and antibody reactivity to bovine casein than those from patients with other neurologic diseases. This reactivity correlated with the B cell response to a mixture of CNS antigens and could again be attributed to MAG reactivity. While we acknowledge disease heterogeneity among individuals with MS, we believe that consumption of cow's milk in a subset of patients with MS who have experienced a previous loss of tolerance to bovine casein may aggravate the disease. Our data suggest that patients with antibodies to bovine casein might benefit from restricting dairy products from their diet.

antibodies | casein | cross-reactivity | multiple sclerosis | myelin-associated glycoprotein

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) in genetically susceptible individuals. It is a clinically heterogenous disease, with the classic variants of MS being relapsing–remitting MS (RRMS) which accounts for the majority of all MS cases, secondary progressive MS which follows up to 70% of RRMS cases after 10 y of disease onset, and primary progressive MS which is the rarer variant (1, 2). Although there is a definite pathogenic role of inflammation in MS (3, 4), the precise roles played by the different cell populations remain controversial. Moreover, another level of complexity is added by the diverse mechanisms driving the development of the disease not just between the different subtypes but also between patients within one type of MS (5, 6). Evidently, the pathophysiology of MS—which is both complex and incompletely understood—involves genetic and environmental factors that interact to disrupt immunological self-tolerance to components of CNS myelin (7). Specific patterns in the epidemiology of MS suggest environmental determinants, including insufficient sun exposure, smoking, and dietary intake (8), play an important role in disease initiation and modulation. Mounting evidence implies that food habits and the gut microbiome, in particular, influence the disease course of MS (9, 10). While the impact of gut microbiota as a potential triggering factor in MS has been widely discussed (11–13), how certain dietary factors may be related to brain autoimmunity remains poorly investigated.

One of the reports as early as in the 1970s suggested milk consumption as an etiological factor in MS (14). Furthermore, epidemiological evidence from another study indicated a correlation between cow's milk consumption and the prevalence of MS (15). Yet, how milk consumption might trigger autoimmune responses to CNS antigens and contribute to disease development remains obscure. Stefferl et al. (16) have previously reported sequence homologies between a CNS myelin-specific

Significance

Multiple sclerosis (MS) is the most prevalent autoimmune disease of the central nervous system (CNS), leading to irreversible deficits in young adults. Its pathophysiology is believed to be influenced by environmental determinants. As far back as the 1990s, it had been suggested that there is a correlation between the consumption of cow's milk and the prevalence of MS. Here, we not only demonstrate that a high percentage of MS patients harbor antibodies to bovine casein but also that antibody cross-reactivity between cow's milk and CNS antigens can exacerbate demyelination. Our data broaden the current understanding of how diet influences the etiology of MS and set the stage for combining personalized diet plans with disease-modifying treatment strategies.

Author contributions: R.C., C.K., W.H.R., T.V.L., and S.K. designed research; R.C., A.W., H.M., N.L., M.E., S.T., J.B.-G., R.I., P.P.H., T.V.L., and S.K. performed research; G.L., T.H., and A.S. contributed new reagents/analytic tools; R.C., A.W., H.M., M.E., J.B.-G., T.V.L., and S.K. analyzed data; and R.C. and S.K. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: stefanie.kuerten@uni-bonn.de.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2117034119/-/DCSupplemental.

Published March 2, 2022.

antigen, namely, myelin oligodendrocyte glycoprotein (MOG), and butyrophilin (BTN), a protein of milk fat. In a follow-up study, it was further demonstrated that antibodies specific to the extracellular domain of MOG cross-reacted with bovine milk protein BTN in a mouse model of MS (17).

In the present study, we provide evidence of how an immune response against casein—another common protein component from bovine milk—can aggravate the demyelinating pathology of MS as a result of sequence homology with myelin-associated glycoprotein (MAG). Furthermore, our data suggest an antibody- and complement-dependent mechanism.

Together, these results identify how consumption of milk and milk products may exacerbate the autoimmune response in MS.

Results

Mice Immunized with Bovine Casein Show Spinal Cord Pathology in a Time-Dependent Manner. Mice immunized with bovine casein were killed at different time points (i.e., day 13, day 20, and day 40) after immunization (n = 6 to 9 in each cohort). Mice that had been immunized with either α-lactalbumin or β-lactoglobulin were killed on day 40. Every cohort was observed daily for the development of any clinical signs like cramping and hind limb weakness. While none of the mice immunized with noncasein milk antigens developed any signs of physical impairment, casein-immunized mice exhibited a range of symptoms that were broadly grouped under "weakness" (including grabbing and gait abnormalities and weakness of the limbs) and "disorientation" (including running around in circles, moving backward, or hesitant movement). A more detailed list of the signs and symptoms, the scoring system, and the clinical score progression of the different cohorts of casein-immunized mice are shown in Table 1 and *SI Appendix*, Fig. S1.

Subsequently, sections of the lumbar region of the spinal cord from each mouse of the different cohorts were processed for electron microscopy (EM) (Fig. 1*A*). Prominent changes were observed only in those cohorts that had been immunized with casein (Fig. 1 *A* and *B*). Furthermore, time kinetics studies of the different casein-immunized cohorts revealed progressive myelin degeneration characterized by swelling of the myelin sheaths with the myelin lamellae diverging widely from each other. In some mice, particularly those killed at day 40, there was severe detachment of the myelin sheath from its axon, indicating maximum pathology (Fig. 1*C*).

To focus on cellular infiltrates that could possibly explain the EM pathology, we subsequently performed hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining for infiltrating T and B cells on five to eight sections per spinal cord tissue per mouse of every casein-immunized cohort. However, despite the identification of sites of demyelination by EM, no corresponding immune cell clusters or perivascular infiltrates in the spinal cord of casein-immunized mice at day 40 were observed (*SI Appendix*, Fig. S2). These findings indicated that casein immunization can provoke demyelinating pathology in a time-dependent manner in the absence of any obvious immune cell infiltration.

Pathology Is Accompanied by IgG Deposition in Casein-Immunized Mice. The lack of cellular infiltration spatially associated with sites of spinal pathology in casein-immunized mice suggested that the observed pathology was not caused by a direct effect of inflammatory T or B cells, but, possibly, by an antibody-mediated mechanism.

Accordingly, we stained spinal cord sections of mice killed at the three time points after immunization to visualize immunoglobulin (Ig) deposition. While we observed only minimal amounts of IgG around the axonal tracts of the spinal cord in casein-immunized mice killed at earlier time points, there was evidence of marked IgG deposition in the spinal cord of animals killed at day 40 (*SI Appendix*, Fig. S3*A*).

To confirm that the pathology observed in casein-immunized mice was mediated by an antigen-specific Ig, IgHEL mice (n = 6) were similarly immunized with casein and killed 40 d after immunization. In these mice, B cells express a transgenic B cell receptor for hen egg lysozyme (HEL) and are therefore unable to generate Igs of any other specificities (18, 19). Accordingly, there were no signs of myelin pathology in casein-immunized IgHEL mice when analyzed by EM compared to casein-immunized wild-type (WT) mice (P = 0.0022; Mann–Whitney U test) (*SI Appendix*, Fig. S3*B*).

We also determined the casein-specific serum IgG and IgM titers in both IgHEL and WT mice. IgM was used as additional control Ig isotype, as the IgHEL mice produce only IgM. In accordance with the IHC data, IgG titers to casein were significantly higher in mice killed 40 d after casein immunization than in mice killed on day 20 (P < 0.0001; Mann–Whitney U test) (*SI Appendix*, Fig. S3*C*). In contrast, we detected no casein-specific IgG titers in IgHEL mice and no casein-specific IgM response in the WT or IgHEL mice (*SI Appendix*, Fig. S3*C*).

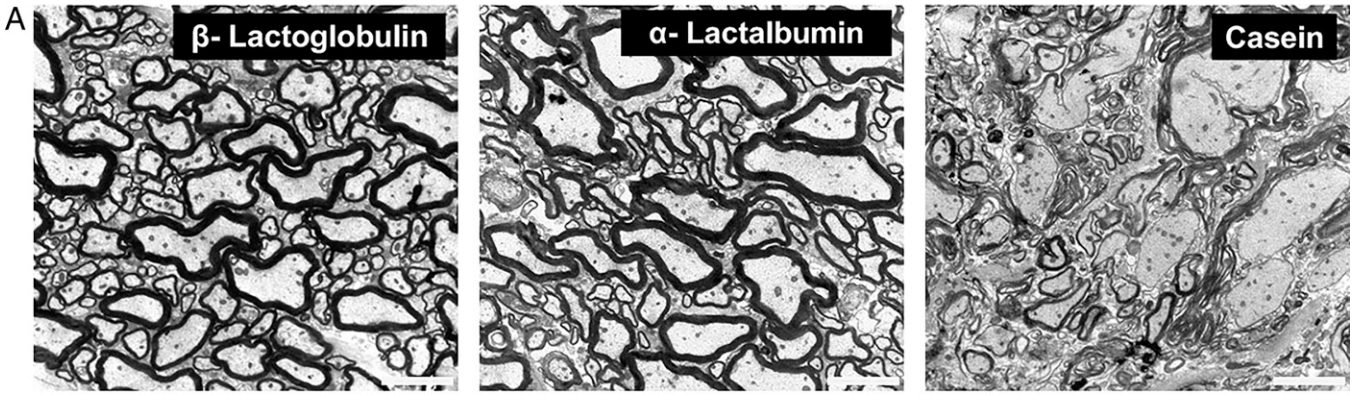
IgG from Casein-Immunized Mice Recognizes Antigens Expressed in the CNS. The occurrence of high titers of IgG to casein in casein-immunized mice prompted us to investigate binding of serum IgG from these mice to CNS tissue. We hypothesized two possibilities: either direct binding of serum IgG to endogenously expressed mouse casein or cross-reactivity to other antigenic structures in the CNS, resulting in spinal cord pathology observed in mice immunized with bovine casein.

