ABSTRACT: Multiple sclerosis has complex pathogenesis encompassing a variety of components (immunologic, genetic, and environmental). The autoimmunogenicity against the host’s myelin basic protein is a major contributor. An increase in myelin basic protein deimination (a post-translational modification) and a change in phospholipid composition have been associated with multiple sclerosis. The interaction of myelin basic protein with phospholipids in the myelin membrane is an important contributor to the stability and maintenance of proper myelin sheath function. The study of this aspect of multiple sclerosis is an area that has yet to be fully explored and that the present study seeks to understand. Several biochemical methods, a capillary electrophoresis coupled system and mass spectrometry, were used in this study. These methods identified four specific phospholipids complexing with myelin basic protein. We show that lysophosphatidylcholine 18:1 provides a robust competitive effect against hyper-deimination. Our data suggest that lysophosphatidylcholine 18:1 has a different biochemical behavior when compared to other phospholipids and lysophosphatidylcholines 14:0, 16:0, and 18:0.

1. INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease that manifests with neurological disabilities that include impaired muscle control, tremors, and visual impairments. This is mainly due to the interruption of signal propagation in different neuronal circuits through the disruption of the myelin sheath. MS is a complex disease with various components contributing to pathogenesis; however, immune reactivity against myelin components seems to be a major contributor. Early efforts sought to identify myelin components associated with MS. It was found that interferences in the interaction of myelin basic protein (MBP) with the rest of the myelin components produce immune-reactive epitopes.

The myelin sheath allows for rapid transduction of nerve impulses through the electrical insulating properties of the plasma membrane. Myelin sheath is composed of two major constituents: proteins and lipids. Among proteins, MBP is the second-most abundant component, whose primary role is to maintain a compact myelin sheath, while lipids provide electrical insulation. There are 14 distinct isoforms of MBP that have been reported in the murine central nervous system (CNS), all of which are the results of different exon compositions of a single MBP gene. The classic family of MBP is the one predominantly expressed in the major dense line of the myelin sheath, and isoform 5 (18.5 kDa) is the isoform primarily associated with MS. The most prominent immune-reactive epitopes found in the cerebrospinal fluid of MS patients are against MBP peptides. These peptides have been documented to be selectively produced by proteolytic cleavage by a number of metalloproteases.

The rate of digestion at which metalloproteases produce these immune-reactive peptides has been directly linked to the levels of deimination (a post-translational modification) in MBP.

Deimination is a post-translational modification mediated by the family of peptidyl arginine deiminases (PADs) that converts protein-bound arginine to protein-bound citrulline. This modification effectively neutralizes the positively charged guanidinium group found in arginine by replacing it with a neutral ureido group. Deimination is considered to be an irreversible post-translational modification given that the enzyme responsible for the reverse reaction has not been found. In addition, due to the longevity of deimination, this poses the potential for long-lasting effects on protein regulation.

Studies focused on the structure of deiminated MBP have documented that deimination renders MBP to an open structure, which allows proteases access to internal residues, increasing the rate of digestion and production of immune-reactive peptides. The elevated levels of MBP...
deimination have been documented to positively correlate with the severity of MS in human subjects, as well as in the experimental autoimmune encephalomyelitis (EAE) mouse. Subsequent studies demonstrated that native MBP takes a C-shape folded structure upon lipid binding, which resulted in an increase in compaction and stability. This highlights the importance that MBP–phospholipid complexation has in maintaining myelin stability.

Lipids in the myelin sheath are arranged in an asymmetric pattern. Sphingolipids are the major class of lipids that face the extracellular surface, and phospholipids are the major class facing the cytoplasmic space. Changes in lipid composition have been documented in human MS brains. Members of...
phospholipid classes [phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), phosphatidylinositols (PIs), and phosphatidylserines (PSs)] have been reported to increase in relative amounts (relative to the total) for specific lipid species and decrease for others. MBP is a cytoplasmic side peripheral membrane protein likely to complex with phospholipids and help maintain an ordered myelin sheath. Although studies have demonstrated that MS pathogenicity is associated with changes in lipid profiles and disruption of MBP structural roles, a study exploring the specific phospholipid interactions that are native to MBP remains to be performed. The effects of MBP–phospholipid complexation on protein deamination also remain to be studied. The aim of the study is to address this gap in the scientific literature, that is, to understand the individual phospholipid that complexes with MBP and plays a potential role toward influencing protein deamination.

2. RESULTS AND DISCUSSION

Demyelinating diseases such as multiple sclerosis converge diverse components resulting in pathogenicity. The immunological events, despite being most well studied, still warrant further intense investigation. To contribute to our understanding of disease pathogenicity, it is crucial to understand the changes in the basic biochemical conditions associated with the myelin sheath. Autoimmunogenicity against host’s MBP is a hallmark of multiple sclerosis. Understanding the native biochemistry can shed light on how the state of antigenicity becomes perturbed in disease conditions to result in autoimmunogenicity.

The animal models available for the study of MS include the toxin-induced models (cuprizone and lysolecithin, useful for understanding demyelination and remyelination processes), viral-induced model (Theiler’s murine encephalomyelitis, useful for understanding axonal damage and inflammation), and chronic EAE model. The EAE mouse model is one of the most characterized models in the literature. The benefits of this model include chronic demyelination and its effects on the central nervous system, allowing for the study of immunological and neuroinflammatory processes.

