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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**LECTIN COMPLEMENT ACTIVATION PATHWAY IN
MULTIPLE SCLEROSIS**

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Puneet Vinod Rana

Committee in charge:

Professor Kumud K. Singh, Chair
Professor Yang Xu, Co-Chair
Professor Michael David

2012

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The Thesis of Puneet Vinod Rana is approved, and is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2012

DEDICATION

This is dedicated to my family and friends. Thank you all for always being so encouraging, supportive, and loving.

EPIGRAPH

“Logic will get you from A to B. Imagination will take you everywhere.”

Albert Einstein

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ABSTRACT OF THE THESIS

LECTIN COMPLEMENT ACTIVATION PATHWAY IN MULTIPLE SCLEROSIS

by

Puneet Vinod Rana

Master of Science in Biology

University of California, San Diego, 2012

Professor Kumud Singh, Chair

Multiple sclerosis (MS) is an inflammatory autoimmune disease leading to myelin loss and axon damage. Studies have shown the complement to be a major player in MS damage, however no research has evaluated the novel role of the lectin mediated complement activation pathway in post-mortem brain tissue with MS. These presented studies have provided insight on the role of the lectin complement activation proteins in MS brain. Our analyses show an increased expression of mannose binding lectin (MBL) and MBL-associated serine protease 2 (MASP-2) in both the white matter and gray matter of MS brain compared to healthy controls. Co-localization of MBL with markers

of axonal damage, neuroinflammation, and immune complexes suggests MBL and associated proteins may play an important role in the development of neuropathological characteristics of MS. Genetic variants of *MBL2* and *MASP-2* that lead to altered protein expression and function were not associated with the risk of the MS, however the small sample size did not provide high statistical power. Finally, Autoantibodies detected against MBL in the MS vs. non-MS healthy brain tissue may also lead to further tissue damage through antibody-dependent complement activation. Altogether, this study suggests that the lectin complement activation pathway could promote and prime aggressive immune response leading to brain tissue damage in MS and might be developed as a biomarker for MS. MBL based therapeutics could also be developed against MS related neuroinflammation and neurodegeneration.

I:

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune and neurodegenerative disorder of the central nervous system (CNS). It is characterized by neuroinflammation, demyelination, axonal damage, and neuron loss (Calabresi P, 2004; Petersen T, 2009). Affecting 2.1 million people worldwide and 400,000 in the United States alone, MS is the most common neurological disability in young adults (National Multiple Sclerosis Society, 2010).

In MS, the body's immune system attacks myelinated axons, destroying myelin and damaging the axons in the white matter of the brain (Weinshenker B, 1996; Olek M, 2011). This attack disturbs and distorts information transfer via nerve impulses leading to a variety of symptoms. Common symptoms of MS include muscle weakness, paralysis, sensory disturbances, lack of coordination, and visual impairment (Steinman L, 2001).

MS is typically categorized into four disease courses: relapsing-remitting (RRMS), primary-progressive (PPMS), secondary-progressive (SPMS), and progressive-relapsing (PRMS). Of these the most common is RRMS, affecting about 85% of MS patients. RRMS is characterized by periods of exacerbations, relapses, followed by periods of remission, when symptoms disappear (Figure 1a). Most patients who initially have RRMS may eventually enter SPMS. SPMS is characterized by a continuous progression with or without superimposed relapses (Figure 1b). PPMS is characterized by continuously progressing disability without any relapses, but sometimes patients have periods of plateaus or minor improvement (Figure 1c). PPMS affects about 10-20% of the patients with MS. Finally, the least common course of MS affecting fewer than 5% of

patients, PRMS is characterized by progression of the disease from onset with periods of relapses (Figure 1d) (Goldenberg MM, 2012; Bitsch A, 2002).

Though there still is no cure for MS, there are effective strategies in altering the disease course. Current FDA-approved disease-modifying agents include interferon beta-1alpha and 1beta therapy, glatiramer acetate treatment, fingolimod treatment, mitoxantrone treatment, and natalizumab treatment. The first-line immunomodulatory therapies for the relapsing forms of MS include interferon beta 1 and glatiramer acetate treatment. The second-line of therapy for MS patients include immunosuppressive drugs such as natalizumab and mitoxantrone. Finally, fingolimod was the first oral medication for relapsing forms of MS (Sperandeo K, 2011). All four drugs alter the disease course by modulating immune response, but do not inhibit disease progression.

Although the exact immunopathology of MS remains uncertain, evidence suggests that MS is a multifactorial disease involving viral infection (e.g., herpes simplex virus), genetic factors (e.g., HLA alleles), environmental factors (e.g., vitamin D synthesis from sunlight), and impairment of the immune system (Hogancamp WE, 1997). MS linked autoimmunity has been mostly attributed to adaptive immune system dysfunction, but recent advances have also implicated innate immune mechanisms (Coles AJ, 2008; Franciotta D, 2008). It has been reported that complement proteins, major contributors of the innate immune system, are found in elevated levels in MS plaques (Compston DA, 1989; Lumsden CE, 1971). MS associated demyelination can be induced by direct complement activation after its binding to myelin by recruiting inflammatory cells and subsequently activating the membrane attack complex. In the brain, this can

result in the damage and lysis of oligodendrocytes and also the chemoattraction and infiltration of macrophages (Veerhuis R, 2011; Rus H, 2006).

Complement is a key component of the innate immune system and consists of a complex network of over 30 soluble and cell associated factors that contribute to both innate and adaptive immune control (Carroll MC, 2004). Complement is also an important player in handling of altered self in response to injury (necrosis or apoptosis) (Korb LC, 1997; Botto M, 1998). Activated complement components act via inflammatory mechanisms to clear and remove infectious agents and injured cells, however excessive complement activation can lead to tissue damage (Kemper C, 2007; Walport MJ, 2001, Carroll MV, 2011).

The complement cascade is initiated through three pathways: classical pathway, alternative pathway, and lectin pathway (Figure 2). The classical, antibody dependent pathway is activated by the binding of C1q with antibodies bound to pathogen surface whereas the alternative and lectin pathways are antibody independent (Wallis R, 2002; Friec G, 2009). Activation of the alternative pathway occurs when C3b complexes with pathogens directly. The lectin pathway involves use of collectin proteins that attach to carbohydrate patterns on the surface of pathogens. In MS, all three pathways seem to play a role in complement activation (Ramaglia V, 2012; Weber MS, 2011; Bouwman LH, 2006).

Out of these three pathways, the lectin mediated complement activation pathway is important because it involves a soluble pattern recognition molecule named mannose-binding lectin (MBL) that can play an important role in promoting autoimmunity. MBL is

primarily synthesized in the liver and is distributed throughout the body via the bloodstream. MBL RNA and protein expression has been shown to also be produced by brain cells (Yanai I, 2005).

MBL is an oligomer of polypeptide chains that are composed of a collagen like region linked to a calcium-dependent carbohydrate recognition domain (CRD) (Tsutsumi A, 2005). Circulating MBL can be composed of higher order oligomeric structures including dimer, trimers, tetramers, pentamers, and hexamers (Figure 3a). The cluster like array of multiple CRD binding sites allows activation of complement to be most effective (Tsutsumi A, 2005). After encountering a pathogen, MBL binds the antigen via carbohydrate recognition domain which complex with D-mannose, N-acetylglucosamine, and glucose residues present on the surface of pathogens, activates MBL-associated serine protease 2 (MASP-2) and leads to C3 activation, MBL-mediated opsonization and phagocytosis of the pathogen (Endo Y, 2006; Downing I, 2003; Dahl MR, 2001; Thiel S, 1997; Matsushita M, 2000) (Figure 3b). In addition to recognizing foreign antigens, MBL is also involved in clearing of damaged or apoptotic cells (Malhotra R, 1995; Nauta AJ, 2004; Nauta AJ 2003; Roos A, Ogden CA, 2001; Russell R, 1998; Duvall E, 2006). MBL also plays an important role in autoimmunity, too excessive or very low levels of MBL can lead to autoimmunity and tissue injury (Carroll M, 2001; Ezekowitz RA, 2003).

Many studies have suggested that MBL deficiency caused by the presence of *MBL2* gene genetic variants may be associated with the autoimmune disease systemic lupus erythematosus (SLE) (Garred P, 2001). There are four common genetic variants of the *MBL2* gene. Two single nucleotide polymorphisms (SNPs) in the promoter region

H/L (position -50, G to C nucleotide substitution) and X/Y (position -221, C to G nucleotide substitution) and one SNP at the 5' untranslated region P/Q (position +4, C to T nucleotide substitution) are associated with lower MBL levels, X variant being the strongest down regulator of MBL expression (Heitzender S, 2012). There are three SNPs located on exon 1 of the *MBL2* gene: B (codon 54, glycine to aspartic acid), C (codon 57, glycine to glutamic acid), and D (codon 52, arginine to cysteine). All three SNPs have a dominant effect on the level of functional MBL in serum even in heterozygote form, decreasing functional MBL by approximately 90%. However, the effect of the D allele in heterozygotes is less dramatic than B or C. Also, the MBL variant proteins are less stable (shorter half-life) and are easily degraded from higher oligomeric forms (Garred P, 2008). These variants may not only lead to reduced MBL function, but also to reduction in MBL concentration in circulating blood.

MBL function is linked to MBL-associated serine proteases (MASPs). Of the MASPs, MASP-2 is the most relevant serine protease of MBL because it is needed for cleavage of C4 and C2 complement components to generate the C3 convertase (Rossi V, 2001; Vorup-Jensen T, 2000). Genetic variants in the *MASP-2* gene may lead to a reduction in MASP-2 levels and misfolding in the protein leading to inability to associate with MBL (Thiel S, 2007). In such a case, MBL complement activation would be impeded or inhibited.

While a pathogen infection may not be the primary cause of MS, critical injury of the CNS may initiate complement activation and lead to the progression of MS. A neuroinflammatory response to a CNS injury may also result in MS related

neurodegeneration (Yong VW, 2010). Limited studies have evaluated the role of MBL mediated complement pathway in innate immune response in MS disease (Petersen T, 2009; Christensen T, 2007), however these studies assessed the role of MBL or MASPs only in the context of MS cases with human endogenous retrovirus infection (Christensen T, 2007) or related interferon-beta therapy (Petersen T, 2009). In a recent pilot study (Kwok JY, 2011), Dr. Singh group has evaluated the expression of MBL, MASP-2 and functional MBL (MBL/MASP-2 mediated C4 cleavage activity) in plasma and cerebrospinal fluids from multiple sclerosis patients and analyzed their association with the risk of MS and MS subtypes. Results showed that components of MBL complement pathway are activated in MS disease and could be potentially involved in the risk or progression of multiple sclerosis. This study is focused on understanding the potential role of MBL and associated proteins in the neuroinflammation, neurodegeneration, and immune complex formation leading to the MS neuropathological characteristics studied in post-mortem brain tissues.

II:

MATERIALS AND METHODS

2.1 Characteristics of the post-mortem brain samples

A total of 30 frozen brain tissue samples were obtained from the Human Brain and Spinal Fluid Resource Center in Los Angeles, California (Figure 4). Autopsy was performed within 40 hours of death of all samples received. Of the 30 subjects from whom the postmortem brain tissues were evaluated for these studies, 10 were non-MS healthy brain tissue, and 20 had MS disease. Neuropathology diagnosis of the MS brain revealed 4 cases of primary progressive MS, 11 cases of secondary progressive MS, 2 cases of relapse remitting MS, and 3 unclassified cases of MS. All brain samples were taken from the cortex and separated by normal appearing gray matter (NAGM) and normal appearing white matter (NAWM) and were well characterized. Of the healthy control cases 4 were female and 6 were male aging from 57 to 85 years old with an average age of 74.3. The MS cases were composed of 12 females and 8 males aging 38 to 82 years old with an average age of 63.6. All 30 samples were from Caucasian subjects. Despite the use of drug therapy and their potential effects on neuropathogenesis, diagnostic characteristics of multiple sclerosis were observed in all MS brain tissues.