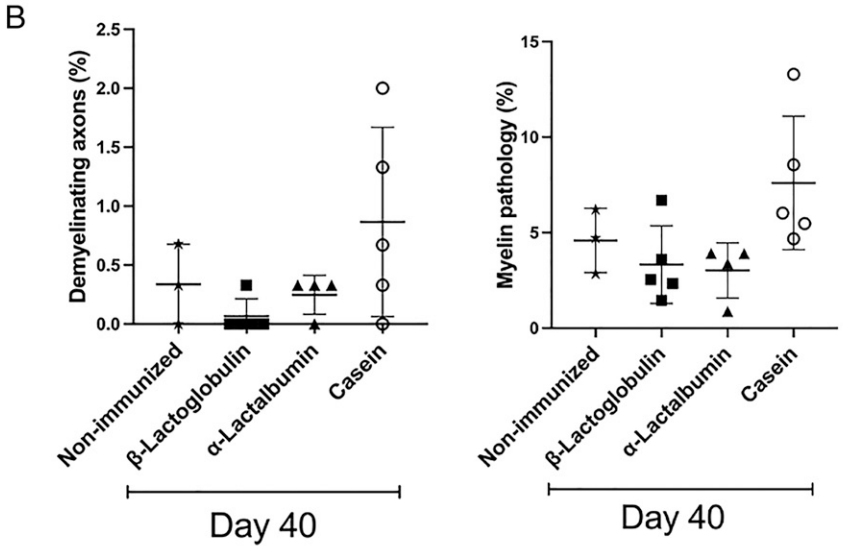
To test the first possibility, we checked the expression of casein by CNS-resident cells. Although there are five functional casein genes in the common house mouse (*Mus musculus*) (20), we looked only at the expression of *CSN1S1*, *CSN2*, and *CSN3* (encoding αS1-, β-, and κ-casein, respectively), because these

Table 1. Clinical scoring strategy in mice immunized with milk antigens

Score	Balance score	Orientation score	Other signs
0	No problems balancing	No signs of spatial disorientation	
0.5	Difficulties in grabbing the cage mesh	No signs of spatial disorientation	
1		Signs of spatial disorientation	
1.5	Slipping off the cage mesh		
2.0			
2.5		Moving around in circles or moving backward	
3.0	Falling off the cage mesh		Cramping

For the balance test, mice were allowed to walk on their cage mesh while the mesh was gently rotated in a 360° manner. Mice that displayed no signs of balance problems were able to turn along as the mesh was rotated. Those which slipped, were hesitant with their grabbing, or fell were scored accordingly. Mice also displayed signs of disorientation, when they were placed on the cage mesh, that ranged from running around in circles to moving backward. A combination of the disorientation problems and the balance problems was used for the complete scoring of mice.





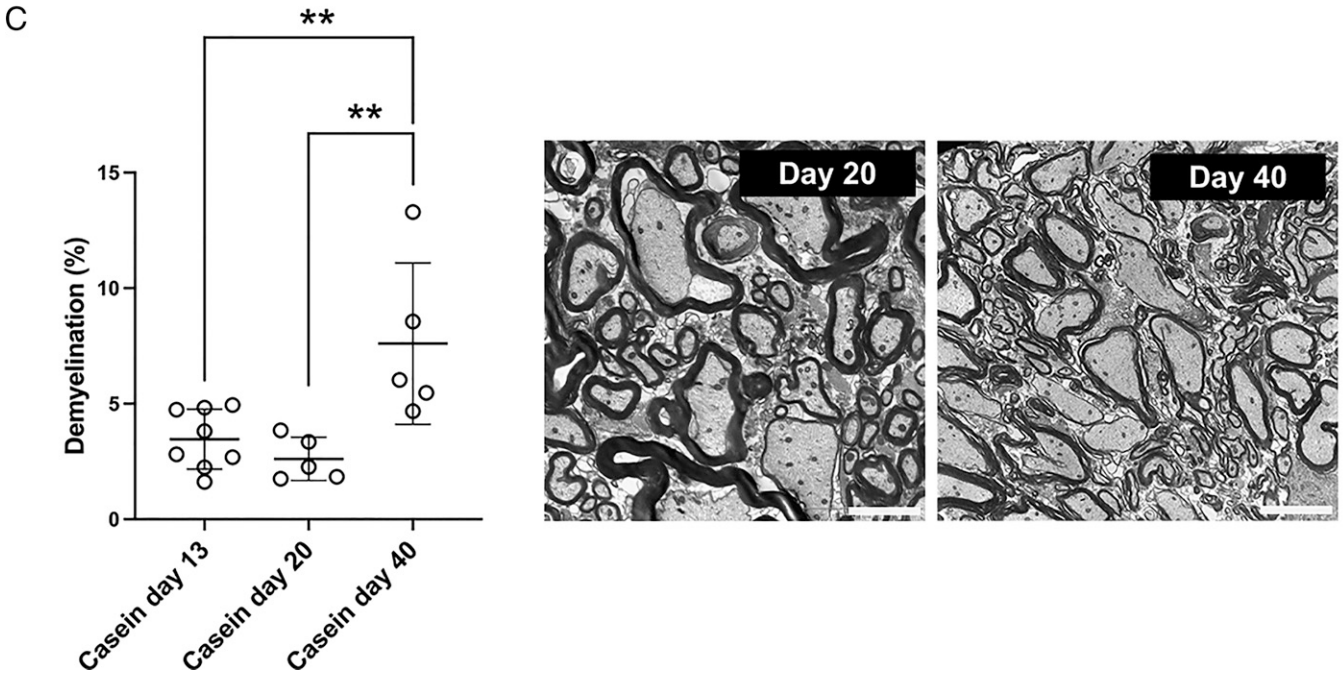


Fig. 1. Spinal cord pathology in casein-immunized WT B6 mice is time-dependent. (*A*) Representative EM images of lumbar regions of the spinal cord of casein-immunized, α-lactalbumin–immunized, and β-lactoglobulin–immunized WT mice. (Scale bar, 10 μm.) (*B*) Myelin pathology in the different cohorts of mice (killed at day 40). (*C*) Time-dependent exacerbation of demyelination (***P* = 0.0045 and ***P* = 0.0082 for groups day 40 vs. d 20 and day 40 vs. d 13, respectively; one-way ANOVA). Each data point represents the mean ± SD for each mouse. Myelin pathology was quantified by counting the number of demyelinating axons per mouse (from a total of 10 representative images per spinal cord per mouse) as a proportion of the total number of axons. (Scale bar, 10 μm.)

are expressed in cattle breeds originating from Europe. No expression of the casein genes was detected at the messenger RNA level in the mouse CNS under physiological conditions as shown by endpoint and qPCR (Fig. 2*A* and *SI Appendix*, Table S1, respectively).

As this argued against caseins as an antigenic target, we investigated the possibility of alternative target(s) for the binding of serum IgG from casein-immunized mice. As previously discussed, IgG titers to casein were significantly higher in mice killed 40 d versus 13 and 20 d after immunization, which was also associated with more pronounced spinal cord pathology in the mice killed at the later time point. We therefore proposed a "cross-reactive" IgG response in casein-immunized mice as the etiologic agent of the aggravated pathology.

Serum from casein-immunized mice was incubated on spinal cord tissues from nonimmunized B6 mice, revealing an