We present findings that (1) PAD-mediated hyper-deimination is associated with EAE (Figure 1B), (2) there are broad changes of phospholipid composition in EAE (30 days post injection; Figure 2C), (3) there is a differential complexation of phospholipids with MBP in EAE (Figure 3B,C), and (4) lysophosphatidylcholine (LPC) 18:1 likely competes for hyper-deimination of MBP (Figures 4A,B, 5A, and 7A). Our findings in the EAE model are parallel to the reports of phospholipid deficiency in human multiple sclerosis subjects only in the PC class. Although there were overall changes in the general classes of phospholipids, we focused on the specific phospholipids that were deficient in the EAE group, thus enabling the selection of a finite number of species that can be pursued with ease. Specific phospholipid species have been reported to be deficient in human MS samples despite increases in the overall class of phospholipids.
2.1. Confirmation of the Experimental Autoimmune Encephalomyelitis (EAE) Mouse Model. To confirm that the multiple sclerosis mouse model utilized for this study (EAE) exhibited its characteristic traits, animals were clinically scored to monitor the progression of EAE (Figure S1A). The clinical score graph demonstrated that animals maintained an elevated clinical score and corresponded to the chronic phase of disease progression (day 30). Additionally, visual impairment was monitored by measuring the inner retinal function (retinal ganglion cell activity) using pattern electroretinogram (PERG). The PERG measurements demonstrated that 30 days post immunization, EAE mice exhibited a decrease in amplitude when compared to the no-injection control and sham-injection control groups (Figure S1B). Flash electroretinogram (FERG) was utilized to determine if the photoreceptor activity was contributing to visual impairment. The FERG demonstrated no change in any of the groups, suggesting that visual impairment was mediated through the inner retina (data not shown). To eliminate the possibility that an aberrant retinal morphology was contributing to visual decline, optical coherence tomography (OCT) was utilized and demonstrated no change in retinal thickness (Figure S1C). Immunohistochemical analysis of optic nerves demonstrated that EAE mice were suffering from optic nerve demyelination as it was observed by a decrease in the MBP signal and topographical distribution (Figure S1D). The blood antibody analysis specific for myelin oligodendrocyte glycoprotein (MOG) further corroborated that experimental animals exhibited characteristic traits of the EAE mouse model (Figure S1E). Thus, our EAE animals demonstrated all clinical neurological deficits, as well as visual function impairment, in parallel to that in human MS.

2.2. Isolation of the MBP–Phospholipid Complex and Deimination in EAE. Several studies have highlighted the importance of MBP–phospholipid complexation; however, the specific lipid species that complex with MBP have not been explored in detail. The isolation of intact complex using inert biochemical methods is the first critical step toward the identification of native MBP–phospholipid complex constituents. The inert methods preserve the intermolecular interactions within the complex. From confirmed EAE mice, we collected neuronal tissue (brain) and subjected the sample to homogenization and fractionation through sucrose gradient ultracentrifugation. Sucrose fractions were then evaluated using
immunoblotting for the identification of the fractions containing MBP (Figure 1A). The distribution of MBP was rather uniform throughout many different fractions (fractions 14–36; Figure 1A). Sucrose fraction 32, taken as a representative, was analyzed for verification of the presence of MBP and levels of deimination. Coomassie staining demonstrated the same pattern of MBP within the gel as observed in the Western blot analysis (Figure 1B, upper panel); the latter also showed an increase in levels of deimination in the EAE sucrose fraction (Figure 1B, lower panel). Bands from coomassie gel were excised and analyzed, verifying the presence of MBP in the fractions (Figure 1C). Sucrose fraction 32 (SF 32) from all groups was further fractionated through capillary electrophoresis (CE). The CE electropherogram displayed large peaks, as indicated by the arrows. The peaks did not demonstrate a constant retention time; however, they typically eluted between 55 and 65 min (Figure 2A). Peaks were collected as well as the eluents 10 min before and after the peak (each for 10 min duration). They were subjected to dot immunoblot analysis using the antibody against MBP. Results demonstrated the presence of MBP in the peak but not in the other fractions, confirming the presence of MBP and its constituents in the peaks (Figure 2B). We therefore collected MBP complexes at the end of CE fractionation.

2.3. MBP–Phospholipid Complexation. Mass spectrometric lipidomic analysis of the CE peak fractions demonstrated a decrease in phosphatidylcholines (PCs), no change in phosphatidylethanolamines (PEs), and an increase in phosphatidylinositols (PIs) and phosphatidylserines (PSs) in the EAE group when compared to that of control groups (Figure 2C). The following criteria were used to identify candidate phospholipids that potentially complex with MBP: (1) phospholipids had to be present in both the no-injection control and sham-injection control groups but (2) absent in the EAE group. We found five candidate lipids that met these criteria (Table 1 and Figure 3A).

Figure 5. LPC 18:1 competes with hyper-deimination of MBP arginine residues and has differential complexation with MBP. (A) Mass spectrometry analysis of MBP arginine residues that are being deiminated in vitro deimination assay (Millipore, 13-104, Burlington, MA). The deiminated Myelin basic protein (UniProt accession: P02687) peptides were identified by mass spectrometry (see Table 3). Gray highlight, deiminated arginine; red bold square, arginine not deiminated in LPC 18:1 reaction (residues 63, 112, and 129). (B) Protein–lipid overlay assay of LPC 18:1, LPC 14:0, LPC 16:0, and LPC 18:0 MBP was probed with an anti-MBP antibody (Abcam, ab7349).
To corroborate their direct complexation with MBP, the candidate phospholipids were analyzed using the liposome flotation assay (LFA) and protein–lipid overlay assay (PLOA) (Figure 3B,C). For this purpose, liposomes/micelles were prepared using extrusion and were confirmed for being within the appropriate size range, using nanoparticle tracking analysis.45 The assay revealed that there are various degrees of complexation that exist between MBP and candidate phospholipids, as revealed by the movement of MBP to either the middle or top layer (Figure 3B).

The PLOA analysis demonstrated and confirmed the same pattern observed in the LFA (Figure 3C). However, PC 16:0/22:6 consistently presented different moving behavior adhering to the membrane during dot immunoblot in the PLOA and was not pursued for further analysis (Table 2). The PLOA demonstrated that LPC 14:0, LPC 16:0, and LPC 18:0 had equal complexation to MBP between the control groups and EAE group. This contrasts to the complexation of LPC 18:1, which showed a difference between MBP derived from controls or EAE group.