2.2 SDS Page and Western Blot

Total protein extracts from MS and normal postmortem brain tissue were prepared by using an AllPrep DNA/RNA mini kit (Catalog # 80204, Qiagen Inc., Valencia, CA) in conjunction with the supplementary protocol for protein purification via acetone precipitation. 4 volumes of ice-cold acetone were added to the flow-through of

the RNA column. Samples were incubated on ice for 30 minutes and centrifuged at max speed in a bench top centrifuge for 10 minutes.

Protein was quantified using bicinchoninic acid kit (Catalog # 23227, Thermo Scientific). A total protein of 15 µg from each brain tissue was separated on 4% to 12% NuPAGE Bis-Tris Gel electrophoresis (Catalog # NP0322BOX, Invitrogen) for 45 minutes at constant 200V. Completed gels were transferred onto nitrocellulose membrane using iBlot Gel Transfer Device (Catalog # IB1001, Life Technology Corporation) and developed with MBL and MASP2 antibodies. A mouse monoclonal anti-β-actin antibody was used as a reference housekeeping protein, a loading control and for MBL/MASP2 quantitation by band intensity normalization using Image J software (NIH, USA). Magic Mark XP western protein standard (Catalog # LC5602, Invitrogen) and Novex Sharp Prestain Protein Marker (Catalog # LC5800, Invitrogen) were used for locating protein bands.

Primary antibodies used included rabbit polyclonal anti-MBL2 antibody (Catalog #HPA002027, Sigma-Aldrich) diluted 1:1000, rabbit polyclonal anti-MASP2 antibody (Catalog # AB65897, Abcam) diluted 1:5000, and 1:4000 diluted mouse monoclonal anti-β-actin (Catalog # A2228, Sigma-Aldrich).

Secondary antibodies anti- mouse/rabbit IgG conjugated alkaline phosphatase were obtained from the WesternBreeze Chemiluminescent kit (Catalog # WB7106, Life Technologies Corporation). Detection was accomplished with CDP-Star Chemiluminescent substrate for alkaline phosphatase. Protein bands were captured by Kodak X-OMAT Blue film (Catalog # NEF596, PerkinElmer).

2.3.1 Immunofluorescence analysis of MBL and MASP-2

Brain tissue samples were sliced into 3mm³ thick sections using a sterile razor blade. Samples were then incubated in formalin for 24 hours and sent to the UCSD Cancer Center Histology Lab for further processing, paraffin block formation and tissue sectioning.

Paraffinized tissue sections were incubated at 60°C for 1 hour, washed with citrisolve clearing agent (Catalog #22143975, Fischer Scientific) and rehydrated in decreasing concentrations of ethanol (50:50 citrisolve and ethanol, 100%, 75%, 50%, 25%, and phosphate buffer saline, PBS) Sections were treated with permeabilization buffer containing saponin (Catalog # PB001, Invitrogen) for 5 minutes at room temperature followed by heat induced epitope retrieval in 10mM sodium citrate buffer, pH 6.0 (Catalog # AP9003-500, Thermo Scientific). For epitope retrieval, tissue sections were placed in a 650W microwave oven and heated intermittently for 10 minutes and allowed to cool to room temperature. Sections were washed with PBS for 20 minutes and nonspecific sites were blocked in 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.

Rabbit polyclonal anti-MBL2 (Catalog #HPA002027, Sigma-Aldrich), marker of axonal damage Goat polyclonal anti-Amyloid Precursor Protein (APP) (Catalog # Ab105122, Abcam), goat polyclonal anti-MASP2 (Catalog #SC17905, Santa Cruz Biotechnologies Inc.), marker for monocyte chemoattractant protein (MCP1) a marker of neuroinflammation (Catalog # SC1305, Santa Cruz Biotechnologies Inc.) and one of the brain cell makers, mouse monoclonal anti-microtubule associated protein (MAP2) for

neurons (Catalog # SC74421, Santa Cruz Biotechnology Inc.) and mouse monoclonal anti-myelin oligodendrocyte glycoprotein (MOG) for oligodendrocytes (Catalog # SC73330, Santa Cruz Biotechnology Inc.) or rat monoclonal anti-myelin basic protein (MBP) (Catalog # ab7349, Abcam) (were diluted 1:200 in PBS with 1% BSA and were incubated overnight at 4°C. Next day, after washing for 20 minutes with PBS, sections were incubated in the dark for 1 hour with donkey anti-rabbit IgG conjugate Alexa Fluor 488 (Catalog # A21206, Invitrogen) for probing MBL antibody, donkey anti-mouse IgG conjugate Alexa Fluor 568 (Catalog # A10037, Invitrogen) for probing either MAP2 or MOG, donkey anti-goat IgG conjugate Alexa Fluor 647 (Catalog # A21447, Invitrogen) for probing either APP or MASP2, and chicken anti-rat IgG conjugate Alexa Fluor 647 (Catalog # A21472, Invitrogen) for probing C3d were diluted 1:200 in PBS with 1% BSA. After incubation with secondary antibodies, sections were washed for 20 minutes in PBS, air dried for 3 hours and were mounted with Prolong Gold anti-fade reagent (Catalog # P36931, Invitrogen) containing DAPI (4', 6-diamidino-2-phenylindole) and covered with a cover slip. Mounted sections were air dried overnight and analyzed by confocal microscope. In each case, control experiments were conducted with IgG antibody, serum, primary or secondary antibody alone.

Appropriate care was taken to avoid the cross-reactivity and nonspecific binding of antibodies. We used confocal microscopy (Olympus FV1000, UC San Diego Neuroscience Microscopy Facility) to capture emission wavelength of individual fluorescent dyes. Fluorescent dyes used, such as DAPI (358 nm excitation and 461 nm emission) for nuclei, Alexa Fluor 488 (496 nm excitation and 519 nm emission) for MBL,

Alexa Fluor 568 (578 nm excitation and 603 nm emission) for cell marker, and Alexa Fluor 647 (650 nm excitation and 665 nm emission) for APP have significant gaps between the emission wavelengths ruling out the possibility of cross interference. All secondary antibodies were selected from different species (e.g., donkey, chicken) compared with the primary antibodies (e.g., rabbit, mouse, goat, rat) to further eliminate any cross-reactivity between the species in which the antibodies were raised. Also, immunofluorescence-staining experiments were repeated several times with different cases to ensure reproducibility of results.

2.3.2 Confocal Microscopy

The immunostained sections were visualized with Olympus FV1000 Confocal Microscope with appropriate excitation/emission filter pairs (403 nm laser window 410–483 nm, 488 nm laser window 493–538 nm, 543 nm laser window 548–628 nm, 647 nm laser window 651–672 nm). Images were collected using the microscope in sequential plane mode with an average of 4 and a format of 1024×1024 pixels using X100 oil immersion lens. Images were exported to Olympus FV1000 Viewer software Ver.02.00 to generate the final figures. Immunofluorescence of the positive cells co-localized with MBL and respective cell markers were counted in 5 areas of a section (4 corner areas and 1 central area of a section in at least 3 slides per case). Percent increase for the expression of MBL from non-MS to MS cases was calculated and *P* value was determined using the 2-tailed unpaired *t*-test.

2.4 DNA Genotyping Real Time PCR

Genomic DNA was extracted from post-mortem brain tissue using AllPrep DNA/RNA Mini Kit (Catalog # 80204, Qiagen Inc., Valencia, CA). DNA was genotyped for genetic variants in the exon and promoter regions using hybrid probes in real-time PCR melting curve analyses using LightCycler 1.0 (Roche Diagnostics Inc.). Mutants were distinguished from wild-type alleles by a distinct melting peak temperature (T_m). T_m will vary due to a single nucleotide mismatch between the amplification product and hybridization probe (Figure 5). The LightCycler software generated a melting curve (dF/dT vs. $^{\circ}C$) using the change in fluorescence resonance energy transfer (FRET) between the detection and anchor probe.

2.4.1 *MBL2* Genotyping

Single nucleotide polymorphisms (SNP) in the exon 1 region of *MBL2* gene were studied (Steffensen, 2003). Three genetic variants occurred at codons 53, 54, and 57 at the nucleotide position 223 (C to T, Arg53Cys, A/D allele), 230 (G to A, Gly54Asp, A/B allele), and 239 (G to A, Gly57Glu, A/C allele). A/A is wild type and A/O is a heterozygotes including A/B, A/C, or A/D genotypes. Homozygous mutant, O/O, includes B/B, B/C, B/D, C/C, C/D, and D/D genotypes.

SNPs at the promoter region -550 (G to C, X/Y variant) and -221 (G to C, H/L variant) along with SNP in the 5' untranslated region +5 (C to T, P/Q variant) were also studied. The Y, H and P alleles are more common.

The following pair of primers/probes for detecting A/O polymorphisms was used: Forward primer- 5' – CATCAACggCTTCCCAGgC and reverse primer- 5' – TgggCTggCAAgACAACCTATTA_g. The following pair of probes was used to detect the A/O alleles: EX1FITC- 5'-CAGCCCAACACgTACCTggTTCCCCCT—FL and EX1LC640- 5'-LC640-TTCTTCCTTggTgCCATCACgCCCA—PH.

The following pair of primers/probes was used for detecting the X/Y, H/L, and P/Q polymorphisms was used: Forward primer- 5' – CCTgCCAgAAAgTAgAgAgg and reverse primer- 5' – CCTCACCTTggTgTgAgAAA. The following pair of probes was used to detect the X/Y alleles: XYFITC- 5'-TCTCACTgCCACggAAgCAT—FL and XYLC705- 5'-LC705-TTTATAgTCTTCCAgCAgCAACgCCA—PH. The following pair of probes was used to detect the H/L alleles: HLFITC- 5'-TTTTAgACA_ggCTTgCCTgggT—FL and HLLC640- 5'-LC640-AgCATTTTCTCTggAAATTTCTTACTACgTTgg—PH. The following pair of probes was used to detect the P/Q alleles: PQFITC- 5'-CAGAgggCATgCTCggTAA—FL and PQLC640- 5'-LC640-ATgTgTTCATTACTgAgATTAACCTTCCCTgAg—PH.

2.4.2 *MASP2* Genotyping

A SNP in the exon region of the *MASP-2* gene was studied (Tulio S, 2011). The genetic variant occurred at codon 120 at exon 3 (A to G, Asp120Gly).

As above, the polymorphism was genotyped by real time PCR melting curve analysis. The following pair of primers/probes was used: Forward primer- 5' – CCTggCAAggACACTTTC and reverse primer- 5' – ATgTTgCAGgACCCCTCTT. The following pair of probes was used to detect the A/G alleles: MASP2Anchor- 5'-LC640-ggTAATgTCCAggCTggAgCCCAg—PH and MASP2Sensor- 5'-TTggAgTAgCCggAgCggA—FL.

2.4.3 *MASP2* Sequencing

PCR amplified DNA was run on a 2% agarose gel and visualized using ethidium bromide (Figure 6). The separated DNA of interest was excised and extracted using Gel extraction kit (Catalog # 28706, Qiagen Inc.) and was subsequently analyzed by sequencing.