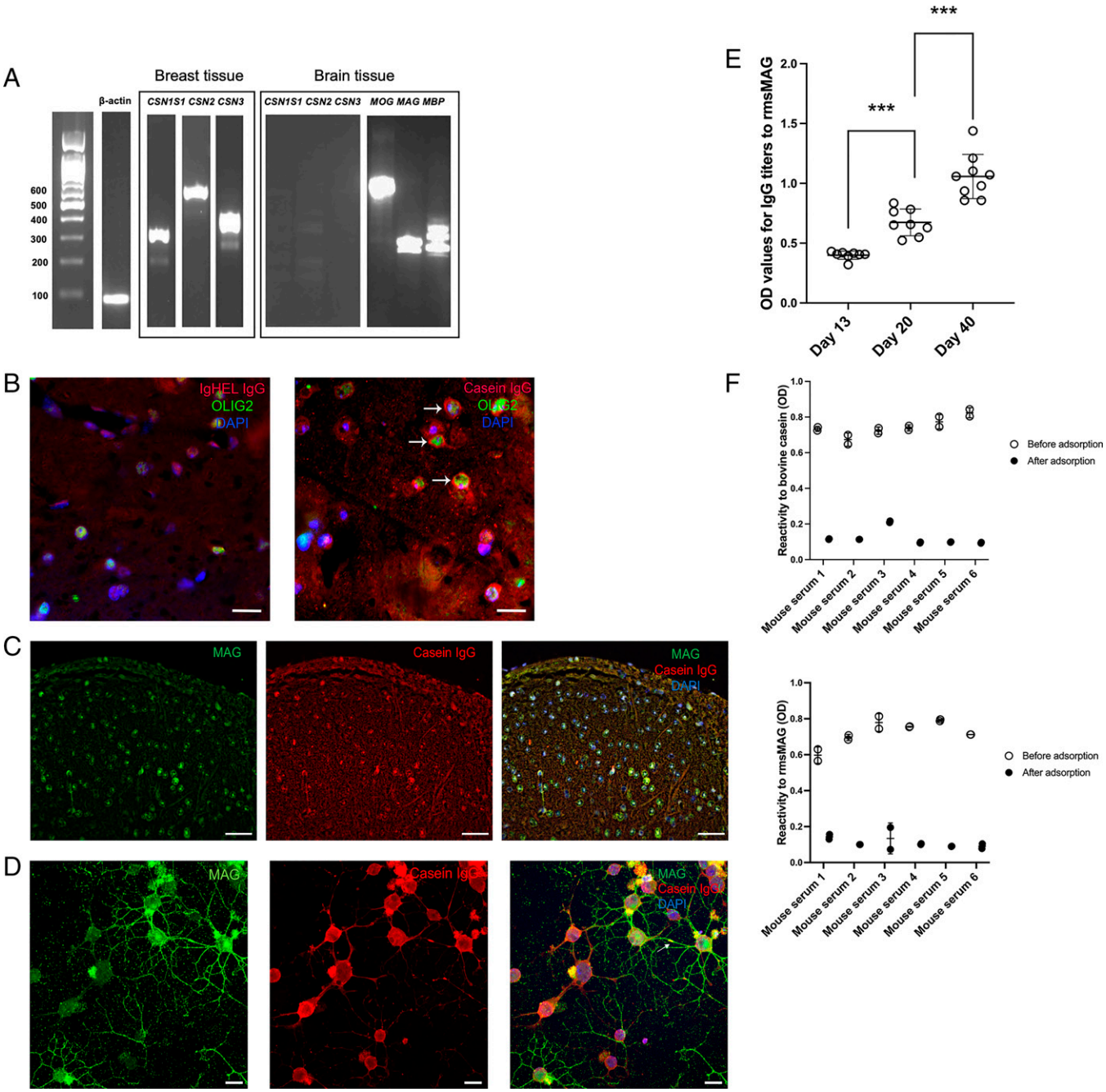


Fig. 2. Serum IgG from bovine casein-immunized mice recognizes MAG expressed by oligodendrocytes. (*A*) Total RNA was extracted from whole brain tissue lysate of 8- to 9-wk-old healthy WT B6 mice ($n = 3$), and RT-PCR was conducted for the detection of casein genes (*CSN1S1*, *CSN2*, and *CSN3*), as well as an array of CNS-related genes. Lysate from breast tissue was used as positive control for the casein genes. (*B*) Incubation of serum IgG from casein-immunized mice on murine spinal cord sections, counterstaining for $OLIG2^+$ oligodendrocytes. (Scale bars represent 10 μm.) (*C*) Colocalization of MAG and anti-casein sera on spinal cord sections from mice. (Scale bars, 50 μm.) (*D*) Double staining of the Oli-Neu cell line with an anti-MAG antibody and purified Ig from casein-immunized WT mice. (Scale bars, 10 μm.) (*E*) The rmsMAG reactivity of serum samples from casein-immunized mice killed on days 13, 20, and 40 analyzed by ELISA. Mean (± SD) OD values are shown. ***$P < 0.0001$. (*F*) Adsorption analysis of serum from casein-immunized WT mice with bovine casein. Mean OD ± SD values for binding to recombinant MAG and casein are shown.

array of staining patterns. We detected homogenous cytoplasmic staining of some cells and a more granular and dotted staining of others. This range of antigen–antibody binding patterns was, however, only seen with serum from casein-immunized mice that had been killed at the later time point (*SI Appendix*, Fig. S4).

To further characterize these individual patterns, the reactivity of serum IgG from mice killed at day 40 to murine spinal cord tissue was analyzed. Although there was a certain degree of heterogeneity in the staining patterns, serum from three out of six mice (3/6) bound to oligodendrocyte transcription factor-2 $(OLIG2)^+$ cells in spinal cord sections. OLIG2 was used as a marker to screen for oligodendrocytes. In contrast, no reactivity to glial fibrillary acidic protein $(GFAP)^+$ astrocytes was detected. This binding of serum IgG to $OLIG2^+$ cells was our first line of evidence indicating a "cross-reactive response" to oligodendrocytes (identified as $OLIG2^+$ cells) in at least half of the casein-immunized mice that were killed at a later time point (Fig. 2*B*).

In order to identify a specific cross-reacting antigen on oligodendrocytes, we costained murine spinal cord with MAG. MAG was selected as a potential cross-reactive target because of the shared sequence homologies between bovine αS1-casein, β-casein, κ-casein, and mouse MAG. Regions of amino acid sequence homologies between mouse MAG and the different bovine caseins are detailed in *SI Appendix*, Table S2. Accordingly, as shown in Fig. 2*C*, a colocalization of the transmembrane protein MAG and sera from casein-immunized mice was observed.

To further underscore the binding of anti-casein IgG to oligodendrocytes on a cellular level, specifically, to its cross-reactive antigen MAG, purified Ig from pooled serum of an additional cohort of casein-immunized mice ($n = 10$) killed on day 60 was tested on Oli-Neu cells, a murine oligodendroglial precursor cell line (21). Oli-Neu cells were cultured for 48 h in the presence of PD174265, which is a selective inhibitor of epidermal growth factor receptor that has been shown as a differentiating agent in these cells (22). When incubated with anti-MAG antibody and purified Ig from casein-immunized mice, differentiated Oli-Neu cells revealed a staining colocalization as shown in Fig. 2*D*.

Following these sets of observations, we tested the reactivity of casein-specific serum samples to recombinant mouse (rms)MAG. Similar to the increasing trend of IgG titers to bovine casein, we observed that the titers to rmsMAG also increased over the time course of the casein immunization, from lower titers at day 13 to high titers in mice killed at the later time point, that is, 40 d after immunization (Fig. 2*E*).

In a last set of experiments to confirm cross-reactivity of casein-specific antibodies with MAG, we adsorbed sera from casein-immunized mice killed after 40 d to bovine casein and measured the degree of IgG binding (using an enzyme-linked immunosorbent assay [ELISA]) when the sera were exposed to casein and rmsMAG before and after adsorption. Casein-adsorbed serum presented significantly reduced optical density (OD) values to both casein ($P < 0.0001$; paired t test) and MAG ($P < 0.0001$; paired t test) (Fig. 2*F*).

Casein-Specific Antibodies Are Cytotoxic to Oligodendrocytes and Cause Morphological Changes in the Presence of Complement. Having identified that spinal cord pathology in casein-immunized mice was accompanied by IgG deposition and serum from these mice cross-reacted with myelin antigens, we further explored the mechanism by which these antibodies can mediate damage. Antibody-induced pathology can depend on a number of mechanisms, including activation of the complement system. Accordingly, we tested the pathogenicity of IgG from mice immunized with casein on differentiated Oli-Neu cells in the presence and absence of 1% rat serum as the source of complement. A lactate dehydrogenase assay was performed on the supernatant of differentiated Oli-Neu cells in the presence of IgG purified from casein-immunized mice to determine the cellular toxicity levels to oligodendrocytes. As shown in *SI Appendix*, Fig. S5*A*, casein-specific IgG in the presence of 1% rat serum was significantly more cytotoxic to Oli-Neu cells compared to serum only ($P = 0.0052$; one-way ANOVA) or the combination of random IgG and serum ($P = 0.02$; one-way ANOVA). Furthermore, to show whether casein antibody-mediated complement-dependent oligodendrocyte cell death followed an apoptotic pathway, we conducted a flow cytometric staining of cells treated with anti-casein IgG and 1% rat serum using annexin V and propidium iodide. Results, as shown in *SI Appendix*, Fig. S5*B*, indicate that Oli-Neu cells underwent apoptosis as a mechanism of cell death in the presence of complement and casein-specific IgG.

Additionally, immunocytochemistry (ICC) done on PLP^+ differentiated Oli-Neu cells (also positive for MAG) in the presence of casein IgG and 1% rat serum revealed morphological changes, including fewer and shorter branchings of the oligodendroglial processes as well as cytoplasmic shrinkage. Cells that were treated with a combination of rat serum and a random anti-mouse IgG did not show any difference in their morphology when compared to medium-only control (*SI Appendix*, Fig. S5*C*). Quantitative analysis of the ratio between cell cytoplasm and nucleus demonstrated that there was a significant amount of cytoplasm shrinkage ($P = 0.0082$; unpaired t test) in oligodendrocytes treated with purified casein IgG and complement (*SI Appendix*, Fig. S5*C*). Together, the data demonstrate that IgG purified from casein-immunized mice that binds oligodendrocytes (shown in Fig. 2*D*) in the presence of complement not only is cytotoxic to the cells but also causes observable changes to the cell morphology.

We also repeated the experiments using primary oligodendrocyte precursor cells (OPCs) which were first checked for their differentiation into oligodendrocytes using neural/glial antigen 2 (NG2) and MOG as maturation markers on culture days 1, 6, and 14 (*SI Appendix*, Fig. S6*A*). OPCs that were in culture with platelet-derived growth factor (PDGF)-AA and fibroblast growth factor (FGF)-2 for 1 or 6 d revealed a significantly higher expression of NG2 compared to day 14 ($P = 0.0065$ for day 1 vs. d 6 and $P = 0.0021$ for day 6 vs. 14; unpaired t test) with an opposite effect observed for MOG comparing days 1 and 6 ($P < 0.0001$; unpaired t test). Additionally, OPCs were treated either with casein- or MOG-specific IgG and 1% rat serum, with casein-specific IgG alone, or with 1% serum alone for 24 h on culture day 1 vs. 14. NG2 staining of these differently treated cells revealed morphological changes between the conditions as shown in *SI Appendix*, Fig. S6*B*. The data indicate that casein-specific IgG preferentially affects mature oligodendrocytes in a complement-dependent manner.

In addition to our in vitro cell culture experiments, we immunized a new cohort of WT B6 mice with casein ($n = 12$). For transient depletion of complement, half of these mice received a single injection of cobra venom factor (CVF) on day 10 after casein immunization. A control group ($n = 6$) received phosphate-buffered saline (PBS) only. On day 6 following treatment with CVF or PBS, mice were killed, and the lumbar regions of their spinal cords were analyzed by EM for demyelination and axonal damage. CVF-injected mice showed less spinal cord pathology, characterized by a trend toward fewer numbers of demyelinated axons and significantly less axonal pathology compared to PBS-injected mice (*SI Appendix*, Fig. S7). The results highlight the involvement of the complement cascade in casein-immunized mice. Taken together, our results provide evidence of a pathogenic role for the humoral arm of a casein-specific immune response generated in the WT mice.