Comparison of phospholipids deficient in the EAE with the control groups helped identify and validate four phospholipids that complex with MBP, of which PC 16:1/16:1 and LPC 18:1 have been reported to be deficient in humans (Figure 3 and Table 2).40,42

Lipids in an aqueous environment can attain different geometries due to their hydrophobic tails and hydrophilic head groups. The packing parameters (P) dictate that the geometry lipids will undertake in an aqueous environment.46,47 Phospholipids (P ≈ 1) and lysophospholipids (P < 1) exhibit a cylindrical and an inverted cone geometry, respectively. In both of these species, the hydrophilic head group will face the outer shell of the liposome (phospholipids) or micelle (lysophospholipids). The myelin sheath mimics the same geometry, that is, the head group is facing the cytoplasmic space and interacting with MBP. Only lipids with a P > 1 (for example, dioleoylphosphocholine) will exhibit an inverted micellar geometry (our study did not identify any such lipid species).

The variable degree of travel observed in the LFA for specific MBP–phospholipid complexes can be accounted for by either the strength of the MBP–phospholipid complexation or the presence of liposomes/micelles of smaller sizes. The smaller-sized liposomes/micelles may fail to move MBP to the top layer due to a lack of attaining sufficient buoyancy. PC 16:0/20:4 was used as a control phospholipid to PC 16:0/22:6. This control was used to determine if reducing the length and saturation of the hydrocarbon chain affected complexation with MBP (Figure S2A,B). LFA and PLOA for PC 16:0/20:4 demonstrated the same pattern observed with PC 16:0/22:6, suggesting that small variations in the length and saturation of the hydrocarbon chain do not affect MBP complexation. Similarly, PC 18:1/18:1 and LPCs (14:0, 16:0, and 18:0) were used as controls for LPC 18:1. These controls were used to determine if the absence of a second hydrocarbon chain and variations in the hydrocarbon chain length affected MBP complexation (Figures S2C,D and 5B). LFA and PLOA demonstrated that PC 18:1/18:1 did not complex with MBP, suggesting that the number of hydrocarbon chains has an effect in complexation with MBP. PLOA of other LPCs demonstrated no preference for MBP complexation derived from controls or EAE group.

### Table 1. Candidate Phospholipids after CE

| lipid molecule | calculated mass |
|----------------|----------------|
| PC 16:0/22:6   | 805.5622       |
| PC 16:1/16:1   | 729.5309       |
| PI 18:0/20:4   | 858.5258       |
| PS 18:0/18:1   | 789.552        |
| LPC 18:1       | 521.3481       |

“Candidate phospholipids that are present in the noninject and sham groups but absent in the EAE group.”

### Table 2. Curated Phospholipids after LFA and PLOA

| lipid molecule | calculated mass |
|----------------|----------------|
| PC 16:1/16:1   | 729.5309       |
| PI 18:0/20:4   | 858.5258       |
| PS 18:0/18:1   | 789.552        |
| LPC 18:1       | 521.3481       |

“Lipids demonstrating direct complexation with MBP. Liposome flotation assay (LFA) and protein–lipid overlay assay (PLOA).”

Lipids in an aqueous environment can attain different geometries due to their hydrophobic tails and hydrophilic head groups. The packing parameters (P) dictate that the geometry lipids will undertake in an aqueous environment.46,47 Phospholipids (P ≈ 1) and lysophospholipids (P < 1) exhibit a cylindrical and an inverted cone geometry, respectively. In both of these species, the hydrophilic head group will face the outer shell of the liposome (phospholipids) or micelle (lysophospholipids). The myelin sheath mimics the same geometry, that is, the head group is facing the cytoplasmic space and interacting with MBP. Only lipids with a P > 1 (for example, dioleoylphosphocholine) will exhibit an inverted micellar geometry (our study did not identify any such lipid species).

The variable degree of travel observed in the LFA for specific MBP–phospholipid complexes can be accounted for by either the strength of the MBP–phospholipid complexation or the presence of liposomes/micelles of smaller sizes. The smaller-sized liposomes/micelles may fail to move MBP to the top layer due to a lack of attaining sufficient buoyancy. PC 16:0/20:4 was used as a control phospholipid to PC 16:0/22:6. This control was used to determine if reducing the length and saturation of the hydrocarbon chain affected complexation with MBP (Figure S2A,B). LFA and PLOA for PC 16:0/20:4 demonstrated the same pattern observed with PC 16:0/22:6, suggesting that small variations in the length and saturation of the hydrocarbon chain do not affect MBP complexation. Similarly, PC 18:1/18:1 and LPCs (14:0, 16:0, and 18:0) were used as controls for LPC 18:1. These controls were used to determine if the absence of a second hydrocarbon chain and variations in the hydrocarbon chain length affected MBP complexation (Figures S2C,D and 5B). LFA and PLOA demonstrated that PC 18:1/18:1 did not complex with MBP, suggesting that the number of hydrocarbon chains has an effect in complexation with MBP. PLOA of other LPCs demonstrated no preference for MBP complexation derived from controls or EAE group.

### Table 3. Arginine Residues Deiminated in MBP–Lipid Challenge Assay

| lipid molecule | total deiminated cites | protein coverage (%) | arginine residue deiminated
|----------------|------------------------|----------------------|-----------------------------|
| MBP + PAD + PC 16:1/16:1 | 13 | 85.80 | 41, 47, 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + PI 18:0/20:4 | 12 | 85.80 | 47, 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + PS 18:0/18:1 | 11 | 85.80 | 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + LPC 18:1 | 9 | 94.08 | 47, 52, 78, 96, 106, 158, 161, 168, 169 |
| MBP + PAD + LPC 18:0 | 13 | 92.90 | 23, 47, 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + LPC 16:0 | 13 | 92.90 | 23, 47, 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + LPC 14:0 | 13 | 92.90 | 47, 52, 63, 78, 96, 106, 112, 129, 158, 161, 168, 169 |
| MBP + PAD + no lipid | 12 | 94.08 | 52, 78, 96, 106, 158, 161, 168, 169 |
| MBP + No PAD + no lipid | 8 | 94.08 | 52, 78, 96, 106, 158, 161, 168, 169 |

“Analysis of arginine deamination of myelin basic protein (MBP). For chymotrypsin-digested MBP (UniProt accession number: P02687; Millipore, 13-104, Burlington, MA), the total number of spectrum matches were 1756 and 20 unique peptides, which were identified by mass spectrometry. The deimination cites had a 99–100% confidence. LPC 18:1 incubation protected residues 63, 112, and 129 from deimination.4 “The sequence positions are in reference to canonical MBP isoform 1 (UniProt accession number: P02687). See Table S1 for more details.”