2.5 ELISA for Autoantibody Detection

Wells were coated overnight with 1µg/mL of recombinant human MBL (R&D Systems, Catalog # 2307-MB-050) in carbonate-bicarbonate buffer at 4°C. Wells were washed with TBS/Tw and then incubated in 1% BSA blocking solution. Wells were then

incubated in 30ug of brain tissue protein extracted via Qproteome Mammalian Protein Prep Kit (Qiagen, Catalog # 37901). Next, wells were washed and incubated in secondary antibody anti IgG conjugated with alkaline phosphatase. Wells were washed and incubated overnight in alkaline phosphatase yellow liquid substrate (Sigma-Aldrich, Catalog # p7998) and then read at 405nm (Figure 7).

III:

RESULTS

3.1 Increased overall MBL expression in MS cases compared to control post-mortem brain tissue as observed by western blots

We determined MBL expression by western blots. The 32 kDa monomers, 51 kDa dimers, and 78 kDa trimers of the MBL present in the post-mortem brain tissues were identified using the Image J software from NIH website (<http://rsb.info.nih.gov/ij/>) for quantitation of MBL expressed in the 2 groups of brain tissues (Figure 8). We found that MBL expression in gray matter was 1.99 fold higher in the post-mortem brain tissues of the MS cases compared with non-MS healthy controls (p value = 0.069). Expression of MBL in white matter was 2.00 fold higher in the post-mortem brain tissues of the MS cases compared to non-MS healthy controls (p value = 0.027). Overall combined MBL expression in both gray and white matter was 1.85 fold higher in MS brain as compared to normal healthy control brain (p value = 0.034). Only two representative cases from each group were shown. A normal housekeeping protein, β -actin, was used as a reference protein and as a loading control for comparing the expression of MBL in the brain tissue (n= 9 for normal cases and n=20 for MS cases).

3.2 Increased MBL Expression in MS Cases Compared to Control Post-Mortem Brain Tissue Observed by Immunofluorescence

Using antibodies against MBL and brain cell markers for oligodendrocytes and neurons we assessed the presence of MBL in the MS brain. In the post-mortem brain tissue from MS individuals, MBL displayed immunoreactivity in both neurons and

oligodendrocytes (Figure 9 and 10). This was determined by co-localization of MBL and the brain cell marker. Additionally, DAPI stain marked the nuclear DNA to ensure only cells with intact nuclei were studied.

Comparison of MBL expression in the brain tissue of MS subjects versus the healthy normal subjects was done. Overall, there was a 5.7-fold increase in the MBL expression of MS cases as compared to the normal cases. Analyzing the gray and white matter separately we see a 7.1-fold increase in the gray matter and a 2.8-fold increase in the white matter as compared to the non-MS healthy control brain (p value = $2e-8$ and $2e-5$, respectively, n=5 for normal cases and n=7 for MS cases).

This increase in MBL expression we observed via immunofluorescence was consistent with the western blot data indicating strong evidence of increased MBL expression in the MS brain.

3.3 MBL Co-localization with Marker of Neuroinflammation

We analyzed the expression of monocyte chemoattractant protein (MCP-1 aka CCL2), a marker of neuroinflammation, in association with MBL in MS brain to understand if MBL may lead to accumulation of MCP-1 (protein expressed in neuroinflammatory conditions including MS) (Conductier G, 2010) in MS brain (Figure 11). We observed a 3.4-fold increase in MCP-1 expression in MS brain as compared to non-MS healthy control brain (p value = $7.2e-10$, n=6 for MS cases and n=4 for normal cases). In addition to this increase of MCP-1 expression, we observed co-localization of

MCP-1 with MBL. This co-localized MBL and MCP-1 proteins suggest an active role MBL plays in the accumulation of MCP-1 in the studied MS brain tissue. We also found co-localization of MBL and MCP-1 specifically on oligodendrocytes, further suggesting MBL involvement in MS linked neuroinflammation (Figure 12). In addition to co-localization of MBL and MCP-1 on oligodendrocytes we also found co-localization on myelin using a marker for myelin basic protein (Figure 13).

3.4 MBL Co-localization with Marker of Axonal Damage

Since we found MBL co-localized with a marker of neuroinflammation we decided to investigate if MBL is linked to axonal damage. Amyloid precursor protein (APP), a marker for axonal damage in multiple sclerosis (Gehrmann J, 1995), was used to study MBL linked axonal damage. In the MS brain tissue there was a 5.2-fold increase in APP deposition as compared to the normal brain tissue (p value = 0.002, n=2 for normal cases and n=2 for MS cases). In addition to the increased APP deposition on neuronal axons, we found MBL to be co-localized with this marker of axonal damage in the MS brain (Figure 14). These findings suggest that MBL may potentially mediate axonal damage in multiple sclerosis.

3.4 MBL Co-localization with Marker of Immune Complexes

We analyzed expression of complement protein C3d, a component of immune complexes (Lood C, 2012), in association with MBL to understand the role of MBL in

formation of immune complexes. We found immune complex deposition in the MS brain in both gray and white matter (Figure 15). In addition to the immune complex formation in MS brain, we also detected MBL co-localization with C3d on neuronal axons. This suggests MBL may be involved in developing immune complexes in the MS brain.

3.5 Increased MASP-2 Expression in MS Cases Compared to Control Post-Mortem Brain Tissue by Western Blot

We determined MASP-2 expression by western blots (Figure 16). In addition to the MASP-2 alpha chain, degraded alpha chain products were identified in the post-mortem brain tissues. Using the Image J software from NIH website (<http://rsb.info.nih.gov/ij/>) for quantitation of MASP-2 expressed in 2 groups of brain tissues, we found that MASP-2 expression in the gray matter of post-mortem brain tissue was 1.89 fold higher the MS cases compared with those from non-MS healthy controls (p value = 0.040). Expression of MASP-2 in white matter was 2.47 fold higher in the post-mortem brain tissues of the MS cases compared to non-MS healthy controls (p value = 0.036). Overall total MASP-2 expression was 2.36 fold higher in MS brain as compared to normal healthy control brain (p value = 0.038).

Expression of MASP-2 in MS brain was 1.82 folds higher in white matter than in gray matter (p value = 0.040). In normal brain, MASP-2 expression was 1.49 fold higher in white matter than in gray matter (p value = 0.279). Only two representative cases from each group were shown. β -actin was used as a housekeeping reference protein and as a

loading control for comparing the expression of MASP-2 in the brain tissue (n=6 for normal cases and n=12 for MS cases).

3.6 Increased MASP-2 Expression in MS Cases Compared to Control Post-Mortem Brain Tissue Observed by Immunofluorescence

Using antibodies against MASP-2 and a cell marker for neurons we assessed the presence of MASP-2 in the MS brain. In the post-mortem brain tissue from MS individuals, MASP-2 displayed immunoreactivity in neurons (Figure 17). This was determined by co-localization of MASP-2 and the neuronal cell marker, MAP-2. Additionally, DAPI stain marked the nuclear DNA to ensure only cells with intact nuclei were studied.

Comparison of MBL expression in the brain tissue of MS subjects versus the healthy normal subjects was done. Overall, in the studied brain tissues, there was a 10-fold increase in the MASP-2 expression of MS cases as compared to the non-MS healthy control brain. Analyzing the gray and white matter separately we observed a 5-fold increase in the gray matter and a 11-fold increase in the white matter as compared to the normal healthy controls (p value 6.6×10^{-6} and 0.0001, respectively).

This increase in MASP-2 expression we observed via immunofluorescence was consistent with the western blot data indicating strong evidence of increased MASP-2 expression in the MS brain.

We also observed the expression patterns of MASP-2 and MCP-1 (Figure 18). We found a similar increase in co-localization of MASP-2 and MCP-1 as we did with MBL and MCP-1.

3.7.1 *MBL2* Genetic Variants Have No Significant Association with the Risk of MS

MBL2 exon 1 and promoter region genotyping was performed on 20 post-mortem brain tissue affected with MS disease and 10 non-MS healthy control post-mortem brain tissues to screen for the A/O, H/L, P/Q, and X/Y functional polymorphisms (Table 1a-d, respectively). Chi square test and Fisher exact test for the association of the alleles/genotypes were computed to determine the association of these polymorphisms with the risk of MS. We found no significant correlation between the genotypes and MS disease. ($p > 0.05$, unpaired t test, $n=10$ for normal cases and $n=20$ for MS cases).

3.7.2 *MASP-2* Genetic Variants Have No Significant Association with the Risk of MS

MASP-2 exon 3 genotyping was performed on 20 post-mortem brain tissue affected with MS disease and 10 non-MS healthy control post-mortem brain tissue to screen for A/G function polymorphism (Table 2). Chi square test and fisher exact test for the association of *MASP2* A/G alleles/genotype with the risk of MS did not show any significant correlation. ($p > 0.05$, $n=10$ for normal cases and $n=20$ for MS cases).

3.8 Anti-MBL Autoantibody Detection in MS Brain Tissue

Using an ELISA assay we developed in the lab, we detected anti-MBL autoantibodies in the MS brain. There was a 4-fold increase in anti-MBL autoantibodies detected in the gray matter and a 2-fold increase in the white matter of the MS brain (Figure 19). Overall there was a significant increase in anti-MBL autoantibodies produced in the MS brain (p value = 0.04, n=15 for MS samples and n=4 for normal samples).

IV:

DISCUSSION

Mannose-binding lectin is an important factor of the innate immunity, playing a key role in the clearance of bacterial, viral, fungal, and as well as necrotic or apoptotic cells. Although MBL is a key player in the clearance of altered self-cells, it can also act to prime and promote aggressive immune response that may lead to autoimmunity and tissue damage (Figure 20). Initial tissue damage due to inflammation or autoimmune attack in MS can lead to development of apoptosis of cells and excessive lectin pathway activation. Apoptotic cells can further initiate lectin pathway through changes on surface glycosylation pattern. Excessive lectin complement pathway activation may lead to priming and promoting aggressive immune response. Excessive debris and apoptotic cells can also lead to an impaired clearance, which can then form immune complexes that further promote immune responses. An aggressive immune response can result in enhanced neuroinflammation, axonal damage, demyelination, tissue injury, and autoimmunity.

A change in the serum MBL levels can contribute to autoimmunity in the following ways: low serum MBL levels result in impaired clearance of apoptotic and damaged cells while high serum MBL levels could lead to excessive complement activation. Both levels of serum MBL can potentially lead to promoting immune response resulting in autoimmunity and tissue damage (Bouwman LH, 2006). In studies involving autoimmune disease systemic lupus erythematosus (SLE), deficiencies in MBL have been shown to influence the course of SLE disease and its development by generation of autoantibodies to MBL (Seelen MA, 2003) and enhanced risk of infection while

increased serum MBL levels have been associated with inflammatory processes of SLE (Pradhan V, 2010).

Few groups have evaluated the role MBL plays in MS. In a small study involving one female individual with relapsing-remitting MS, it was found that the patient had undetectable MBL levels (Malhotra GK, 2012). Petersen et al. evaluated the role of the lectin complement pathway in the context of interferon- β therapy (Petersen T, 2012) and Christensen et al., found an increase in MBL levels in MS patients with human endogenous retrovirus infection (Christensen T, 2007). In a recent study, it was found that in the plasma and cerebrospinal fluid (CSF) of MS patients there was an increase in functional MBL and MASP-2 levels (Kwok JY, 2011). These findings suggest a potential involvement of the lectin complement activation proteins with the risk or progression of MS disease. Yet no studies to our knowledge, have evaluated the role MBL-mediated complement activation plays in the brain of MS patients. Brain tissue specific studies that show expression of MBL are more likely to correlate with the extent of neuroinflammation and neurodegeneration in MS.