Antibody Repertoire Analysis Confirms Cross-Reactivity between Casein- and MAG-Specific Antibodies. As another approach to investigate the B cell response against casein and cross-reactivity to MAG, an additional group of mice was immunized twice with casein ($n = 4$), and a control group was immunized twice with HEL ($n = 3$). Plasmablasts were sorted by flow cytometry 10 d after the second immunization, and the single-cell antibody repertoire was sequenced. Plasmablast counts did not differ significantly between the groups (casein: mean [SD] 0.17% [0.04%]; HEL: mean [SD] 0.24% [0.05%]). Repertoire analysis, however, revealed that casein potently induced class switching to IgG, whereas HEL generated an IgA-dominated B cell response (Fig. 3 *A* and *B*; and *SI Appendix*, Fig. S8*A*). Clonality and mutation counts were elevated in the respective dominant Ig classes (Fig. 3 *C–F* and *SI Appendix*, Fig. S8*B*). The repertoire in the casein group was skewed toward the preferential use of a few heavy chain (HC) V genes (IGHV), most significantly IGHV9-3 and IGHV8-8 (*SI Appendix*, Fig. S8*C*).

To identify anti-casein antibodies that might cross-react with MAG, we aligned all HC and light chain (LC) complementarity-determining regions 3 (CDR3), and then identified clusters that contained sequences from casein-immunized but not from HEL-immunized or nonimmunized mice (Fig. 3*G* and *SI Appendix*, Fig. S8*D*). A total of 17 representative antibody sequences from 14 casein clusters were selected and expressed as recombinant monoclonal antibodies (mAbs) (Fig. 3 *G*, *i–iv*). Three of the 17 expressed mAbs bound strongly to casein (Fig. 3*H*) and two of those showed significant cross-reactivity to recombinant human MAG (rhuMAG) (Fig. 3*I*). The cross-reactive antibodies originated in several mice, indicating that casein/MAG cross-reactivity might be a commonly occurring phenomenon. The antibodies shared the same HC with high similarity in all three CDR regions, indicative of binding to the same epitope (*SI Appendix*, Fig. S8*E*). The LCs were substantially different (*SI Appendix*, Fig. S8*F*), suggesting that specificity was mostly determined by the HC.

Patients with MS Harbor Bovine Casein-Specific Antibodies and B Cells. To translate our findings to humans, we investigated whether patients with MS have a high titer of antibodies to bovine casein. We selected a group of 39 patients with MS and 23 patients with other neurological diseases (ONDs) to test for their serum IgG reactivity to casein (Table 2 and *SI Appendix*, Tables S3 and S4). Mean IgG titers to casein in patients with MS were significantly higher than in patients with ONDs ($P = 0.032$; Mann–Whitney U test) (Fig. 4*A*).

To confirm the specificity of serum IgG to casein in patients with MS, we performed an adsorption assay using a selected number of serum samples ($n = 10$) and tested the IgG titers before and after adsorption on bovine casein. Although our selected cohort of MS serum samples had varying IgG titers to casein before adsorption, an ELISA showed that all sera had minimal baseline reactivity to casein after adsorption ($P = 0.0008$; paired t test) (Fig. 4*B*). This indicated that a subset of patients with MS harbor casein-specific antibodies. To provide evidence of cross-reactivity of anti-casein antibodies with MAG in patients with MS, we used the bovine casein-adsorbed serum samples ($n = 10$) and tested their reactivity to rhuMAG by ELISA before and after adsorption. In general, all patients with MS from our selected cohort displayed low levels of humoral immunity to MAG in comparison with casein. Although the serum samples showed inconsistent results, an overall significant decrease was observed in OD values (corresponding to the IgG titers) after adsorption with bovine casein ($P = 0.0142$; paired t test).

To further complement our data, we tested the CNS- and casein-specific B cell response in patients with MS ($n = 45$) and those with ONDs ($n = 35$) (Table 2 and *SI Appendix*, Tables S3 and S4) by performing B cell enzyme-linked immunosorbent spot (ELISPOT) assays. Polyclonally stimulated peripheral blood mononuclear cells (PBMCs) from patients were seeded onto ELISPOT assay plates in which B cells producing antibodies to CNS antigens and bovine casein could be detected. While the number of casein-specific B cell spots/patient was similar in the two patient groups, the number of patients positive for casein-specific B cells was higher in the MS (19/45; 42%) than in the OND group (10/35; 28%) ($P = 0.0379$; χ^2 test). In addition, a Pearson's correlation coefficient of $r = 0.605$ ($P < 0.0001$) was obtained when the numbers of CNS- and casein-specific B cell spots were plotted against each other for the patients in the MS group (Fig. 4*C*). Taken together, these findings corroborated the link between casein- and MAG-specific antibodies and MS.

Discussion

Alterations of the immune repertoire by environmental and dietary factors have been implicated in the etiology of several diseases with an underlying autoimmune component, including autoimmune uveitis and type I diabetes (10, 23–26). In this study, we propose that an immune response to casein from bovine milk can contribute to the pathology of MS.

The allergenicity and antigenicity of bovine casein has been well documented in different reports (23, 27–29). While oral tolerance to casein may be the default response following gastrointestinal exposure to bovine milk, a breakdown of this tolerance in MS patients can be initiated by disturbance in antigen uptake and presentation of the antigenic epitopes in individuals with a genetic predisposition (30, 31). The exact mechanisms by which oral tolerance is broken are not well understood. However, exposure early in life to opioid peptides from casein, which are a major component of bovine milk (27), could act as a destabilizer for oral tolerance (32, 33). In general, milk proteins are suspected of having the potential to trigger autoimmune responses (16, 34), with humans being the only species that consumes milk products from other species through adulthood.

We speculate that, once tolerance to an otherwise harmless food antigen like casein is broken, it might exacerbate ongoing autoimmunity like in MS, as a result of cross-reactivity to self-antigens (17). In the present study, we have identified MAG as a potential cross-reactive self-antigen, an important myelin component localized in the periaxonal membrane of the CNS and peripheral nervous system (PNS) myelin sheaths. Despite the wider distribution of MAG in Schwann cell membranes than in oligodendroglial membranes, the total amount of MAG in the CNS is much greater than in the PNS (35). This would be an argument for why consumption of bovine milk could specifically aggravate CNS myelin pathology in the event where antibodies against bovine casein cross-react with human MAG. Nonetheless, pathophysiological consequences of drinking milk by MS patients are difficult to predict, as they will be influenced by multiple factors, including an individual's immunological repertoire as well as the health state of the gastrointestinal tract (16). However, it may also be interesting to note that, while one of the patterns of demyelination (pattern II) in MS lesions is characterized by antibody and complement deposition, a second type of pattern, pattern III, can be described by its preferential loss of MAG (36). In our cohort of casein-immunized mice, we have demonstrated that antibodies to casein cross-react with MAG and that the pathology is complement-dependent. One could speculate whether, in MS patients with a loss of tolerance to milk antigens, there is anti-casein antibody-mediated loss of MAG within the lesion.

Our current study has focused on immunization experiments in B6 mice. Typically, in mice susceptible to developing experimental autoimmune encephalomyelitis, immunization with complete