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deimination of arginine residues in MBP when compared to the other phospholipids (Figures 4A,B and SA). In the present study, LPC 18:1 was identified to be present in healthy, but absent in chronic EAE mice. The absence in EAE suggests that endogenous LPC 18:1 can potentially have important biological functions in healthy animals. The complexation of LPC 18:1 is likely to be beneficial for MBP stability, preventing MBP from further deimination when challenged with PAD. It is likely that LPC 18:1 complexation occurs prior to deimination and thus prevents hyper-deimination. In normal humans, MBP is known to be deiminated in 6 of a total of 19 arginine residues (UniProt accession: P02686-5, in MBP isoform 5; Figure S5). However, deimination in MBP isoform 5 goes beyond these six sites in MS and/or in demyelinating diseases.46 In other words, hyper-deimination is prevented in normal conditions but occurs only in demyelinating diseases/MS.8,10,24,28,49 The more severe the demyelinating disease, the more intense the hyper-deimination.23 In mouse and bovine, four and eight residues are found deiminated in normal or wild-type animals, respectively. The number of sites (beyond four and eight, respectively) increases in mouse and bovine in demyelinating disease conditions consistent with hyper-deimination in humans (Figure S5).

The complexation of LPC 18:1 with MBP is partial in vitro as demonstrated by LFA of MBP in the middle layer and the partial signal in the PLOA, but more complete complexation in vivo cannot be ruled out. When LPC 18:1—MBP complex is challenged by PAD, only nine arginine residues are deiminated (one more residue compared to eight endogenous residues in MBP). In contrast, all other LPCs (14:0, 16:0, and even 18:0) and other phospholipids demonstrated deimination of 13 and 11–13 arginine residues, respectively (Figure SA and Table 3). This supports the hypothesis that MBP–LPC 18:1 complexation prevents hyper-deimination of arginine residues by PAD (Figure 7B,C). LPC 18:1 complexation with MBP derived from the EAE group (Figure 3B,C) is consistent with the availability of strong binding sites (vacant sites) for LPC 18:1 in hyper-deiminated MBP. In vivo, the EAE animals (or human MS CNS samples) are deficient in LPC 18:1, leaving such binding sites vacant in MBP derived from these sources. This is consistent with our CE experiments, where we found a lack of bound LPC 18:1 with the EAE-derived MBP (Table 1). LPC 18:1 confers protection against hyper-deimination, contrasting other LPCs (14:0, 16:0, and 18:0). These LPCs are the ones frequently used for demyelination (at or beyond 100 μM, they induce toxicity).50–53 A comparative study of the degree of demyelination caused by various LPC species remains to be performed.

2.5. Structural Analysis of LPC 18:1–MBP Complex.

Sequence analysis-based computer methods and full-atomic-level molecular dynamics (MD) simulations are consistent with MBP being an intrinsically disordered protein (IDP). MBP exhibiting a disordered structure (Movie S1 and Figure S3) is supported by previous literature reports.43,54,55 This was further corroborated by circular dichroism (CD) studies, which demonstrated that despite MBP being an intrinsically disordered protein (IDP), it retains a partial secondary structure. The CD showed altered spectra when MBP was denatured at 105 °C (Figure 4C, top left panel) compared to that at 20 °C. The LPC 18:1 binding does not cause any change in the secondary structure (Figure 4C, bottom left panel). In hyper-deiminated MBP, the loss of secondary structure (Figure 4C, top right panel) is quite pronounced (Figure 4C, top right panel), as expected. However, the loss of secondary structure in hyper-deiminated MBP is somewhat protected (not statistically significant) when complexed with LPC 18:1 (Figure 4C, top right panel and bottom left panel, respectively).

Computer simulations indicated that MBP is a highly flexible protein that shows a variable conformational landscape, a behavior characteristic of IDPs (see Movies S1–S3 in the Supporting Information). MD simulations indicated that the presence of lipids could change the level of deimination of arginine residues due to accessibility by PAD. This change could be due to a combination of two possible factors. The first factor is the presence of the arginine residues on the MBP surface rendering accessibility by PAD. The presence of lipid molecules around the arginine residues could make them inaccessible to PAD. The second factor is the overall conformation of the protein, which is altered by the presence of the lipid. In the extended conformation of MBP, more arginine residues are accessible or present on an accessible surface (Figure S4). The water-only simulations indicated that the arginine residues are solvent accessible (located on the surface) and could be easily accessed by PAD (Figure 6). The alternate case would be when these residues are not accessible to the solvent due to salt linkages or being buried inside the protein. In the LPC 18:1 simulations, the lipid molecules surround the arginine residues, possibly making it difficult for deimination by PAD to occur.

The behavior in the PC simulations is significantly different. First, in the presence of PC, there are several more arginine residues that appear to be solvent accessible. Second, there is a change in the structure of MBP. The averaged radius of gyration of MBP in the presence of PC is 19.1 Å, compared to 18.2 Å in the case of water-only simulations and 18.7 Å in the
presence of LPC 18:1. The more extended conformations in the presence of PC allow more arginine residues to be accessible by other biomolecules, including PAD. This further supports that steric hindrance provided by LPC 18:1 competes against PAD-mediated hyper-deimination.

3. CONCLUSIONS
Together, the data presented in this study demonstrate that LPC 18:1 undergoes complexation with MBP. This complexation prevents hyper-deimination when challenged with PAD. The severity of MS has shown strong and positive correlation with hyper-deimination of MBP. Hyper-deiminated MBP is more easily unfolded and becomes vulnerable to autolysis and proteolysis, resulting in the release of MBP peptides, which in turn triggers autoimmunogenicity. Our findings reported here expands our understanding of the biochemistry associated with the previously recorded molecular events in MS pathogenicity (Figure 7).