The current studies had several strengths such as availability of well characterized brain tissue from MS cases and also availability of both gray and white matter from the same autopsy patient. Our work analyzed the MBL-mediated complement activation components in gray and white matter of multiple sclerosis brain. Through western blots and immunofluorescence, we have shown an increase in MBL and MASP-2 expression in the brains of MS subjects compared to normal healthy brain. Also, we show a change in MBL and MASP-2 expression patterns in gray and white matter of both MS and Normal

brain. Finally, using real-time PCR we see that there seems to be no correlation between the genetic variants of MBL and MASP-2 and developing multiple sclerosis, however this data may be inconclusive due to the small sample size and low statistical power.

This work is important because it adds to the much-needed research on investigating the pathogenesis of MS. These studies provided important results and pave a path for further research on potentially developing biomarkers for MS or MBL associated drugs therapies that may help to manage the neurodegeneration and demyelination in MS.

While evaluating the expression of MBL and MASP-2 proteins in MS post-mortem brain tissues, we found that MBL and MASP-2 showed an increased expression in both gray and white matter of MS brain as compared to normal healthy brain controls, emphasizing their importance in the innate immune aspect of the MS disease. The increase of lectin complement activation proteins implies a potential association of MBL and MASP-2 with MS associated neuronal injury. Increased MBL expression and deposition in neuronal cells and axons may not only mount an enhanced MBL-mediated complement activation and cytokine response, but it can also lead to axonal damage, thus aiding in the neurodegeneration and demyelination in the MS disease.

Through immunofluorescence we not only observed an increase in MBL and MASP-2 expression in MS brain compared to non-MS healthy brain, but we also found MBL and MASP-2 co-localization with MCP-1 in the studied MS brain tissues. MCP-1 is a marker of neuroinflammation and it is important in recruiting monocytes, macrophages, natural killer cells, dendritic cells, mast cells, basophils, and T lymphocytes to sites of

damage. Elevated levels of expression of MCP-1 are found in both acute and chronic MS plaques (Conductier G, 2010; Simpson JE, 1998). In addition to finding co-localization of MBL with MCP-1 on neurons, we also found co-localization of these proteins on oligodendrocytes as well as myelin itself. Finding lectin complement activation proteins in addition to markers of inflammation on sites where major MS attack occurs, further supports the claim that the lectin complement system plays in the tissue injury through induction of MCP-1 expression.

In addition to the MBL co-localization with a marker of neuroinflammation, we also found MBL co-localization with a marker of axonal damage, amyloid precursor protein (APP). APP has been found in multiple sclerosis lesions and it can potentially be used as a marker of axonal damage (Gehrmann J, 1995).

Furthermore, these data provide the first evidence of differential expression patterns of MBL and related lectin components in gray and white matter in non-MS normal healthy brain as well as multiple sclerosis brain in humans.

It is known that MS is a neurodegenerative disease targeting the white matter of the CNS. The brain white matter is primarily composed of neuronal axons that are wrapped in myelin, the major target for attack in MS. From our study, we found more than a 2-fold increase of both MBL and MASP-2 in the white matter of MS brain as compared to normal brain. As the white matter is the major site of axonal damage, it is not surprising to see an accumulation of such a large increase of proteins that activate the complement system. The deposition of complement proteins on myelin in MS lesions has been reported by a number of groups. Complement activation products have been found

on myelin sheaths, degraded myelin, as well as microglia and macrophages containing myelin in multiple sclerosis brain (Veerhuis R, 2011; Lumsden CE, 1971; Brink BP, 2005; Barnett MH, 2009; Compston DA, 1989). This large deposition or expression of MBL and MASP-2 in the white matter suggests that the lectin proteins may play an important role in activating complement in sites of demyelination and axon damage and loss in MS.

The gray matter of the brain is primarily composed of neuronal cell bodies. Analyzing the lectin components in the gray matter of MS brain also show an increase in the expression or deposition of MBL and MASP-2. This increase suggests an increase in the production of the lectin complement activation proteins in the neurons of MS subjects.

We did not observe any significant association between the *MBL2* and *MASP-2* genotypes and the risk of MS disease, however our sample size was not statically powerful. *MBL2* genotypes potentially associated with MBL deficiency were present in brain tissues; however their presence was not significantly associated with MS. *MASP-2* genotype associated with altered MASP-2 function and lower expression was found in one of the studied MS brain however it was not significantly associated with altered MASP-2 expression in the studied MS brain. This data suggest that the change in MBL and MASP-2 expression is not occurring at the DNA level.

MBL2 genotypes associated with MBL deficiency could lead to priming and promoting autoimmunity and tissue injury in MS brain because of an impaired clearance of damaged or apoptotic cells and myelin debris (Bouwman LH, 2006). However in the studied MS samples, we observed an increase in MBL and MASP2 expression. This

increase in lectin component deposition or expression can lead to excessive activation of complement and thus tissue damage.

The change in protein expression of MBL led us to evaluate if autoantibodies against MBL could be present in the MS brain. Upon investigation, we found that there were in fact autoantibodies produced against MBL in the MS brain. These autoantibodies may have been produced in consequence of the elevated levels of MBL. However, in addition to the MBL mediated neuroinflammation and axonal degeneration, these MBL autoantibodies can induce further brain tissue damage.

This study focused on understanding the novel role MBL potentially plays in the MS disease by conducting an end point study of MS post mortem brain tissue. One of the major drawbacks to this study was examining only those cases that were enrolled for this study. Future studies should validate these findings in a larger cohort and further evaluate the detailed mechanism of the novel role of MBL complement activation pathway in MS.

Increased expression of MBL and MASP-2 at the translational level suggest a chronic activation of complement mediated through the lectin pathway. This increase in complement deposition can contribute to the neuroinflammation and neurodegeneration observed in MS brain (Zajick J, 1995; Rus H, 2006). Enhanced complement synthesis and chronic activation have been implicated in brain damage and progression of several neuroinflammatory and neurodegenerative diseases including multiple sclerosis (Horstman LL, 2011; Veerhuis R, 2011). Potential mechanisms of MBL-mediated brain damage may also include opsonization of surrounding brain cells and MBL-dependent lysis following formation of the membrane attack complex (MAC).

In summary, we found an increase in the expression of lectin complement activation pathway proteins in MS cases compared to non-MS healthy controls. Furthermore, we found that there was an increased expression of MBL proteins in white matter in MS brain compared to white matter of non-MS brain tissues. These findings may potentially be helpful in development of MBL based therapeutics for treating MS disease.

V:

FIGURES AND TABLES

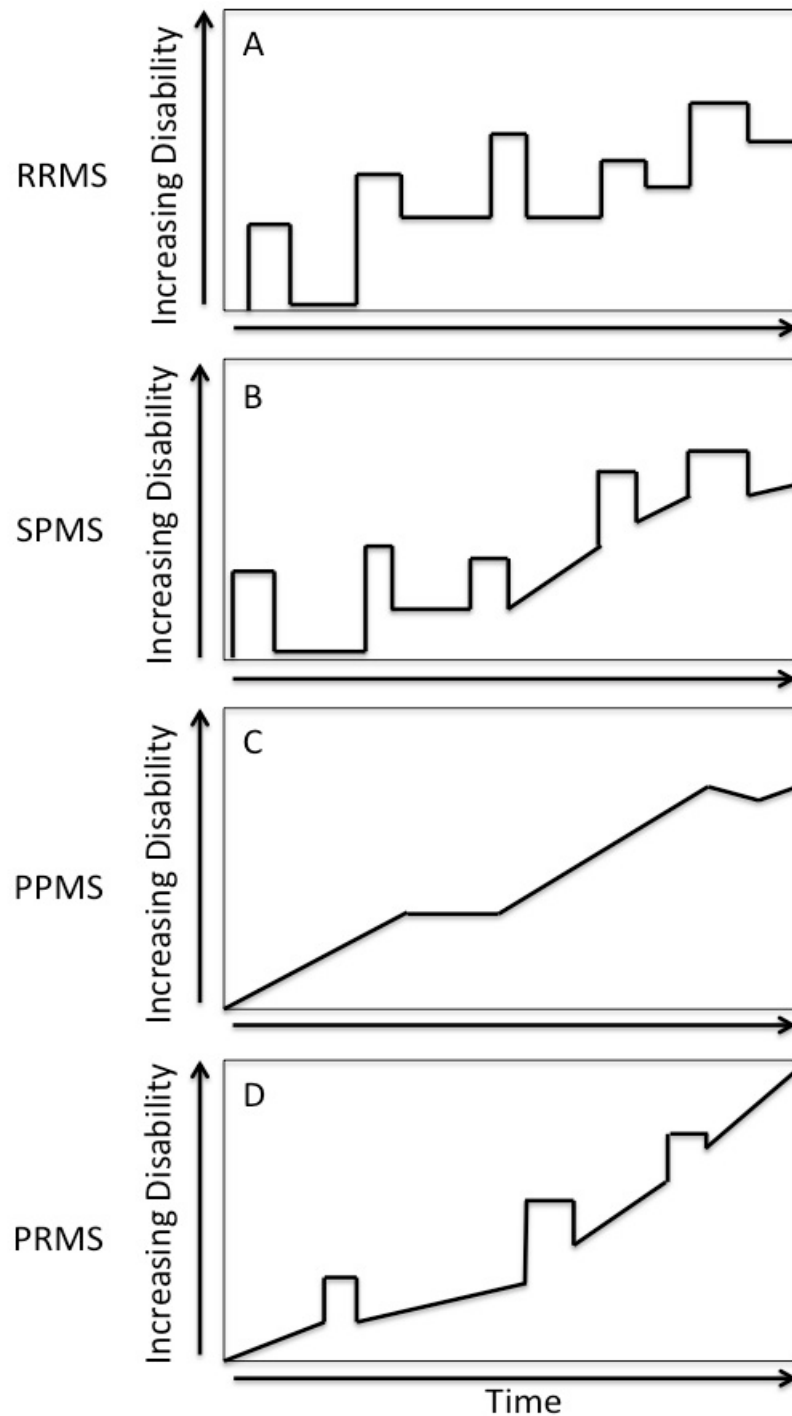


Figure 1a-d. The four disease courses of MS. A) Schematic representing Relapsing-Remitting MS. B) Schematic representing Secondary-Progressive MS. C) Schematic representing Primary-Progressive MS. D) Schematic representing Progressive-Relapsing MS. All schematics are shown as progression of the disease on *y-axis* and time on *x-axis*. (Modified from A. Bitsch, et al., 2002)

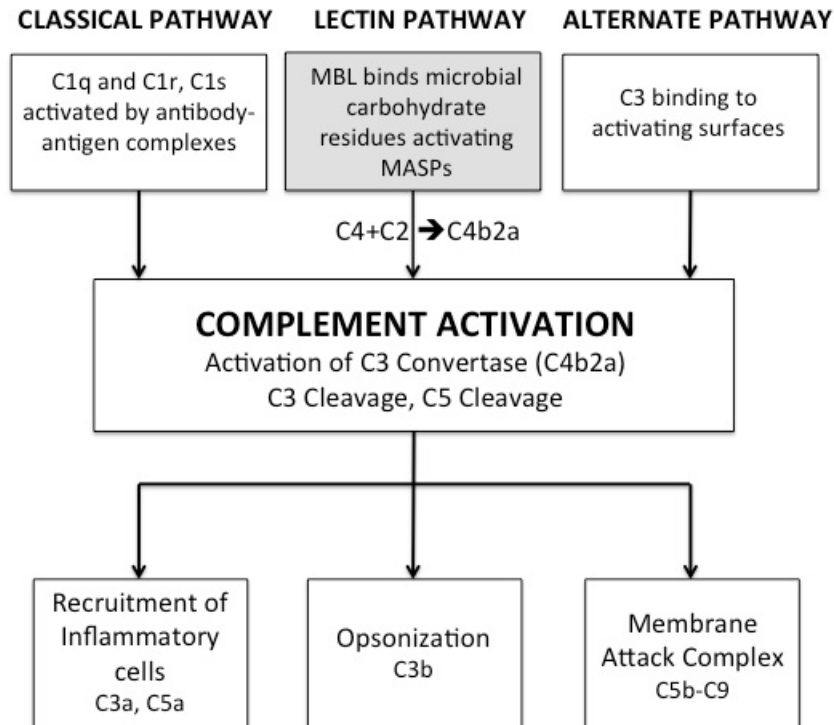


Figure 2. Activation of complement system. Classical pathway utilizes C1q, which recognize and bind antibody-antigen complexes. Lectin pathway uses a protein named mannose binding lectin, which can recognize and bind specific carbohydrate residues. Alternate pathway uses direct C3 binding to activating surfaces on antigen to activate complement. Activation via classical, lectin, and alternative pathways lead to complement activation, formation of C3 convertase. Once complement is active, C3a and C5a recruit inflammatory cells while C3b opsonizes pathogens for macrophage engulfment and C5b-9 forms the membrane attack complex.