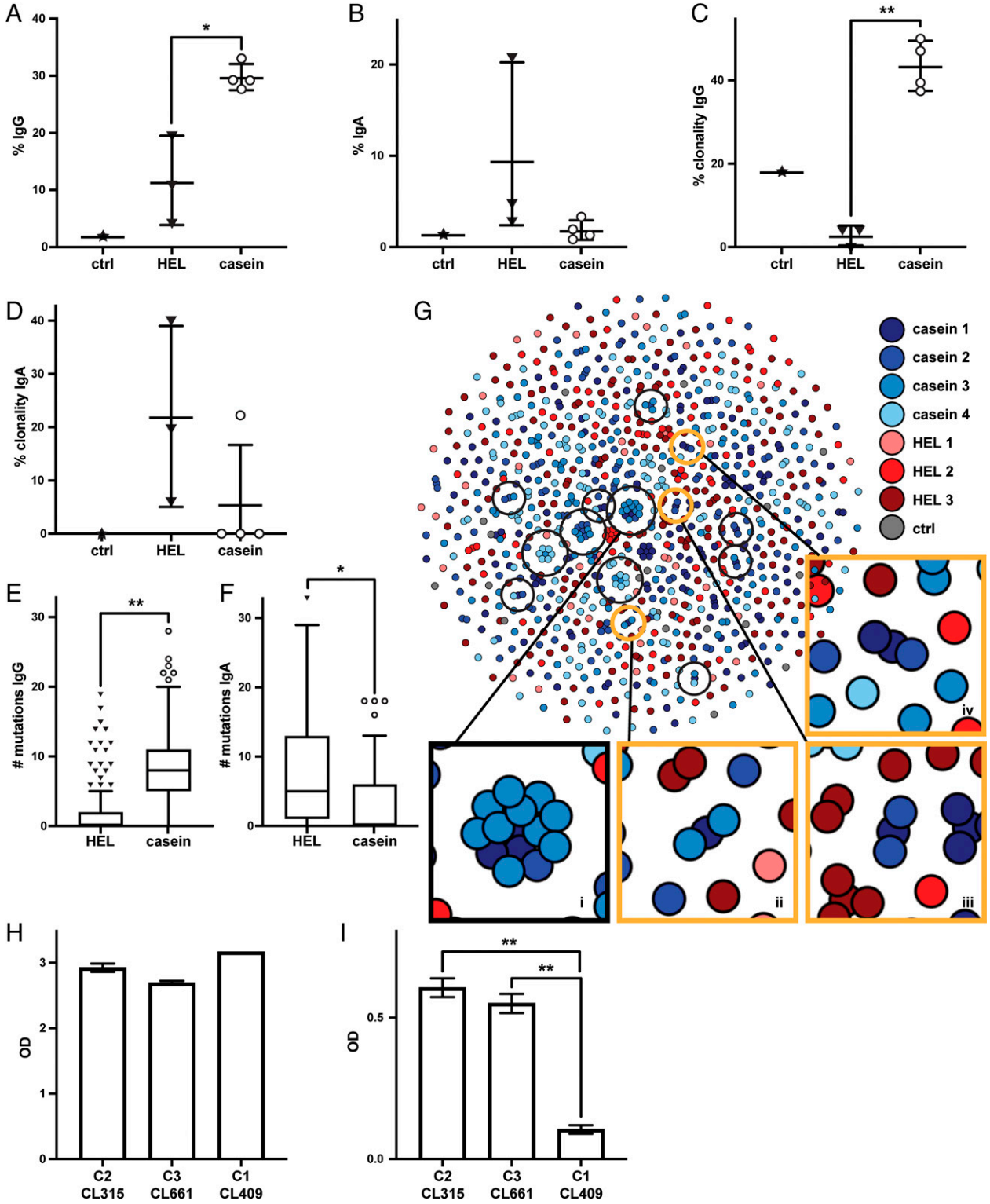


Fig. 3. B cell repertoire analysis and identification of casein/MAG cross-reactive antibodies. (*A–F*) Repertoire analyses, for the groups of casein-immunized ($n = 4$) and HEL-immunized ($n = 3$) mice, and the control mouse ($n = 1$). Number of (*A*) IgG and (*B*) IgA sequences, represented as percentage of all antibody sequences; $*P < 0.05$; Student's *t* test. Clonality of (*C*) IgG and (*D*) IgA sequences; $**P < 0.005$; unpaired *t* test. (*A–D*) Bar and whiskers represent mean ± SD for each group; each data point represents mean of all respective sequences from one mouse. (*E* and *F*) HC and LC V-gene mutation counts in (*E*) IgG-expressing and (*F*) IgA-expressing B cells. Mean ± SD are shown for all sequences of all mice in each group; $*P < 0.05$, $**P < 0.005$; unpaired *t* test. (*G*) Visualization of clustering of HC and LC CDR3 sequences of all IgG and IgA. Blue tones represent antibody sequences from casein-immunized mice, red represent antibody sequences from HEL-immunized mice, and gray represent antibody sequences from control mouse. Circles indicate the clusters that were selected for mAb expression. (*i–iv*) Magnifications of (*i*) the largest cluster, and (*ii–iv*) the clusters containing casein-binding antibodies (*ii*, cluster containing C3_CL661; *iii*, cluster containing C2_315; *iv*, cluster containing C1_CL409). (*H* and *I*) ELISA data of the three highly reactive mAbs reacting to (*H*) casein and (*I*) MAG. Mean ± SD of triplicate measurements are shown from one representative of three independent experiments; $**P < 0.005$; unpaired *t* test.

Table 2. Demographics and disease characteristics of patients

Characteristic	Patients with ONDs	Patients with MS
Total, *n*	35	45
ELISA	23	39
ELISPOT	35	45
Female sex, %	60	67.5
Age, median (range), y	56 (24–88)	48 (20–70)
Time since diagnosis, median (range), y	NA	11 (0.75–33)
EDSS score (range)	NA	2.5 (1–5.5)
Consumption of milk,* median (range), mL/d	42.85 (0–400)	53.35 (0–1,000)
Consumption of cream,* median (range), g/d	0 (0–35)	0 (0–28)
Consumption of cheese,* median (range), g/d	14.28 (0–100)	14.28 (0–71)
ELISPOT casein responders, %†	28	42
ELISA OD value > 1.0, %†	21.7	41.02

EDSS, expanded disability status scale; NA, not applicable.
*Data for 35 patients with ONDs and 39 patients with MS.
†The table indicates values for the ELISA and ELISPOT assays that were both directed against bovine casein.

Freund's adjuvant (CFA) emulsified with different myelin antigens along with pertussis toxin (PTx) overcomes the tolerant/anergic state and induces an inflammatory response in the CNS (37–39). Here we immunized B6 mice with antigens from bovine milk and CFA and Ptx to potentiate a milk antigen-specific proinflammatory/humoral immune response. Serum IgG from mice that were immunized specifically with casein presented a diverse and unique range of immunohistological staining patterns when incubated on murine spinal cord sections or human brain sections. This heterogeneity of response can be explained by the nature of immunization where the immunizing antigens include all casein types (α-, β-, κ-casein), the hydrolyzed products of casein and partially dephosphorylated casein. Because of the complexity of the casein proteins/immunizing antigens, differences in antigen processing and presentation between individual mice could essentially lead to the release of multiple and dissimilar epitopes (40–42). This immune response to one or multiple casein proteins or their hydrolyzed products could induce cross-reactive antibodies to more than one epitope or candidate antigen. While further analysis needs to be done to carefully dissect this diverse response to casein, these observations can be translated to the human disease itself, where patients who were seropositive for casein but negative for titers to MAG could potentially have higher titers to other casein cross-reactive myelin or neuronal antigens.

To summarize, we hypothesize that, in the event of ongoing CNS inflammation, there is a loss of tolerance to the group of milk protein casein(s), one or several of which share sequence similarities with CNS antigens such as MAG, resulting in antibody cross-reactivity between the two. While there is also an epidemiological correlation involving milk consumption and the prevalence of MS (14, 15, 43), whether this is related to the cross-reactivity between casein and MAG remains a matter of speculation. We acknowledge that, in addition to the experiments performed, further studies need to be conducted, including isolation of casein-specific antibodies from MS patients with high seropositivity to bovine casein, to provide stronger evidence that anti-casein antibodies cross-react with MAG. Nevertheless, our observations call for the need to consider personalized dietary restrictions in the treatment of individual patients with MS.

Materials and Methods

Mice and Immunizations. Eight-week-old female WT B6 mice (Charles River) were kept in specific pathogen-free conditions at the animal facility of the Franz-Penzoldt-Zentrum, Erlangen, Germany (approval by the Regierung von Unterfranken, RUF-55.2.2-2532-2-575-5). All animal experiments complied with the German Law on the Protection of Animals, the "Principles of laboratory animal care" (44) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (45). For immunization, incomplete Freund's adjuvant (IFA) was prepared by mixing paraffin oil and mannide monooleate (both Sigma Aldrich) in a 9:1 ratio. CFA was subsequently obtained by adding 5 mg/mL *Mycobacterium tuberculosis* H37 Ra (Difco) to IFA. A total of six cohorts of B6 mice ($n = 6$ to 10 per cohort) were immunized subcutaneously in both sides of the flank with casein solution from bovine milk (Sigma Aldrich) emulsified (1:1) in CFA, resulting in a total injection of 200 μg of antigen per mouse. Additionally, 200 ng PTx (List Biological Laboratories) was injected intraperitoneally on the day of immunization and 48 h later. One cohort each of casein-immunized mice was killed at day 13 or day 20, three cohorts were killed at day 40, and the last cohort was killed at day 60.

The three control groups comprised nonimmunized mice or mice immunized with β-lactoglobulin ($n = 5$) (Sigma Aldrich) or α-lactalbumin ($n = 4$) from bovine milk (Sigma Aldrich). The same protocol and amount of protein (i.e., 200 μg antigen per mouse) was used as for casein immunization.

Additionally, another cohort of casein-immunized ($n = 18$) WT B6 was used for complement depletion studies. A summary of the different cohorts of mice and the respective immunizations are mentioned in *SI Appendix*, Table S5. A clinical scoring system was developed to check the mice of the different cohorts for any signs of weakness or disability, as shown in Table 1.

IgHEL Mice. IgHEL mice carry the *IghelMD4* transgene, that is, Igkc (κ chain constant region) and rearranged Ighm (IgM) and Ighd (IgD) HC specific for HEL, integrated at chromosome 17. Over 90% of splenic B cells derived from the transgene are specific for HEL and predominantly express IgM; therefore, these mice are unable to generate Igs of any other specificities (18, 19). All mice were bred in specific pathogen-free conditions at the central animal facility of the University Clinic of Bonn, Bonn, Germany, and were used at 8 to 12 wk of age. All animal experiments were approved by a government ethics board of the German state of North Rhine-Westphalia, with approval from the Bezirksregierung Köln of the German state of North Rhine-Westphalia (File # 8.87-50.10.31.09.027) and were performed in strict accordance with the recommendations of the Federation of Laboratory Animal Science Associations. To confirm that the pathology observed in casein-immunized WT mice was IgG-mediated, IgHEL mice ($n = 6$) were immunized with bovine casein as above and killed 40 d later. Spinal cords from casein-immunized IgHEL mice were examined by EM.