4. EXPERIMENTAL SECTION

4.1. Generation of Experimental Autoimmune Encephalomyelitis (EAE) Model. All animals were housed at the McKnight vivarium, University of Miami, FL, and animals utilized in this study were under the University of Miami Institutional Animal Care and Use Committee-approved protocols (IACUC protocol number: 16-235). Method was adapted from other studies. Immunizing neuroantigen was prepared by the emulsion of 2 mg of myelin oligodendrocyte glycoprotein (MOG35-55) and 1 mg of M. tuberculosis. Pertussis toxin (Biological Laboratories, 181, Campbell, CA) was prepared at a concentration of 50 μg/mL in sterile phosphate-buffered saline (PBS), pH 7.4, with an intraperitoneal (i.p.) administration of 200 ng per injection. Female C57Bl6/J mice were injected i.p. with pertussis toxin the day before injection. They were then immunized subcutaneously (s.c.) at 2 months of age with 200 μL of neuroantigen (MOG35-55) followed by an i.p. injection of pertussis toxin 2 days post immunization. Three groups were created for this study, an immunized group with MOG35-55 (EAE, 15 animals), an immunized group without MOG35-55 (sham-injection control, 10 animals), and an injection control that received no injections (no-injection control, 10 animals). Animals were monitored for decline in body weight as well as manifestation of clinical signs indicative of encephalomyelitis following the same criteria as previous studies.

4.2. Electroretinogram Recordings. Pattern electroretinogram (PERG) and flash electroretinogram (FERG) were recorded utilizing the Jörvec PERG system (Jörvec PERG Visual Stimulation Box, M014760L, Miami, FL). The experimental setup for FERG was as described in previously published protocols; however, there was a slight modification for the experimental setup for PERG. The modification consisted of the subcutaneous insertion of the recording electrode between the two eyes, the reference electrode in the scalp and the ground electrode in the lower back adjacent to the tail. For both PERG and FERG, animals were anesthetized by the i.p. injection of 100 μL of ketamine and xylazine cocktail (1.5 mg/0.3 mg per 100 μL) per 20 g of body weight. A drop of the balanced saline solution was delivered to the eye to prevent dryness for the duration of the procedure. Animals were situated in a heating pad (Physitemp TCAT-2LV controller) with an anal reference thermometer set at 37 °C.

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to stabilize the internal body temperature. PERG was recorded by three consecutive responses of 600 contrast reversals, and recording settings consisted of 10.0 K gain, 1.0 Hz high pass, 100.0 Hz low pass, and 360.0 μV rejection. FERG was recorded by three consecutive measurements of a flash, with a strength of 20.0 CDS/SM and a frequency of 1.0 Hz. Amplitude for PERG and FERG was measured as the difference in microvolts between the highest peak and the consecutive lowest trough. Latency for PERG and FERG was measured as the time (milliseconds) it took to reach the highest peak (from time zero to the time when the highest peak was recorded).

4.3. Optical Coherence Tomography (OCT). Animals were anesthetized as described above, followed by the application of one drop of tropicamide ophthalmic solution (Akorn, 17478-102-12) to dilate the pupil and a drop of the balanced saline solution to prevent eye dryness. A 3.5 mm diameter mini contact lens (Ocuscience, ERGACC3) was placed for the visualization of retina. OCT images were taken with the Engineering Spectralis HRA + OCT (Franklin, MA) and analyzed with the Spectralis HRA + OCT software version 6.6.

4.4. Enzyme-Linked Immunosorbent Assay (ELISA). Animals were anesthetized as described above, followed by exposure of the thoracic cavity. Using a 26G1/2 needle, about 1 mL of blood was aspirated from the left atrium. Animals were immediately decapitated to ensure euthanasia and prevent recovery from anesthesia. The blood was treated with 0.25 mL 4% paraformaldehyde in PBS and incubated overnight at 4 °C. Tissue was washed using PBS, followed by cryopreservation using a gradient of 10% sucrose (overnight at 4 °C), 20% sucrose (overnight at 4 °C), and 30% sucrose (overnight at 4 °C). Tissue was then embedded in an optimized cutting temperature compound (VWR, 25608-930) and stored at −80 °C. Utilizing the AnaSpec Anti-MOG35-55 Quantitative ELISA kit (VWR, AS-S446S) and following the manufacturer’s protocol, antibodies against MOG35-55 were detected and normalized to the total protein concentration in isolated blood plasma.

4.5. Immunohistochemistry. Dissected optic nerves were immersed fixed in 4% paraformaldehyde in PBS and incubated overnight at 4 °C. Tissue was washed using PBS, followed by cryopreservation using a gradient of 10% sucrose (overnight at 4 °C), 20% sucrose (overnight at 4 °C), and 30% sucrose (overnight at 4 °C). Tissue was then embedded in an optimized cutting temperature compound (VWR, 25608-930) and stored at −80 °C. Immunohistochemistry standard protocol was used to detect MBP (Abcam, ab7349) and CD90 (Cymbus Biotech, CBL1354), and the tissue was mounted using Vectashield with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, H-1200).