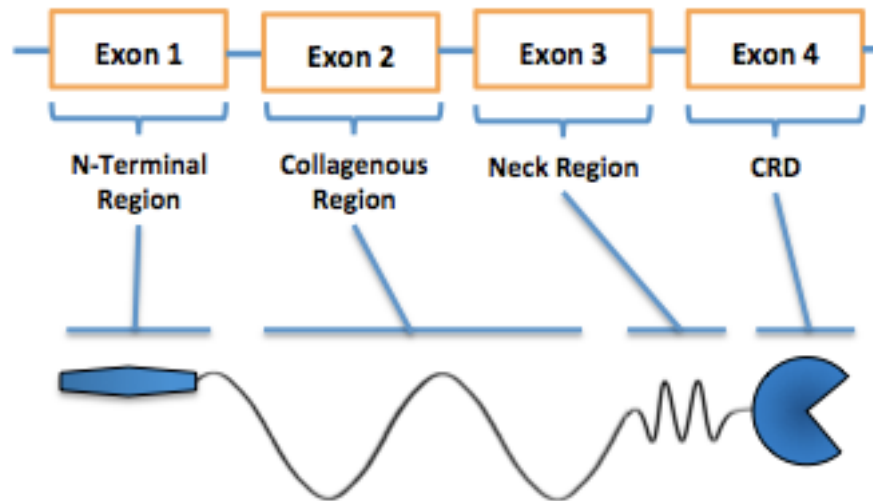


Figure 3a. Structure of mannose binding lectin (MBL). CRD stands for carbohydrate recognition domain. MBL protein is encoded from *MBL2* gene shown above. The figure shows MBL as a single subunit, however MBL protein is found in higher oligomeric forms.

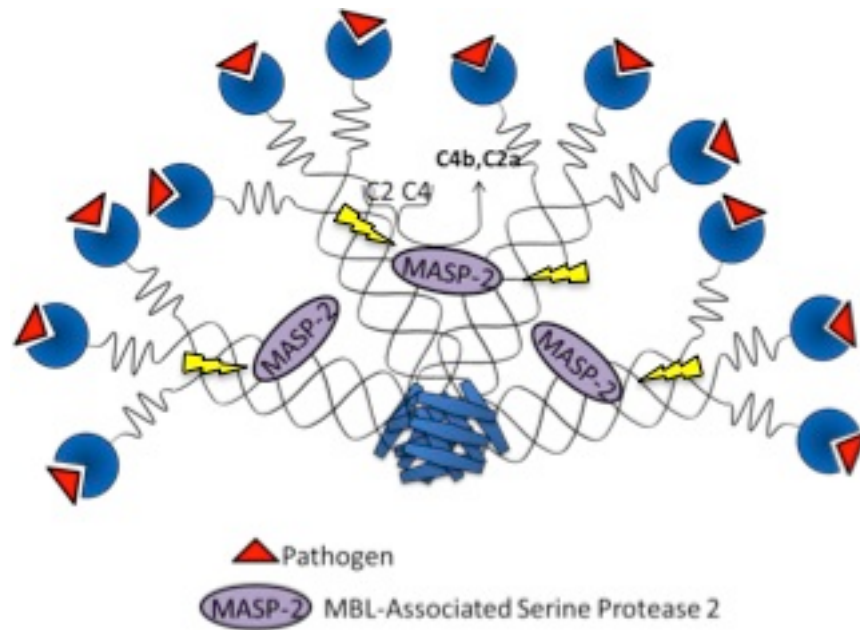


Figure 3b. Activation of the lectin pathway. MBL binding to pathogen (shown as red triangles) activates MBL-associated serine proteases (MASP-2). MASP-2 plays the functional role of MBL pathway by cleaving C2 and C4 into C4b,2a (C3 convertase), a major component in activating the complement system.

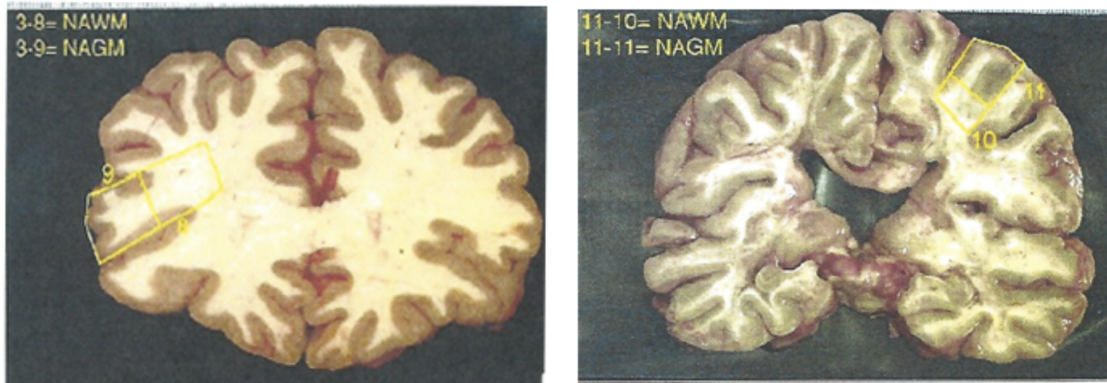


Figure 4. An example of clinical samples used in the study. The images above show non-MS normal (left) and multiple sclerosis (right) post-mortem human brain tissues that were used in this study. Photographs for normal appearing gray matter (NAGM) and normal appearing white matter (NAWM) brain sections were obtained from the Human Brain and Spinal Fluid Resource Center, Los Angeles, CA.

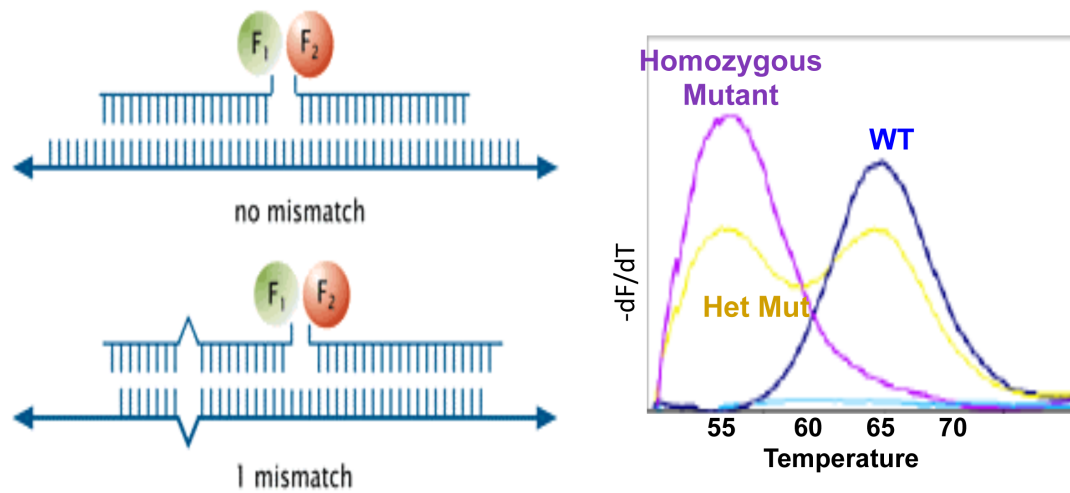


Figure 5. Real-time PCR genotyping by melting curve analysis. Panel on the left demonstrates binding of probes in the case of no nucleotide mismatch and in the case of 1 mismatch. Mismatches will lead to unstable hybrids and lower melting temperature (T_m). Panel on the right shows a typical melting curve with a wild-type T_m of 65°C and a homozygous mutant with a T_m of 55°C. Heterozygote mutant has a double peak spanning the T_m of the homozygous mutant and the wild type.

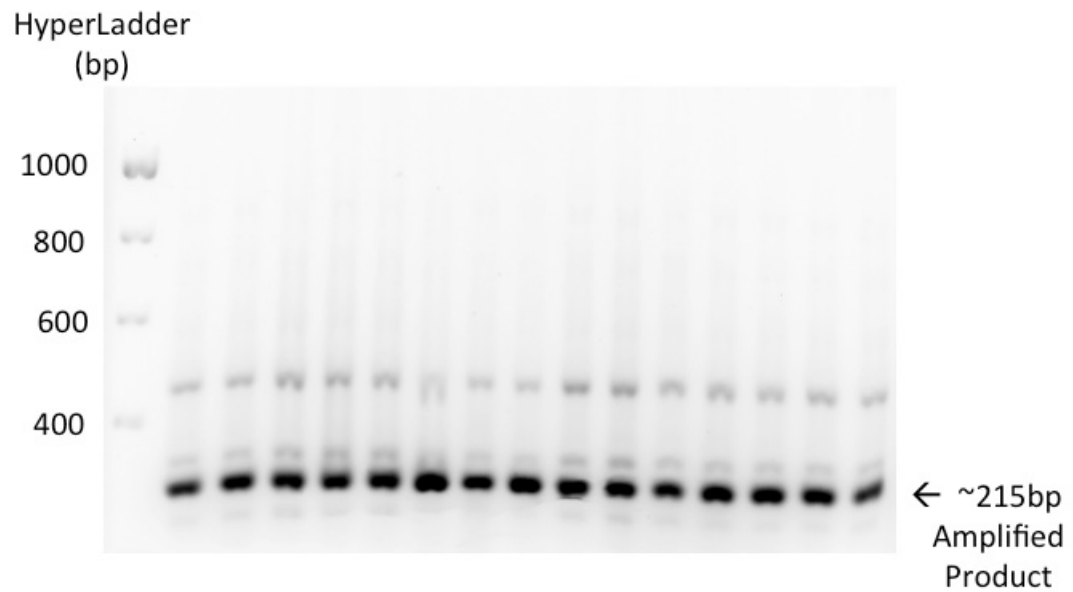


Figure 6. DNA gel for *MASP-2* Genotype Sequencing. 2% Agarose DNA gel running *MASP-2* gene PCR amplified product. *MASP-2* gene amplified by primers is approximately 215 base pairs.