Electron Microscopic Assessment. Tissue preparation and subsequent EM analysis of the spinal cord were performed as previously described (46). Briefly, immunized mice and controls were killed by CO_2 asphyxiation and perfused with 4% paraformaldehyde (PFA) (wt/vol) in PBS. The lumbar region of the spinal cord was removed from the vertebral canal and post-fixed overnight at 4 °C in 4% glutaraldehyde/4% PFA/0.2% picric acid in phosphate buffer/cacodylate buffer. Following fixation, the specimens were rinsed in PBS and postfixed in 1% (vol/vol) osmium tetroxide and 1.5% (vol/vol) potassium ferricyanide at 4 °C. The tissues were rinsed again in PBS and dehydrated through an ascending ethanol series. The samples were infused with an ethanol/acetone mixture, pure acetone, an acetone/Epon 812 substitute mixture, and pure Epon 812 substitute (Carl Roth). After addition of 2% (vol/vol) 2,4,6-Tris(dimethylaminomethyl)phenol (Carl Roth) to the final volume of the Epon 812 substitute, tissue specimens were placed in resin-filled BEEM® capsules and polymerized at 60 °C and 80 °C. Single ultrathin sections were cut, mounted on single Pioloform-

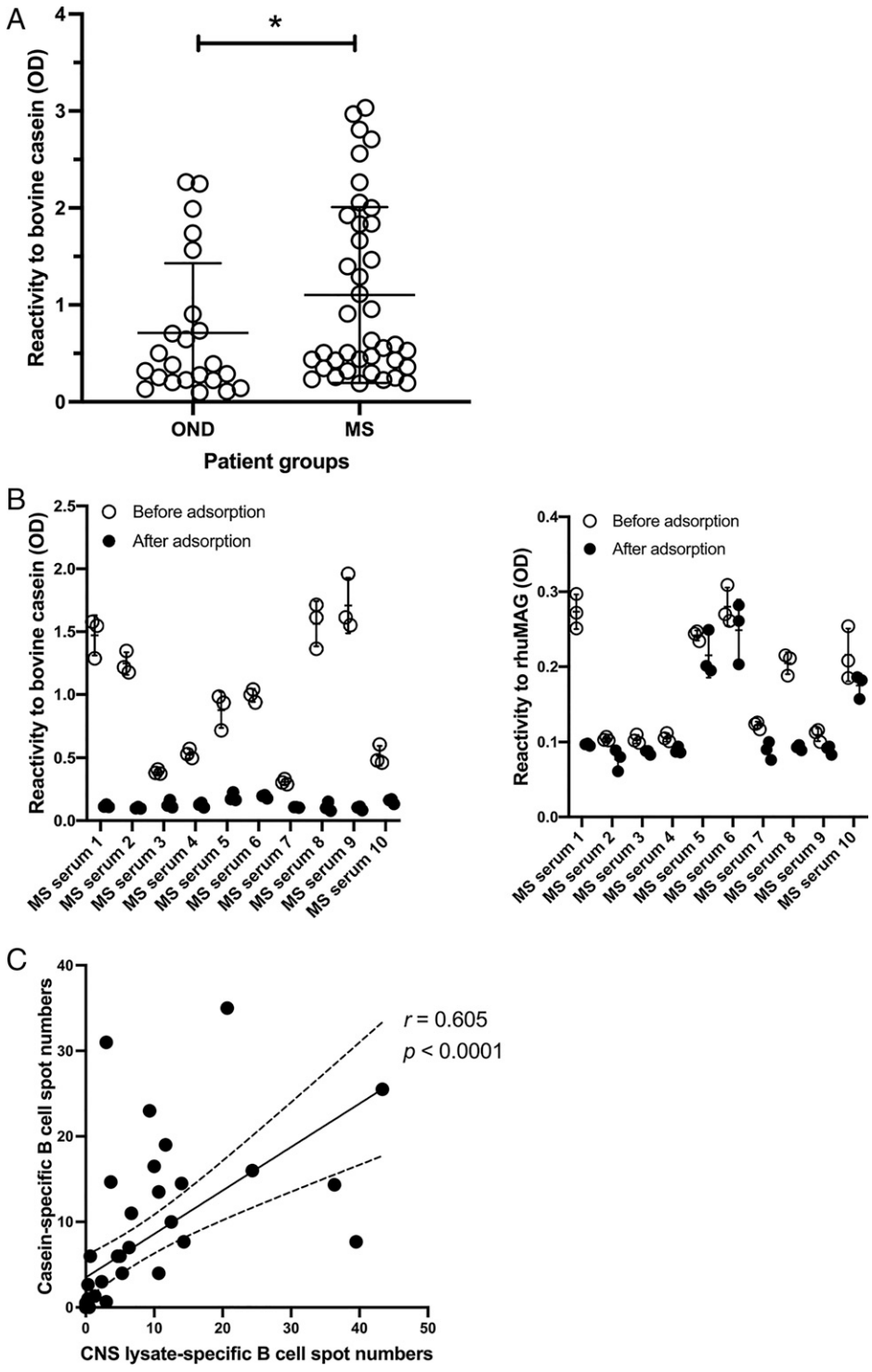


Fig. 4. Patients with MS show elevated numbers of B cell responses and IgG titers to bovine casein in the blood. (*A*) Casein-specific IgG reactivity in patients with MS versus patients with ONDs, measured by ELISA. Each data point for the OD represents the mean ± SD of triplicates for every serum sample. *$P < 0.05$ (Mann–Whitney *U* test). (*B*) IgG titers to bovine casein and MAG before and after adsorption against bovine casein of serum samples from patients with MS. Mean values ± SDs are shown for the individual samples. (*C*) Correlation analysis between B cell reactivity to whole normal human brain lysate and bovine casein in the MS group; the dashed lines are the 95% CIs to the correlation (solid line) (Pearson's correlation coefficient $r = 0.605$; $P < 0.0001$).

coated copper slot grids (Plano), and stained with lead citrate and uranyl acetate. Ultrathin sections of 50 nm were examined with a Zeiss 906 electron microscope (Carl Zeiss). Ten images were acquired per mouse, and the percentage of nerve fibers showing myelin pathology was quantified using ImageJ according to previously published criteria (47). Analysis was performed blinded.

IHC. Immunized and control mice were killed by CO_2 asphyxiation and transcardially perfused with 4% PFA (wt/vol) in PBS. For histological analysis and IHC, spinal cord tissue from the lumbar region was carefully dissected out and fixed overnight in 4% (wt/vol) PFA, following which it was processed according to standard protocols for paraffin embedding. Paraffin-embedded spinal cord sections were cut to 5-μm thickness using a semiautomated microtome (Leica),

rehydrated in a descending series of ethanol solutions, and stained with H&E according to standard histologic protocols. For IHC, heat-induced antigen retrieval of murine spinal cords was performed in 10 mM sodium citrate buffer (pH 6.0). For all IHC stainings, a technical negative control was included where the tissue section was incubated with secondary antibody only.

Staining for IgG Deposition. To confirm the deposition of IgG, sections of spinal cord tissues from immunized WT and IgHEL mice were blocked in 5% normal goat serum (vol/vol) in Tris-buffered saline (TBS) with 0.05% (wt/vol) Tween 20 (TBS-T) for 1 h at room temperature and then incubated with either a fluorescein isothiocyanate-conjugated goat anti-mouse IgG F(ab′)2 antibody (Invitrogen) or biotinylated goat anti-mouse Igs (Agilent Dako) (dilution 1:1,000) in 0.5% (vol/vol) blocking buffer for 2 h to 3 h at room temperature. Sections incubated with goat anti-mouse Igs (biotin) were developed using streptavidin conjugated with Alexa Fluor 647 (Invitrogen).

Mouse ELISA for Bovine Casein and Recombinant MAG. ELISA Microlon medium binding plates (Greiner Bio-One) were coated overnight at 4 °C with casein solution from bovine milk (Sigma Aldrich) or rmsMAG (Sino Biological) in PBS. The concentration of the coating antigens was optimized at 10 μg/mL. Plates were blocked with 10% (vol/vol) fetal bovine serum (FBS) (BD Biosciences) in 0.05% (wt/vol) Tween 20 in PBS (PBS-T). Serum samples were diluted in 0.5% FBS in PBS-T, and secondary antibodies were diluted in PBS-T. Biotinylated goat anti-mouse IgG (Abcam) or biotinylated mouse anti-mouse IgM (BD Biosciences) served as the secondary antibody at dilutions of 1:10,000 and 1:1,000, respectively. For development, plates were incubated with streptavidin–alkaline phosphatase (ALP) (Mabtech) diluted in PBS-T for 2 h before paranitrophenyl phosphate (pNPP) ELISA substrate (Mabtech) was applied. The OD in the wells was read at 405 nm on an MRXII microplate reader (Dynex Technologies) with Dynex Revelation software (version 4.22).

RNA Extraction, PCR, and qPCR for Casein Gene Expression in Mice. Total RNA was isolated from unfixed snap-frozen homogenized murine tissues (brain and breast, where breast tissue was used as a positive control for the detection of casein genes) from $n = 3$ nonimmunized WT B6 mice using TRIzol reagent (Invitrogen). The lysate was incubated in chloroform (Sigma Aldrich) at room temperature and centrifuged at 4 °C. The aqueous phase was precipitated by isopropanol (Sigma Aldrich) at room temperature; 75% (vol/vol) ethanol was added to the RNA pellet, mixed well, and centrifuged. The pellet was dried, resuspended in diethylpyrocarbonate-treated water (Invitrogen), and incubated in a heat block at 57 °C, before quantification using a photometer. The RNA was reverse transcribed to complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was used as a template for subsequent endpoint and qPCR analysis. β-actin was used as a loading control and housekeeping gene control for the endpoint and qPCRs, respectively. The qPCR runs included a no-template control for every primer set, and data were analyzed using the ΔΔcycle threshold method. A list for the primer pairs used for the different types of PCR is provided in *SI Appendix*, Table S6.

Detection of Casein-Specific Serum Reactivity to Mouse CNS Tissue. A Mouse-on-Mouse (MOM) Immunodetection Kit (Vector Laboratories) was used to identify antigen–antibody binding patterns on mouse spinal cord tissue using sera from casein-immunized mice. Briefly, antigen retrieval was performed as described above, following which mouse spinal cord sections were treated as per the instructions in the MOM kit. Dilutions of serum from immunized mice were used as the primary antibody. For fluorescence detection, streptavidin conjugated with Alexa Fluor 647 (Invitrogen) was used following incubation with MOM biotinylated anti-mouse IgG reagent.

To confirm binding of serum IgG to specific cell populations, costaining was performed using either anti-GFAP or anti-OLIG2 antibodies (both Abcam), which recognize astrocytes and oligodendrocytes, respectively. Additionally, a MAG antibody (Abcam) was used to confirm colocalization of serum IgG and its cross-reactive antigen, MAG, on oligodendrocytes. Following use of the MOM kit, spinal cord sections were incubated with either anti-GFAP, anti-OLIG2, or anti-MAG antibodies in TBS-T. Donkey anti-chicken Cy2 or donkey anti-rabbit Cy3 (both Jackson ImmunoResearch) were used as the corresponding secondary antibodies for the detection of GFAP or OLIG2 and MAG, respectively. All sections were counterstained with DAPI (Sigma Aldrich). Images were acquired using a Leica DM6 B fluorescence microscope equipped with Las X software (Leica), a Nikon D-Eclipse C1 confocal microscope, or a Leica DMi8 inverted microscope (Thunder Imager, Leica).