4.6. Tissue Preparation and Density Ultracentrifugation. Method was adapted from other studies.30 Brains from 30 days postinjection mice (8–10 brains, ~3.5 g total weight, when they manifested clinical signs indicative of encephalomyelitis) were rapidly dissected, flash-frozen by liquid nitrogen immersion followed by equilibration on dry ice, and stored at −80 °C. Tissue was homogenized at 4 °C utilizing 0.32 M sucrose solution with a protease inhibitor (5% w/v homogenate). Gradient I was established by layering the homogenate (18 mL) over 18 mL of 0.85 M sucrose at 4 °C and ultracentrifuged at 140 000 g at 4 °C for 1 h (Beckman Rotor SW28). The low-density band was discarded, and the main band along with the dispersion band was collected. They were homogenized and diluted to 200 mL, utilizing a 10 mM ethylene glycol tetracetic acid (EGTA) solution followed by centrifugation at 141 000 g at 4 °C for 40 min (Beckman Rotor SW28). The supernatant was discarded, and the pellet was resuspended in 120 mL of 10 mM EGTA solution, homogenized, and ultracentrifuged at 35 000 g at 4 °C for 15 min (Beckman Rotor SW28). The low-density band was discarded, and the main band along with the dispersion band was collected followed by processing as described above; however, the pellet was finally resuspended in 13 mL with 0.85 M sucrose solution. Gradient II was established by layering from bottom to top over 12 mL of 0.85 M sucrose-containing pellet, 12 mL of 0.75 M sucrose, and 13 mL of 0.32 M sucrose at 4 °C followed by ultracentrifugation at 14 000 g at 4 °C for 16 h (Beckman Rotor SW28). Fractions were collected in separate tubes (1 mL each, 37 fractions total) along with the pellet. Fractions were washed with 2 mL of 2 mM EGTA solution followed by centrifugation at 18 000 g at 4 °C for 25 min (Beckman Rotor GH-3.8). The supernatant was discarded, and the pellets were resuspended in 1 mL of 50 mM Tris solution with a protease inhibitor and stored at −80 °C.

4.7. Gel Electrophoresis and Immunoblotting. Fractions from density centrifugation were heated to 85 °C for 3 min and diluted using Laemmli loading buffer (Amresco, M337). Equal volume of samples was loaded in 4–20% Tris–glycine gel (Bio-Rad, 5671095) to maximize the protein content in lower fractions and ran using Tris–glycine–sodium dodecyl sulfate (SDS) running buffer at 200 V for 33 min. Poly(vinyliodene difluoride) (PVDF) membrane was activated with methanol and washed with ultrapure water and with transfer buffer (TGS-20% methanol) before transfer. Semidy transfer was carried out using the Trans-Blot Turbo Transfer System (Bio-Rad, 1704150). Membrane was blocked with a blocking buffer (Bio-Rad, 170-6404) and probed for either MBP using antibodies (Abcam, ab7349) and CD90 (Cymbus Biotech, CBL1354), and the tissue was mounted using Vectashield with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, H-1200).

4.8. Capillary Electrophoresis (CE). After confirming the presence of MBP, fraction 32 was selected for further analysis. Fractions were separated using the Agilent Capillary Electrophoresis 7100 coupled with a bare fused silica capillary tube (Agilent, G1600-63311). CE buffer consisted of 100 mM formic acid, 5 mM ammonium acetate, and 100 mM Tris at a pH of 7.3. The sample was prepared at a 1:10 ratio of density centrifugation fraction to CE buffer. Parameters for the CE system included injection at 50 mbar for 10 s, high voltage at...
30 kV, 25 μA and 6 W, a cassette temperature of 25 °C, coupled with a high-pressure system of 10 mbar for the duration of the separation (70 min). Signal was detected using UV absorbance at 230 nm (bandwidth 4 nm) and a reference at 350 nm (bandwidth 50 nm). Fractions were collected in separate tubes in intervals of 10 min.

4.9. Lipid and Protein Extraction. Capillary electrophoresis fractions were centrifuged at 18 000g for 4 °C for 1 h (Beckman Microfuge 18). The supernatant was discarded, and the pellet (very small) was resuspended in methanol with 0.516 mg/mL butylated hydroxytoluene (BHT). The resuspended pellet was transferred to a glass tube to minimize contact with plastic, and methyl tert-butyl ether (MTBE) was added, maintaining a 10:3 ratio per volume of MTBE/methanol. Extraction was left shaking overnight (~16 h) at 4 °C. Examinations were treated with 0.15 M ammonium acetate for phase separation while maintaining a 20:6:5 ratio per volume of MTBE/methanol/ammonium acetate. Examinations were centrifuged at 2000g for 10 min (Thermo Fisher Megafuge 8R), and the upper organic phase was collected in a separate glass tube. Extraction containers were washed with a 20:6:5 ratio per volume of MTBE/methanol/ammonium acetate, centrifuged, and the organic phase was added to the first collection. Organic phase samples were completely dried, utilizing speed vacuum at a temperature of 37 °C; the dried samples were stored at −80 °C and resuspended in 1:1 chloroform/methanol for mass spectrometry analysis. The aqueous phase was used for liposome flotation assay and protein–lipid overlay assay.

4.10. Sample Preparation for Protein Flotation Assay. Fifteen micrograms of total protein from the aqueous phase sample was added to four times the volume of acetone at 4 °C and incubated at room temperature for 15 min. Samples were centrifuged at 21 000g at 4 °C for 30 min (Thermo Fisher Megafuge 8R). The supernatant was discarded, and the pellet (very small) was air-dried for 10 min. The pellet was resuspended and reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 45 min at room temperature. Following reduction, the resuspended pellet was alkylated with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min while maintained in darkness. Two times the volume of acetone at room temperature was added and centrifuged at 21 000g at room temperature for 15 min (Thermo Fisher Megafuge 8R). The supernatant was discarded, and the pellet (very small) was air-dried for 10 min. The pellet was resuspended in 0.1 μg/μL of chymotrypsin (Promega, V106A) in 15 mM 4-ethylmorpholine and incubated overnight (~16 h) at 37 °C. Samples were centrifuged at 21 000g at room temperature for 5 min (Thermo Fisher Megafuge 8R) and dried, utilizing speed vacuum at a temperature of 40 °C, leaving about 50 μL of sample. Samples were stored at −80 °C.