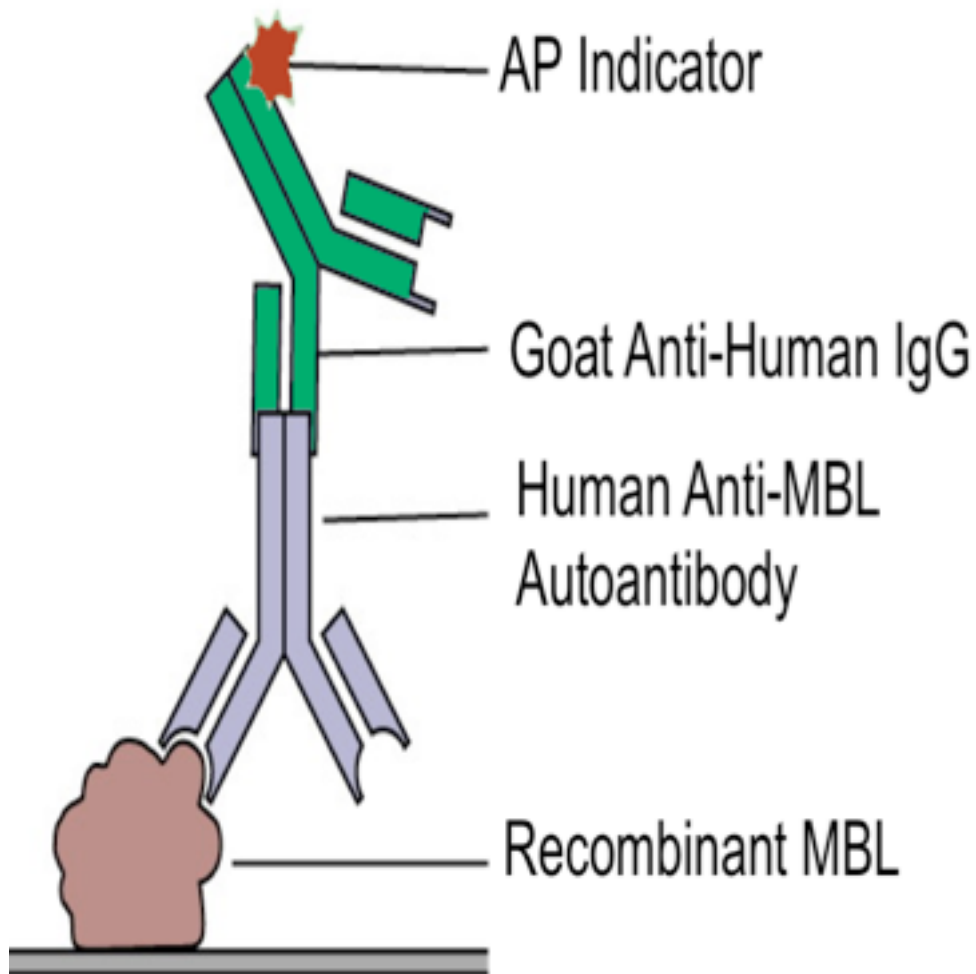


Figure 7. Anti-MBL autoantibody detection ELISA. Plate was coated with recombinant human MBL. Brain tissue protein was added and autoantibodies against MBL bind to recombinant MBL. Next, addition of goat anti-human IgG with alkaline phosphate conjugation allows visualization of autoantibodies present.

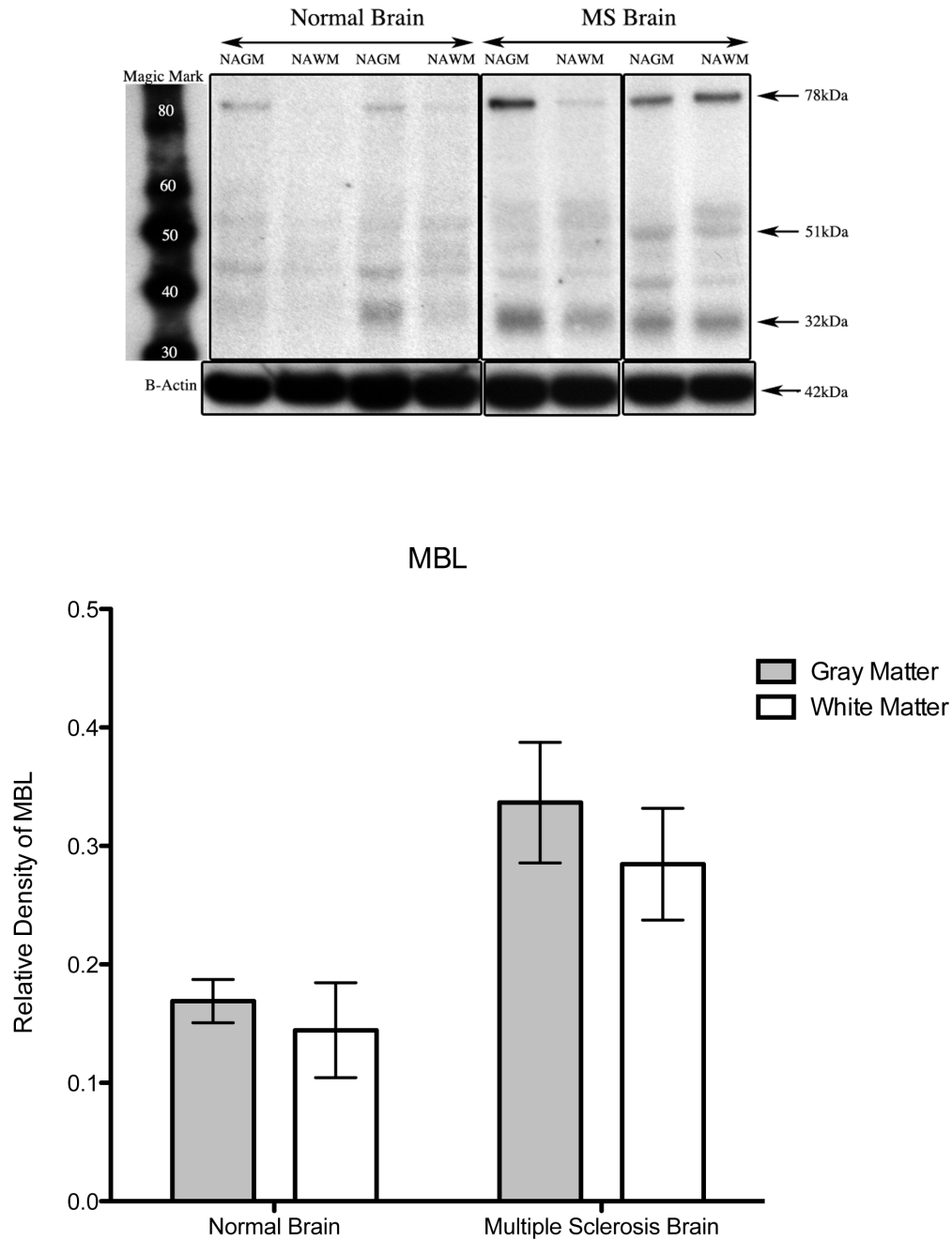


Figure 8. Western blot analysis (top) of MBL shows an increase of MBL expression in MS post-mortem brain tissue compared to non-MS controls. Image J analysis (bottom) using the reference protein beta-actin shows a 2-fold increase in the white matter of MS brain as compared to white matter of normal cases ($p=0.027$, unpaired t-test) Also a 1.7-fold increase in the gray matter of MS brain as compared to gray matter of normal brain ($p=0.103$, unpaired t-test) was observed. ($n=9$ for normal cases and $n=20$ for MS cases)

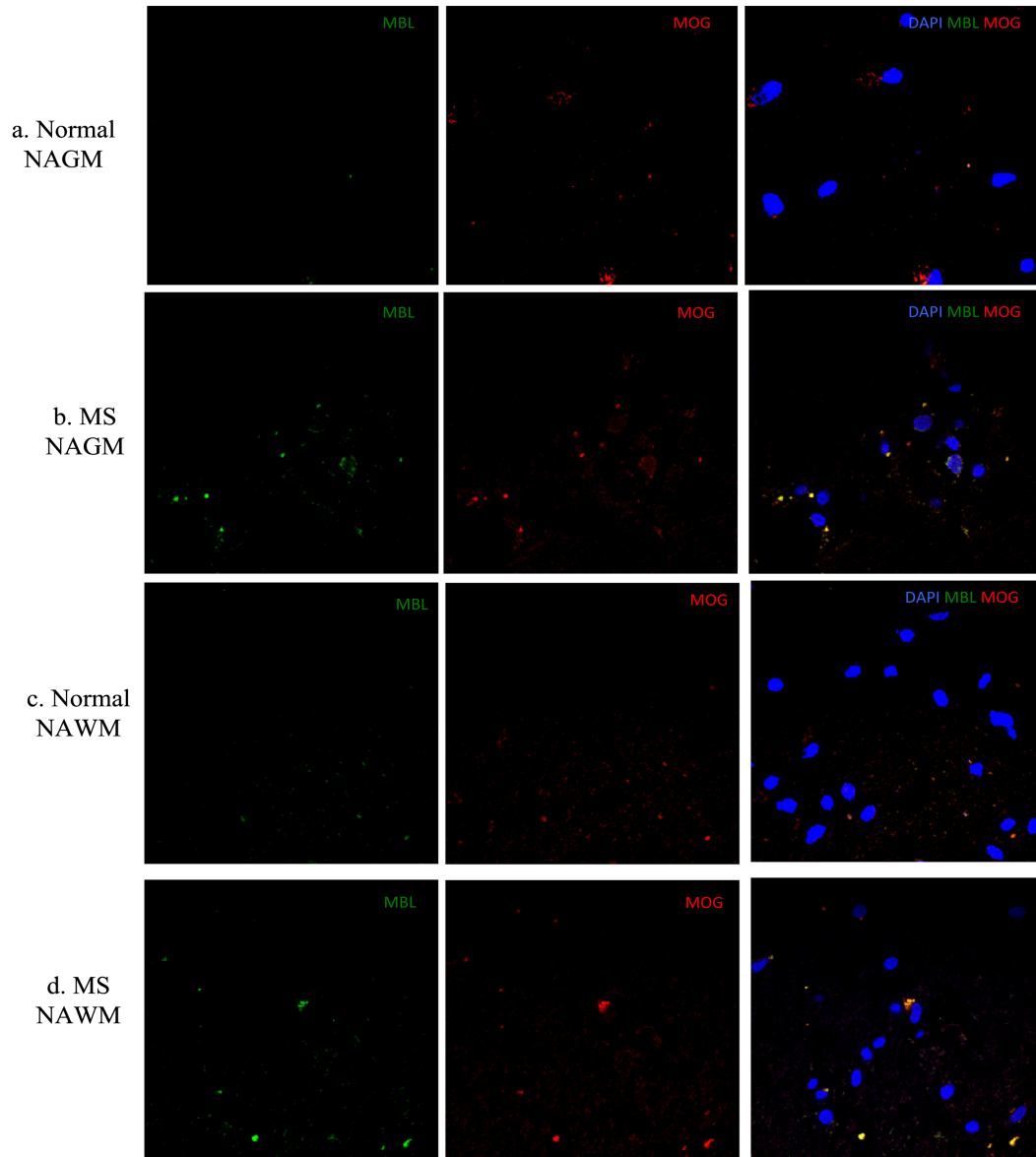


Figure 9a-d. Immunofluorescence showing immunoreactivity of MBL with oligodendrocytes. Immunofluorescence analysis of expression patterns of MBL in gray (a,b) and white (c,d) matter of MS (b,d) and non-MS (a,c) healthy control post-mortem brain tissue. Antibodies against MOG were used as a brain cell marker for oligodendrocytes, shown as red fluorescence. Antibodies against MBL are shown in green fluorescence. Co-localization of MBL and MOG show immunoreactivity of MBL with oligodendrocytes in the brain, this is shown as yellow. There is an increase expression of MBL in both normal appearing gray matter (NAGM) and normal appearing white matter (NAWM) of MS cases compared to normal cases ($p=2.2 \times 10^{-12}$, unpaired t test). $n=4$ for normal cases and $n=6$ for MS cases. DAPI (blue), marker for nuclei, was used to ensure that only intact cells were studied.