Cell Line and ICC. To further confirm the cross-reactive antigen that might be responsible for the binding of anti-casein IgG to oligodendrocytes, purified Ig from serum samples of mice killed at day 60 ($n = 10$) was tested on cultured Oli-Neu cells. Ig from casein-immunized mice was purified using a commercially available mouse antibody purification kit (Abcam) following the manufacturer's protocol. The oligodendroglial precursor cell line Oli-Neu, derived from mouse brain (20), was cultured and maintained at 37 °C and 5% CO_2.

For immunocytochemical staining, Oli-Neu cells were seeded at a density of 25,000 cells on poly-L-ornithine (Sigma Aldrich)-coated coverslips and cultured at 37 °C, 5% CO_2. Cell culturing was done in Bottenstein–Sato medium supplemented with 2% horse serum (ThermoFisher), 5 μg/mL insulin (Sigma), 1% penicillin/streptomycin (ThermoFisher), 1% N2 supplement (ThermoFisher), 5 ng/mL sodium selenite (Sigma S-9133), 25 μg/mL gentamicin (Sigma), 400 nM T3 (Sigma), and 520 nM T4 (Sigma) in Dulbecco's Modified Eagle Medium (GlutaMAX) (Thermo Fisher). After 48 h in culture, they were treated with 1 μM of PD174265 (Abcam) for their differentiation and arborization into oligodendrocytes (22). Cells were fixed with 4% (wt/vol) PFA and blocked with 10% (vol/vol) bovine serum albumin (BSA) in PBS. For double staining, the fixed cells were sequentially incubated with 40 μg/mL of IgG purified from casein-immunized mice (killed at day 60), followed by rabbit anti-MAG antibody (Abcam). Alexa Fluor 488-conjugated goat anti-mouse IgG (Abcam) and donkey anti-rabbit Cy3 (Jackson ImmunoResearch) were used as the corresponding secondary antibodies for the purified mouse IgG and anti-MAG antibody, respectively. After washing, cells were counterstained with DAPI (Sigma Aldrich). Immunofluorescent images were acquired using a Leica DMi8 inverted microscope (Thunder Imager, Leica). Images at a higher magnification were taken using a Nikon A1R laser scanning confocal microscope (Nikon).

Adsorption Assay. For casein adsorption experiments, 20 μL to 30 μL of mouse serum or 100 μL of human serum was incubated overnight with 0.3 mg or 1 mg of powdered casein from bovine milk (Sigma Aldrich) in 150 μL or 500 μL of PBS, respectively, at 4 °C. Casein and bound Igs were pelleted by centrifugation, and the resulting supernatant was tested for residual titers to casein from bovine milk by ELISA.

Plasmablast Sort and Repertoire Sequencing. For plasmablast sort and B cell receptor repertoire sequencing, an additional cohort of mice was immunized with either casein ($n = 4$) or HEL ($n = 3$, control group). Immunizations were performed on day 0 and day 21, as described above, and mice were killed on day 31. One control mouse remained nonimmunized. Splenocytes, blood, and lymph node cells were singularized by mechanic disruption and stained with antibodies against CD19 (BD Biosciences), B220 (ebioscience), CD38 (Miltenyi Biotec), CD138, CD27, MHCII, CXCR4, IgA, IgM (BioLegend), and Sytox Green (ThermoScientific). The Sytox$^-$ CD19$^+$ B220$^{int/low}$ CD38$^+$ CD138$^+$ CXCR4$^+$ MHCII$^+$ population was sorted on a FACSAria II sorter (BD Biosciences) (48). Two thousand sorted cells per sample were used for preparing single-cell immune profiling libraries with 5′-transcription (10× Genomics), yielding 712 to 995 cells per sample, of which 463 to 622 passed filter thresholds. Variable sequences were aligned to the International ImMunoGeneTics (IMGT) database (https://www.imgt.org) using IMGT/HighV-QUEST (49). Data were analyzed with Loupe V(D)J Browser (10× Genomics) as well as R, version 3.6.1 (50), and GraphPad Prism, version 8.4. Clonality was defined as usage of the same HC and LC V and J genes as well as 70% overlap in CDR3 regions (Levenshtein distance). Mutation load is the summed HC and LC V-gene mutation load, as assessed by IMGT. For clustering of CDR3 regions, full-length sequences of HC and LC CDR3 were concatenated with a 10-amino acid spacer between the two regions, and clustered with Cluster Database at High Identity with Tolerance (CD-HIT) using a 90% identity cutoff (51). Visualization of clusters was done in R version 3.6.1 using the iGraph package. Antibodies for recombinant expression were selected on the basis of being a representative sequence of a cluster consisting of sequences from casein-immunized mice but not from HEL-immunized mice or the control mouse. HC and LC variable sequences were cloned into pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hK/hL2 vectors (Invivogen), respectively, expressed in HEK293 cells, and purified with protein A according to standard protocols. Phylogenetic analysis was performed as previously described (52). Briefly, individual sequences were binned according to their HC usage, then concatenated and aligned with multiple sequence alignment (MUSCLE) (53). They were clustered using maximum-likelihood clustering in PhyML (54). All individual phylogenetic trees were arranged by their HC V-gene families, generating the displayed phylogenetic trees. Trees were visualized with iTol (55). The CDR alignment visualization was generated with Geneious Prime, version 2020.1.2 (Biomatters).

Patient Serum ELISA. Using ELISA, sera from 39 patients with MS and 23 patients with ONDs (as control) were tested for their IgG reactivity to casein. The research protocol was approved by the Ethics Committee of the University of Erlangen-Nürnberg, Germany (file 185_18B). The study used pseudonymized

data, and informed written consent was obtained from all patients. To investigate cross-reactivity of anti-casein antibodies with MAG in patients with MS, a subset of serum samples ($n = 10$) was tested for their reactivity to rhuMAG before and after adsorption with bovine casein.

ELISA Microlon medium binding plates (Greiner Bio-One) were coated overnight at 4 °C with 10 μg/mL casein from bovine milk (Sigma Aldrich) or 10 μg/mL rhuMAG (Sino Biological) in PBS. Plates were blocked with 10% FBS in PBS-T (Carl Roth). Patient serum samples were diluted in 0.5% (vol/vol) FBS in PBS-T, and secondary antibodies were diluted in PBS-T. All patient sera were plated in triplicate at 1:100 dilution for both casein- and rhuMAG-coated plates. Biotinylated anti-human IgG (Mabtech) diluted in PBS-T at a concentration of 0.1 μg/mL served as the secondary antibody. For development, plates were first incubated with streptavidin–ALP (Mabtech) diluted in PBS-T before pNPP substrate (Mabtech) was applied. The OD in the wells was read at 450 nm on a MRXII microplate reader (Dynex Technologies) with Dynex Revelation software (version 4.22). To measure the reactivity of recombinant monoclonal antibodies against bovine casein and rhuMAG, the same protocol was followed, with the modification that the primary antibodies were diluted at a concentration of 5 μg/mL.

Patient Sample ELISPOT. PBMCs from 45 patients with MS and 35 patients with ONDs were tested by ELISPOT assay. The research protocol was approved by the Ethics Committee of the University of Erlangen-Nürnberg, Germany (files 185_18B and 74_18B). The study used pseudonymized data, and informed written consent was obtained from all patients. The protocol for this assay has been previously described by our group (56). Briefly, PBMCs were isolated from patients' blood by Biocoll (Merck) density gradient centrifugation. For polyclonal stimulation of B cells, PBMCs were cultured for 6 d before the ELISPOT assay at a concentration of 3×10^6 cells/mL in complete Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2.5 μg/mL R-848 (Enzo Life Sciences), 15 ng/mL interleukin-2 (Peprotech), and 1 μM mercaptoethanol (Sigma Aldrich). Ninety-six-well ELISPOT plates (Merck) were coated overnight with whole normal human brain tissue lysate (Novus) or with casein solution from bovine milk (Sigma Aldrich) at a final concentration of 30 μg/mL diluted in PBS. Wells coated with 10 μg/mL anti-human κIgG1 (SouthernBiotech) served as a positive control. Plates were blocked with 10% (vol/vol) FBS in sterile PBS at room temperature for 2 h. Each patient sample was subsequently plated in triplicate at a density of 1×10^6 polyclonally stimulated PBMCs per well. Biotinylated anti-human IgG (Mabtech) at 0.2 μg/mL in 1% (wt/vol) BSA in PBS solution was used as a detection antibody. All plates were developed with Vector Blue substrate (Vector Laboratories). Spots were counted on an ImmunoSpot Series 6 Analyzer (Cellular Technology Limited). The background reading was calculated from the negative control and was subtracted from the raw sample reading to provide the test sample measurement.

Statistical Analysis. GraphPad Prism 8.0 (GraphPad Software) was used for statistical analysis. A Shapiro–Wilk normality test was used to confirm whether the dataset followed a Gaussian distribution. Accordingly, differences between two parametric groups were assessed using an unpaired *t* test or paired *t* test while ANOVA was used for greater than two groups. For nonparametric datasets, a Mann–Whitney *U* test was used. For both parametric and nonparametric datasets, a significance level of 5% was chosen. Pearson's correlation coefficient was used to assess the correlation between OD values in ELISAs and between spot numbers in ELISPOT assays.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank Professor Winfried L. Neuhuber and Clemens Wagner for help with confocal imaging. We thank Birgit Blanck, Marion Michels, Anita Hecht, Andrea Hilpert, and Stephanie Link for their excellent technical assistance. This research project was sponsored by Bärbel and Berthold Pohl and also funded by the Interdisziplinäres Zentrum für klinische Forschung - Anschubfinanzierung program of the Friedrich-Alexander-Universität (Project P019, grant to S.K.). S.K. is also supported by Deutsche Forschungsgemeinschaft IRTG2168 Grant 272482170. S. K., J.B.-G., and C.K. are members of the Excellence Cluster "ImmunoSensation" EXC2151-390873048.