4.11. High-Performance Liquid Chromatography (HPLC)—Mass Spectrometry. Mass spectrometry lipidomic chromatography and identification were carried out as described in detail in our previous publication. In brief, samples were run through an Acclaim C30 column (particle size 3.0 μm, 150 × 2.1 mm² ID; Thermo Fisher Scientific, Waltham, MA). An HPLC Accela instrument (equipped with an autosampler and a 600 pump) was coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Heated electrospray ionization (ESI) was used as the method of ionization by coupling a HESI probe to the Q-Exactive instrument. The instrument was set at full scan with a collision energy of 30 and 19 eV in positive mode. Lipids were identified using LipidSearch software version 4.1, developed by Ryo Taguchi and Mitsui Knowledge Industry Co. (Tokyo, Japan). Mass spectrometry proteomics was performed on a Q-Exactive instrument after fractionation on a coupled Easy nLC 1000 nano-liquid chromatography system (Thermo Fisher Scientific), as described in our other published reports. Peaks were generated using a Thermo Scientific Xcalibur (version 4.1.31.9, released 2017); proteins were identified using Proteome Discoverer 2.2 (version 2.2.0.388, released 2017). UniProt sequence database was used for the identification of proteins (downloaded July 2019). Proteome Discoverer search parameters for chymotrypsin-digested enzymes (mass missed cleavage sites: 2, min peptide length: 6, max peptide length: 144), post-translational modification: deamidation (+0.984 Da (R), max modification per peptide: 3), precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.02 Da, signal/noise threshold for spectra: 1.5, false discovery rate (FDR): strict for PSMs 0.01, and strict for peptides 0.01. In brief, false discovery rates are calculated as follows: first, the software ascertains whether there are q-values and PEPs available for PSMs. If so, the software uses them and assigns the PSM confidences based on target FDRs for PSMs. Next, the software calculates q-values and PEPs for peptides engaging the quality algorithm. Peptide confidences are then assigned based on target FDRs for peptides. If there are no q-values and PEPs available for PSMs, the PSM confidences are set based on our defined target FDRs for PSM employing the respective search engine scores.

4.12. Liposome Extrusion and Liposome Flotation Assay. Candidate lipids were purchased at Avanti Polar Lipids Inc. (PC 16:0/22:6 cat no. 850461, PC 16:1/16:1 cat no. 850358, PI 18:0/20:4 cat no. 850144, PS 18:0/18:1 cat no. 840039, LPC 18:1 cat no. 855773, LPC 18:0 cat no. 855774, LPC 16:0 cat no. 855675, LPC 14:0 cat no. 855575, PC 16:0/20:4 cat no. 850459, PC 18:1/18:1 cat no. 999989). Lipids were resuspended in chloroform, and 30 μg of lipid was aliquoted in glass vials, desiccated in speed vacuum, and resuspended in 100 μL of PBS using sonication. Lipids were extruded using the NanoSizer MINI extruder kit (T&T Scientific, TT-030-0001) through a 100 nm NanoSizer (T&T Scientific, TT-002-0010). Extruded liposomes (100 μL) were incubated with 2.5 μg (1 μL) of isolated ex vivo mouse protein from the lipid–protein extraction of CE fractions (no-injection control, sham-injection control, and EAE groups; see Section 4.9) for 30 min at room temperature. Liposomes/micelles and protein mixture were then resuspended in 50% sucrose. Sucrose gradient was prepared by layering from bottom to top over 1 mL of lipidic/micelle–protein mixture in 50% sucrose, 2 mL of 25% sucrose in PBS, and 1 mL of PBS. The gradient was ultracentrifuged at 114 000g at 4 °C for 3.5 h. Fractions were collected using a mechanical pum to aspirate 1 mL of the bottom fraction followed by 2 mL of the middle fraction and 1 mL of the top fractions. The collected fractions were washed using PBS followed by centrifugation at 18 000g 4 °C for 25 min (Beckman Rotor GH-3.8). The supernatant was discarded, and the pellet was processed for dot immunoblot analysis, as described above.

4.13. Protein–Lipid Overlay Assay. Candidate lipids listed above were dotted on a PVDF membrane at 30 μg per dot and allowed to completely dry. The membrane was
incubated for 1 h at 4 °C with 2.5 μg of isolated ex vivo mouse protein from the lipid–protein extract of CE fractions (no-injection control, sham-injection control, and EAE groups; see Section 4.9) in PBS, followed by cross-linking at 1200 × 100 μJ for 50 s (Stratagene, La Jolla, CA, Stratagene UV crosslinker, model 1800). The membrane was blocked, washed, and probed using primary and secondary antibodies, as described in Section 4.7.

4.14. MBP–Lipid Deimination Assay. Candidate lipids (30 μg per lipid) listed above were incubated in the presence of 10 μg of a vendor-purified MBP protein (Millipore, 13-104, Burlington, MA) in PBS and allowed to interact for 30 min at room temperature. Sequence alignment of bovine, mouse, and human MBP demonstrated high conservation between species (Figure S5). After incubation, each reaction was adjusted to a final concentration of 1.66 mM CaCl₂ and incubated overnight at 37 °C in the presence of a purified peptidyl arginine deiminase (PAD, Sigma-Aldrich, P1584). Proteins were then precipitated using standard acetone precipitation protocols and analyzed as described in Section 4.7. Densitometry analysis was done using ImageJ program (National Institute of Health, Bethesda, MD). Deiminated MBP (MBP–PAD) densitometry signal was normalized to the MBP signal, and fold change was adjusted to MBP.

4.15. Circular Dichroism. Candidate lipids (PI and LPC, 180 μg per lipid) and 60 μg of a vendor-purified MBP (Millipore, 13-104, Burlington, MA) were separately resuspended in CD buffer (10 mM potassium phosphate, 50 mM ammonium sulfate). Circular dichroism (Jasco, Jasco J-815, Easton, MD) settings included measured range 250–190 nm, data pitch 1 nm, bandwidth 3 nm, scanning speed 20 nm/min, data integration time (D.T.) 2 s, and variable temperatures 20 and 105 °C. Baseline measurements were recorded for the CD buffer, and candidate lipids were resuspended in CD buffer before binding assays were performed. Following these measurements, a vendor-purified MBP was added to the corresponding candidate lipids or CD buffer sample and allowed to interact for 30 min at room temperature. CD spectra were recorded for MBP–lipid samples at room temperature and MBP without the presence of any lipids at two temperatures (20 and 105 °C). Baseline CD buffer and candidate lipid spectra were subtracted from their corresponding sample. Samples were then incubated overnight at 37 °C in the presence of purified PAD and 1 mM calcium phosphate. CD spectra were recoded, and baseline spectra were subtracted for each sample. CD analysis was done on the CD analysis and plotting tool (CAPITO) software (mean with SEM).