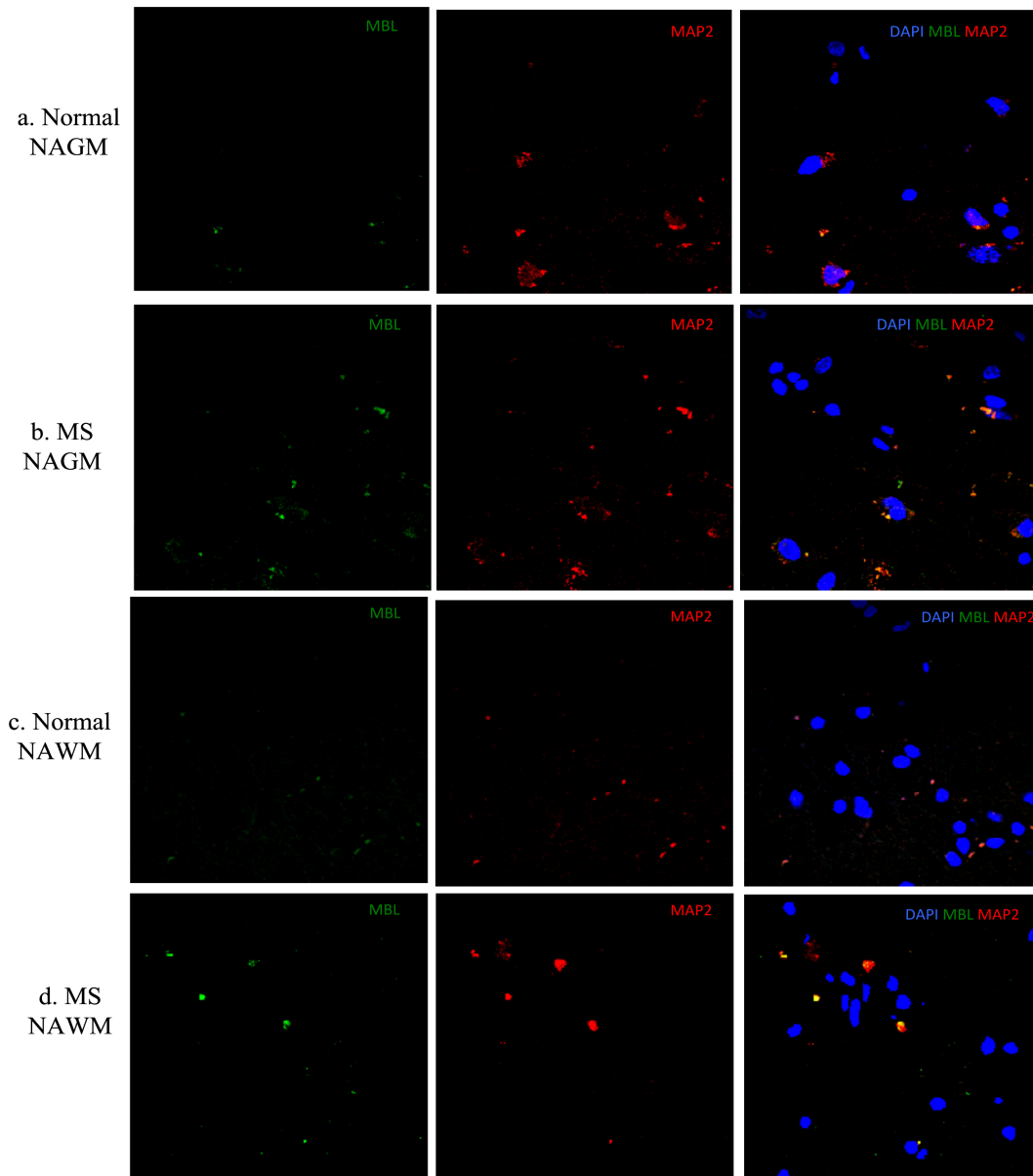


Figure 10a-d. Immunofluorescence analysis showing MBL immunoreactivity with neurons. Immunofluorescence analysis of expression patterns of MBL in gray (a,b) and white (c,d) matter of MS (b,d) and non-MS (a,c) healthy control post-mortem brain tissue. Antibodies against MAP2 were used as a brain cell marker for neurons, shown as red fluorescence. Antibodies against MBL are shown in green fluorescence. Co-localization of MBL and MAP2 show immunoreactivity of MBL with neurons in the brain, this is shown as yellow. There is an increase expression of MBL in both normal appearing gray matter (NAGM) and normal appearing white matter (NAWM) of MS cases compared to normal cases ($p= 2.2 \times 10^{-12}$, unpaired t test). $n=4$ for normal cases and $n=6$ for MS cases. DAPI (blue), marker for nuclei, was used to ensure that only intact cells were studied.

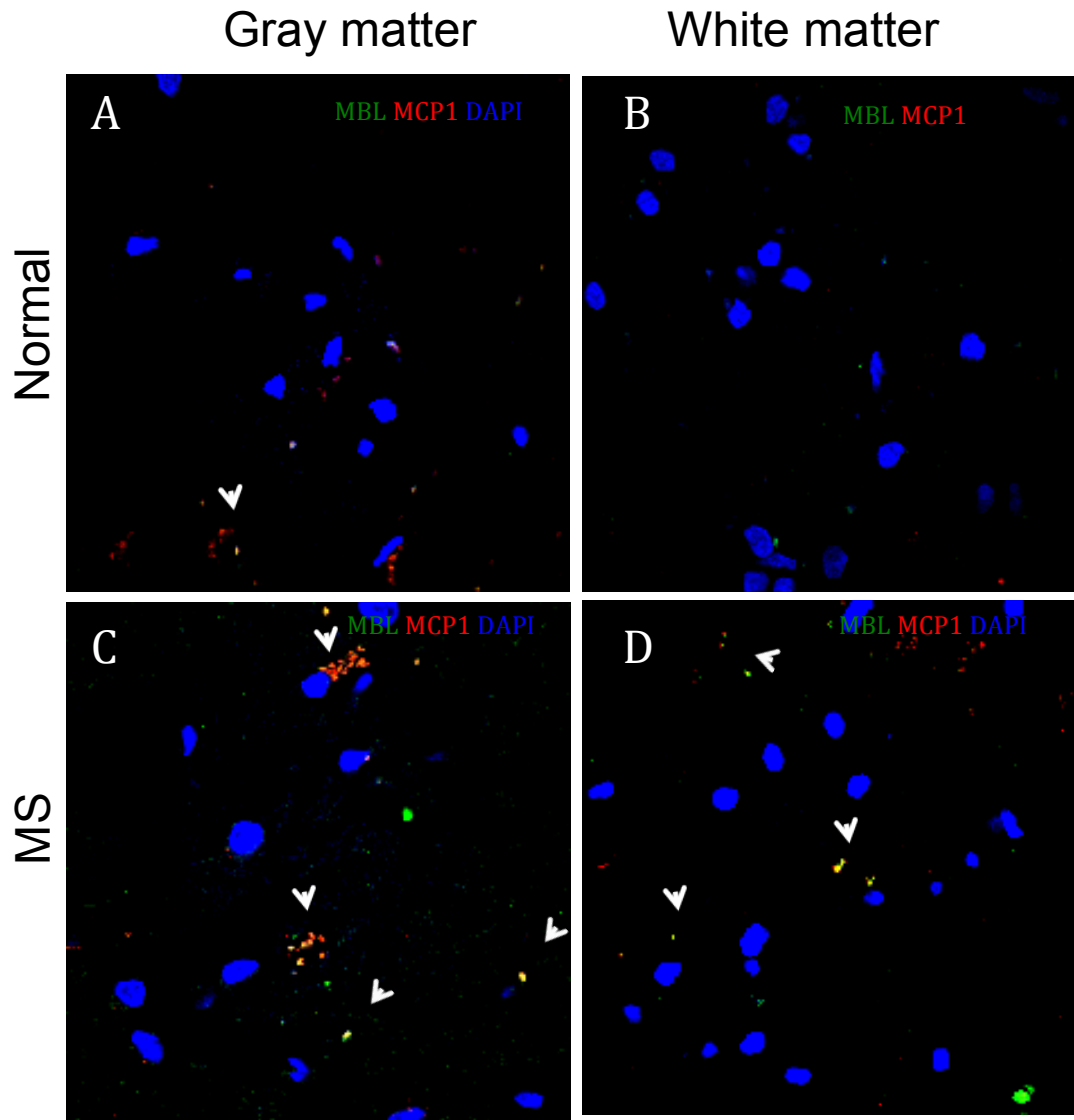


Figure 11a-d. Immunofluorescence analysis of MBL (green) and marker of neuroinflammation MCP-1 (red) expression in MS (C,D) and non-MS healthy (A,D) post-mortem brain tissue, along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MBL and MCP-1 is shown in yellow (white arrows). DAPI (blue), marker of nuclei, was used to ensure that only intact cells were studied. In the studied MS cases, there is a 3.4-fold increase in MCP1 expression ($p=7.2 \times 10^{-10}$, unpaired t test) compared to non-MS healthy controls.