1. G. Disanto *et al.*, Heterogeneity in multiple sclerosis: Scratching the surface of a complex disease. *Autoimmune Dis.* **2011**, 932351 (2010).
2. S. Klineova, F. D. Lublin, Clinical course of multiple sclerosis. *Cold Spring Harb. Perspect. Med.* **8**, a028928 (2018).
3. S. Haase, R. A. Linker, Inflammation in multiple sclerosis. *Ther. Adv. Neurol. Disord.* **14**, 17562864211007687 (2021).
4. T. Kuhlmann *et al.*, An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* **133**, 13–24 (2017).
5. H. Lassmann, Pathogenic mechanisms associated with different clinical courses of multiple sclerosis. *Front. Immunol.* **9**, 3116 (2019).
6. C. A. Dendrou, L. Fugger, M. A. Friese, Immunopathology of multiple sclerosis. *Nat. Rev. Immunol.* **15**, 545–558 (2015).
7. R. Dobson, G. Giovannoni, Multiple sclerosis - A review. *Eur. J. Neurol.* **26**, 27–40 (2019).
8. T. Olsson, L. F. Barcellos, L. Alfredsson, Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat. Rev. Neurol.* **13**, 25–36 (2017).
9. T. Okuno, M. Kinoshita, T. Ishikura, H. Mochizuki, Role of diet, gut microbiota, and metabolism in multiple sclerosis and neuromyelitis optica. *Clin. Exp. Neuroimmunol.* **10**, 12–19 (2019).
10. W. J. van den Hoogen, J. D. Laman, B. A. 't Hart, Modulation of multiple sclerosis and its animal model experimental autoimmune encephalomyelitis by food and gut microbiota. *Front. Immunol.* **8**, 1081 (2017).
11. K. Berer *et al.*, Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 10719–10724 (2017).
12. J. Chen *et al.*, Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci. Rep.* **6**, 28484 (2016).
13. A. C. Tengeler, T. Kozicz, A. J. Kiliaan, Relationship between diet, the gut microbiota, and brain function. *Nutr. Rev.* **76**, 603–617 (2018).
14. P. J. Butcher, Milk consumption and multiple sclerosis—An etiological hypothesis. *Med. Hypotheses* **19**, 169–178 (1986).
15. D. Malosse, H. Perron, A. Sasco, J. M. Seigneurin, Correlation between milk and dairy product consumption and multiple sclerosis prevalence: A worldwide study. *Neuroepidemiology* **11**, 304–312 (1992).
16. A. Stefferl *et al.*, Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis. *J. Immunol.* **165**, 2859–2865 (2000).
17. J. Guggenmos *et al.*, Antibody cross-reactivity between myelin oligodendrocyte glycoprotein and the milk protein butyrophilin in multiple sclerosis. *J. Immunol.* **172**, 661–668 (2004).
18. J. Gotot *et al.*, Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 10468–10473 (2012).
19. D. Y. Mason, M. Jones, C. C. Goodnow, Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. *Int. Immunol.* **4**, 163–175 (1992).
20. A. F. Kolb *et al.*, Milk lacking α-casein leads to permanent reduction in body size in mice. *PLoS One* **6**, e21775 (2011).
21. M. Jung *et al.*, Lines of murine oligodendroglial precursor cells immortalized by an activated neu tyrosine kinase show distinct degrees of interaction with axons in vitro and in vivo. *Eur. J. Neurosci.* **7**, 1245–1265 (1995).
22. K. Simon *et al.*, The orphan G protein-coupled receptor GPR17 negatively regulates oligodendrocyte differentiation via Gαi/o and its downstream effector molecules. *J. Biol. Chem.* **291**, 705–718 (2016).
23. G. Wildner, M. Diedrichs-Möhring, Autoimmune uveitis induced by molecular mimicry of peptides from rotavirus, bovine casein and retinal S-antigen. *Eur. J. Immunol.* **33**, 2577–2587 (2003).
24. J. S. J. Chia *et al.*, A1 beta-casein milk protein and other environmental pre-disposing factors for type 1 diabetes. *Nutr. Diabetes* **7**, e274 (2017).
25. J. Karjalainen *et al.*, A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **327**, 302–307 (1992).
26. S. Winer *et al.*, T cells of multiple sclerosis patients target a common environmental peptide that causes encephalitis in mice. *J. Immunol.* **166**, 4751–4756 (2001).
27. H. Bernard *et al.*, Molecular basis of IgE cross-reactivity between human β-casein and bovine β-casein, a major allergen of milk. *Mol. Immunol.* **37**, 161–167 (2000).
28. I. Nentwich, Z. S. Szépfalusi, C. Kunz, P. Spuergin, R. Urbanek, Antigenicity for humans of cow milk caseins, casein hydrolysate and casein hydrolysate fractions. *Acta Vet. (Beogr.)* **73**, 291–298 (2004).
29. G. H. Docena, R. Fernandez, F. G. Chirdo, C. A. Fossati, Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* **51**, 412–416 (1996).
30. B. J. Pelz, P. J. Bryce, Pathophysiology of food allergy. *Pediatr. Clin. North Am.* **62**, 1363–1375 (2015).
31. M. R. Karlsson, J. Rugtveit, P. Brandtzaeg, Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. *J. Exp. Med.* **199**, 1679–1688 (2004).
32. M. S. Trivedi *et al.*, Food-derived opioid peptides inhibit cysteine uptake with redox and epigenetic consequences. *J. Nutr. Biochem.* **25**, 1011–1018 (2014).
33. M. S. Trivedi *et al.*, Epigenetic effects of casein-derived opioid peptides in SH-SY5Y human neuroblastoma cells. *Nutr. Metab. (Lond.)* **12**, 54 (2015).
34. A. Vojdani *et al.*, Antibodies to neuron-specific antigens in children with autism: Possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae* and Streptococcus group A. *J. Neuroimmunol.* **129**, 168–177 (2002).
35. R. H. Quarles, Myelin-associated glycoprotein (MAG): Past, present and beyond. *J. Neurochem.* **100**, 1431–1448 (2007).

36. C. Lucchinetti *et al.*, Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. *Ann. Neurol.* **47**, 707–717 (2000).
37. X. Chen *et al.*, Pertussis toxin as an adjuvant suppresses the number and function of $CD4^+CD25^+$ T regulatory cells. *Eur. J. Immunol.* **36**, 671–680 (2006).
38. L. Steinman *et al.*, Murine model for pertussis vaccine encephalopathy: Linkage to H-2. *Nature* **299**, 738–740 (1982).
39. L. Steinman *et al.*, Pertussis toxin is required for pertussis vaccine encephalopathy. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8733–8736 (1985).
40. L. Vidard, K. L. Rock, B. Benacerraf, Heterogeneity in antigen processing by different types of antigen-presenting cells. Effect of cell culture on antigen processing ability. *J. Immunol.* **149**, 1905–1911 (1992).
41. P. W. Addis *et al.*, Conformational heterogeneity in antibody-protein antigen recognition: Implications for high affinity protein complex formation. *J. Biol. Chem.* **289**, 7200–7210 (2014).
42. K. L. Rock, E. Reits, J. Neefjes, Present yourself! By MHC class I and MHC class II molecules. *Trends Immunol.* **37**, 724–737 (2016).
43. D. Malosse, H. Perron, Correlation analysis between bovine populations, other farm animals, house pets, and multiple sclerosis prevalence. *Neuroepidemiology* **12**, 15–27 (1993).
44. National Research Council, *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC, ed. 8, 2011).
45. C. Kilkenny, W. J. Browne, I. C. Cuthill, M. Emerson, D. G. Altman, Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412 (2010).
46. J. Wörl, W. L. Neuhuber, Ultrastructural analysis of the smooth-to-striated transition zone in the developing mouse esophagus: Emphasis on apoptosis of smooth and origin and differentiation of striated muscle cells. *Dev. Dyn.* **233**, 964–982 (2005).
47. M. S. Recks *et al.*, Early axonal damage and progressive myelin pathology define the kinetics of CNS histopathology in a mouse model of multiple sclerosis. *Clin. Immunol.* **149**, 32–45 (2013).
48. S. Lacotte *et al.*, Early differentiated CD138(high) MHCII+ IgG+ plasma cells express CXCR3 and localize into inflamed kidneys of lupus mice. *PLoS One* **8**, e58140 (2013).
49. E. Alamyar, V. Giudicelli, S. Li, P. Duroux, M. P. Lefranc, IMGT/HighV-QUEST: The IMGT web portal for immunoglobulin (Ig) or antibody and T cell receptor (TR) analysis from NGS high throughput and deep sequencing. *Immunome Res.* **8**, 26 (2012).
50. R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. Accessed 26 February 2022.
51. Y. Huang, B. Niu, Y. Gao, L. Fu, W. Li, CD-HIT Suite: A web server for clustering and comparing biological sequences. *Bioinformatics* **26**, 680–682 (2010).
52. Y. C. Tan *et al.*, Barcode-enabled sequencing of plasmablast antibody repertoires in rheumatoid arthritis. *Arthritis Rheumatol.* **66**, 2706–2715 (2014).
53. R. C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
54. S. Guindon *et al.*, New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321 (2010).
55. I. Letunic, P. Bork, Interactive Tree Of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Res.* **47**, W256–W259 (2019).
56. D. M. Rovituso *et al.*, The brain antigen-specific B cell response correlates with glatiramer acetate responsiveness in relapsing-remitting multiple sclerosis patients. *Sci. Rep.* **5**, 14265 (2015).