4.16. Computational Modeling. There is no atomic structure available for the full-length MBP. Previous NMR studies had indicated that a part of the sequence adopts a helical structure, while additional parts are unstructured. Previous studies have suggested that MBP may be an intrinsically disordered protein (IDP). A check with the PrDOS disordered structure prediction server indicated that MBP sequence matches the criteria for disordered proteins. Robetta server (http://new.roberta.org/) was used to obtain structure prediction (based on the Rosetta method) for MBP. We used five best predictions provided by the server as the starting point and performed MD simulations, allowing MBP to explore the landscape in the presence of water, as well as in the presence of PC and LPC.

4.17. Model Preparation. AMBER’s LEaP module was used for model preparation. Starting with the five structural predictions obtained from the Robetta server (secondary structure depicted in the Supporting Information; Figure S4), five separate systems were prepared. Each structure was immersed in a rectangular box of explicit solvent (extended simple point charge (SPC/E)) such that the protein coordinates are at least 10 Å from the edge of the box, and counterions were added to make the system charge neutral. AMBER’s f14SB was used for all simulations.

For PC and LPC systems, the coordinates for the protein were surrounded with 25 molecules of LPC or PC, using a model development procedure used and validated in our lab for other protein systems in nonaqueous solvents. These calculations were performed by PackMol such that there was one molecule of MBP for every 25 molecules of lipid, in a cube of 80 Å × 80 Å × 80 Å. The size of this box was selected after trial and error, such that the lipid molecules could easily fit but the cube is smaller in size, possibly to keep the number of atoms in the system at a minimum. Then, the system was immersed in a box of water similar to the case of water-only simulations. The charges for PC and LPC lipids were obtained using the protocol described in AMBER, and f14SB parameters were used.

4.18. MD Simulations. Each of the 15 systems (five in water, five in PC + water, and five in LPC + water) was equilibrated separately using a protocol developed in our laboratory. Equilibration removes vacuum bubbles and equilibrates the density closer to the density of water and was found to be in the range of 1.05–1.08 g/mL for the 15 model systems. The final temperature was 300 K. Following the equilibration, each of these systems was slowly heated to 305, 310, 315, and 320 K to generate conformational diversity in the MD simulation of 1 ns. These four points were separately cooled to 300 K. Therefore, for each of the 15 systems, there were five starting conformations (the equilibrated conformations at 300 K and four additional ones obtained by heating and cooling) to represent the possible conformational diversity.

For each of these 15 systems with five different starting conformations, production MD simulations were performed for a duration 100 ns under constant energy conditions (NVE ensemble). A total of 2.5 μs aggregated MD sampling was obtained for each system (with water, with water + LPC, and water + PC). NVE was used as this ensemble offers better computational stability and performance for longer MD simulations. The production simulations were performed at a temperature of 300 K and a time step of 2 fs (with SHAKE applied to bonds and angles involving hydrogens). As NVE ensemble was used for production runs, these values correspond to the initial temperature at the start of simulations. A temperature adjusting thermostat was not used in simulations; over the course of 200 ns simulations, the temperature fluctuated around 300 K, with the fluctuations between 2 and 4 K, which is typical for well-equilibrated systems. A total of 100 conformational snapshots collected from each MD run was used for analysis.

4.19. Computational Modeling Analysis. The conformational snapshots from five runs with different starting conformations were collected and analyzed. Therefore, a total of 2500 conformations were analyzed for MBP in water, MBP in PC and water, and MBP in LPC and water. The goal here was to capture as much conformational diversity that MBP samples in the disordered state. The radius of gyration was calculated for the three systems separately, for each of the five
structures. Secondary structure was analyzed using AMBER’s PTRAJ program. Previously, we have successfully used the radius of gyration as a measure of conformational flexibility of proteins.

4.20. Statistics. Data are expressed as mean ± standard error (S.E.) of three separate replicates. Statistical significance was assessed using paired (for PERG data) and unpaired (remaining data) t-tests with Prism software version 8 (GraphPad). p < 0.05 was considered statistically significant. Lipid abundance ratios were normalized to the protein concentration found in each samples used for lipidomic analysis. Deimination levels were the measurement of raw densitometry for Cit-MBP normalized to MBP densitometry after membrane stripping. Deimination fold changes reported were all compared and normalized to the endogenous levels of deimination in the MBP sample.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01590.

Confirmation of the EAE model, variation of hydrocarbon chain, computational modeling of MBP, and five model structures of MBP and MBP sequence alignment (Figures S1–S5); mass spectrometry results for arginine residues and confirmation of MBP presence in gel-excised band (Table S1); and computer simulations of MBP (Movies S1–S3) (ZIP)

Accession Codes

MBP (UniProt accession: P02686-5 and P02687). Mass spectrometry data (PRIDE archive accession: PXD015494).

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A.O.V. and S.K.B., conceptualization; A.O.V. and P.K.A., data acquisition and curation; A.O.V., formal analysis; A.O.V. and S.K.B., supervision; S.K.B., funding acquisition; A.O.V., validation; S.K.B., principal investigator; A.O.V. and S.K.B., methodology; A.O.V., writing-original draft; A.O.V. and S.K.B., project administration; and A.O.V. and S.K.B., writing: review and editing.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MS, multiple sclerosis; MBP, myelin basic protein; PE, phosphatidylethanolamines; PC, phosphatidylcholines; PI, phosphatidylinositol; PS, phosphatidylserines; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; PERG, pattern electroretinogram; FERG, flash electroretinogram; BHT, butylated hydroxytoluene; MTBE, methyl tert-butyl ether; OCT, optical coherence tomography; CE, capillary electrophoresis; PAD, peptidyl arginine deiminase; LFA, liposome flotation assay; PLOA, protein–lipid overlay assay; CD, circular dichroism; MD, molecular dynamic; IDP, intrinsic-disordered protein; CNS, central nervous system

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