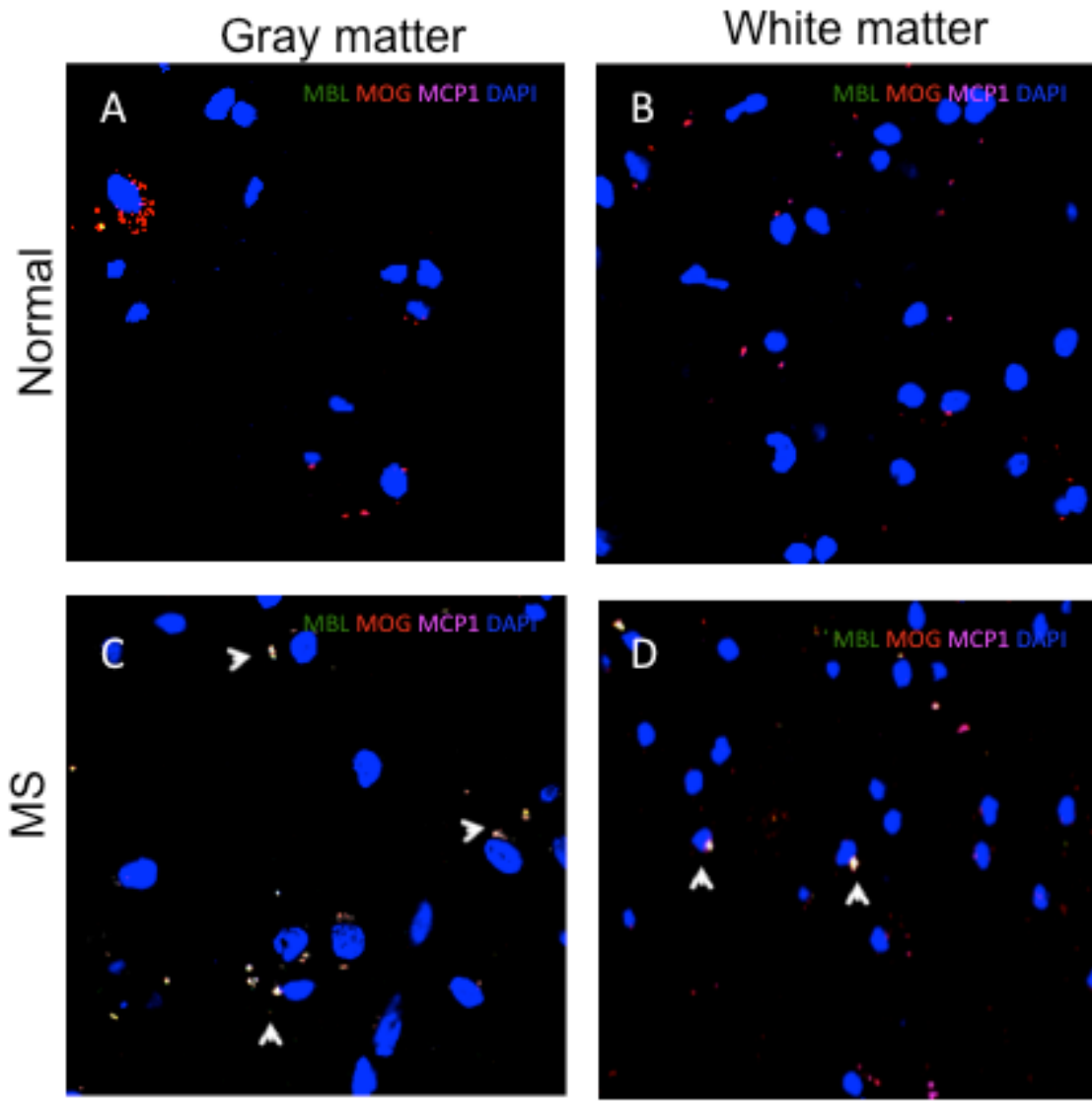


Figure 12a-d. Immunofluorescence analysis of MBL (green) and marker of neuroinflammation MCP-1 (magenta) expression on oligodendrocytes MOG (red) in MS (C,D) and non-MS healthy (A,D) post-mortem brain tissue, along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MBL and MCP-1 is shown in white (also shown with white arrows). DAPI (blue), marker of nuclei, was used to ensure that only intact cells were studied. In the studied MS cases, there is a 3.4-fold increase in MCP-1 expression ($p=7.2 \times 10^{-10}$, unpaired t test) compared to non-MS healthy controls.

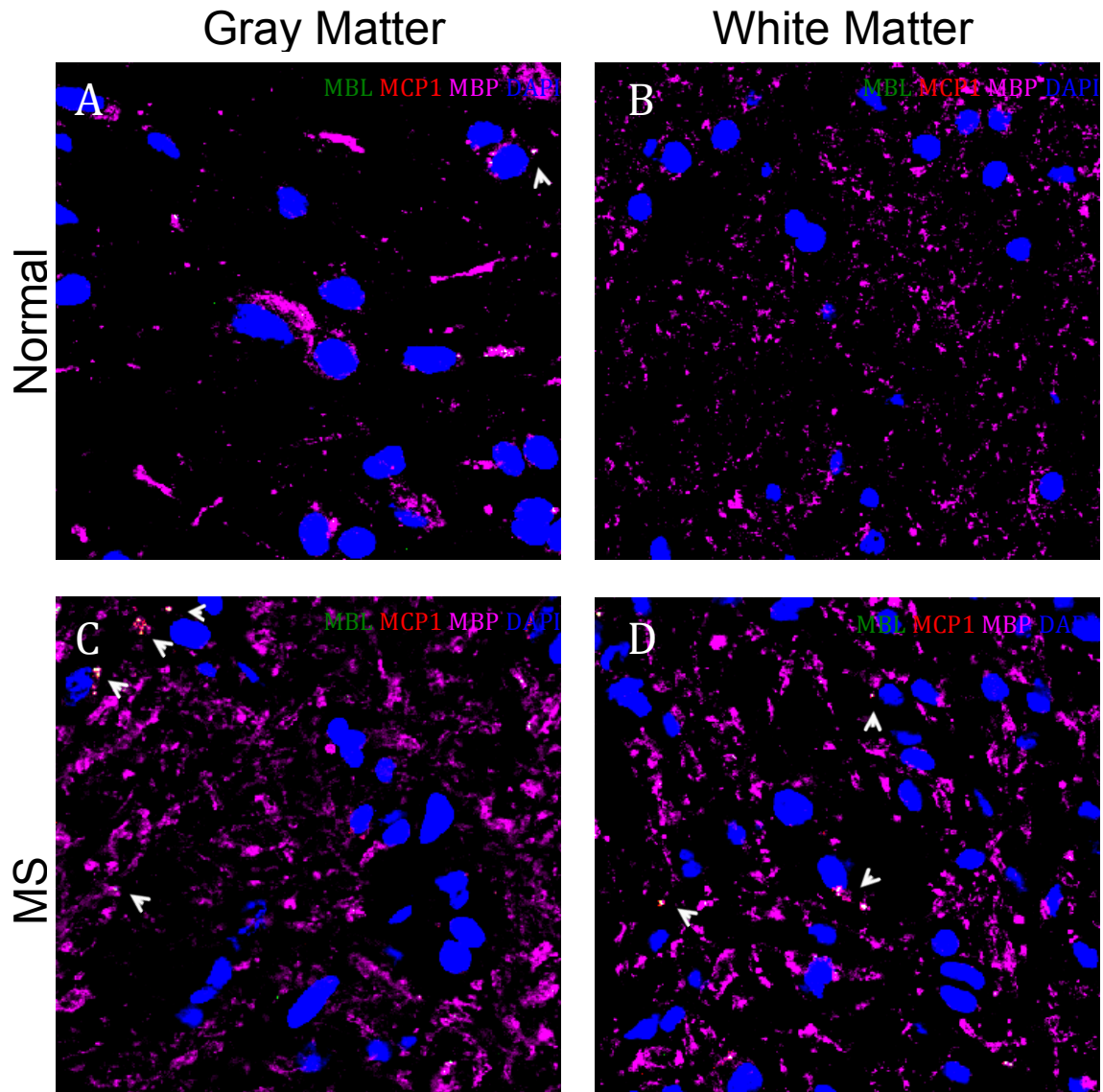


Figure 13a-d. Immunofluorescence analysis of MBL (green) and marker of neuroinflammation MCP-1 (red) expression on myelin MBP (magenta) in MS (C,D) and non-MS healthy (A,D) post-mortem brain tissue, along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MBL and MCP-1 is shown in white (white arrows). DAPI (blue), marker of nuclei, was used to ensure only intact cells were studied.

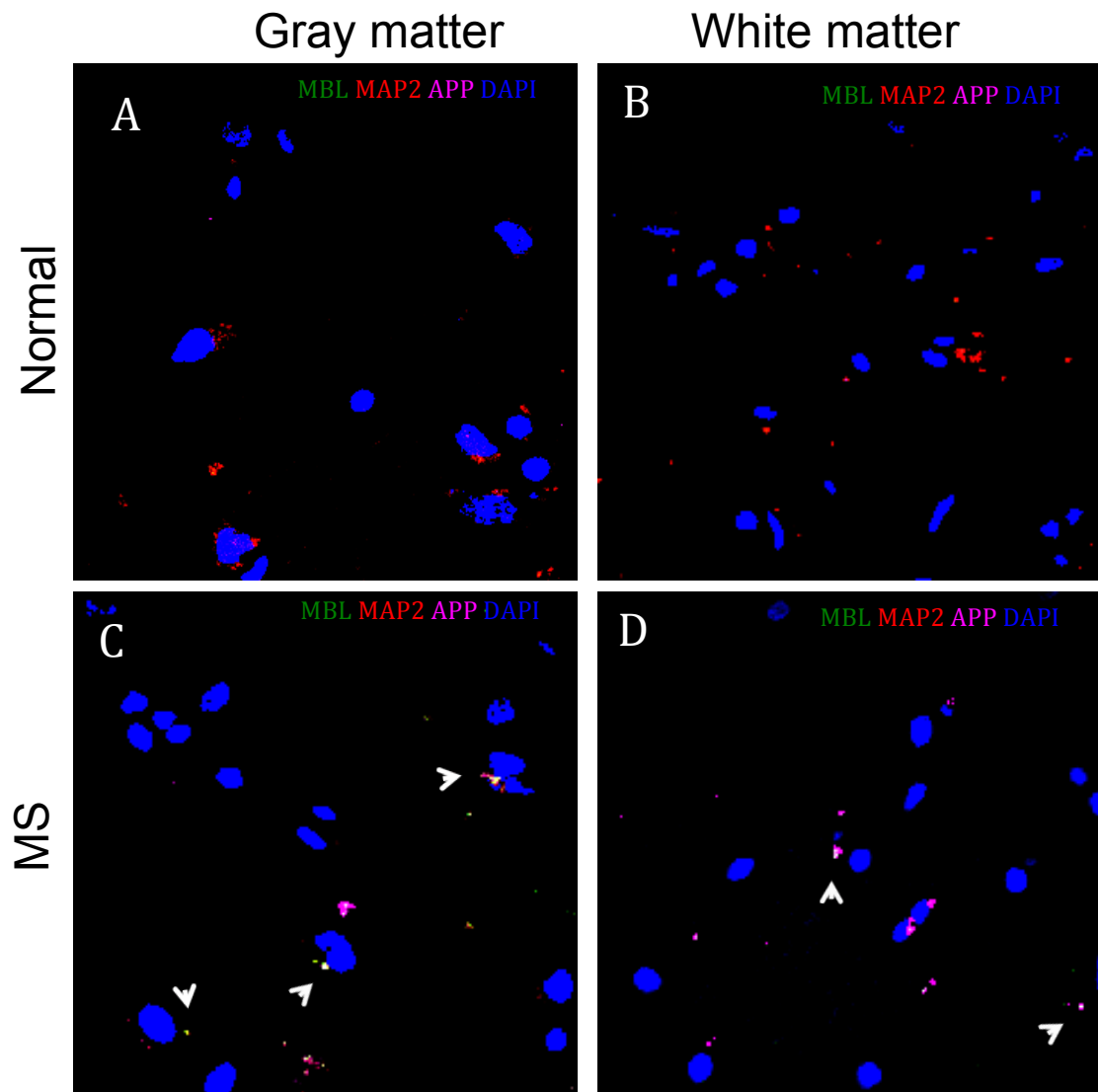


Figure 14a-d. Immunofluorescence analysis of MBL (green) and marker of axonal damage APP (magenta) expression on neuronal axons MAP2 (red) of MS (C,D) and non-MS healthy (A,B) post-mortem brain tissue along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MBL and APP is shown in white (white arrows). DAPI (blue), marker of nuclei, was used to ensure only intact cells were studied. In the studied MS cases, there is an increase in APP expression compared to normal cases. In the studied MS cases there was a 5.1-fold increase in APP expression compared to the normal cases (p value = 0.0015, unpaired t test, n = 2 for MS cases and n = 2 for normal cases).

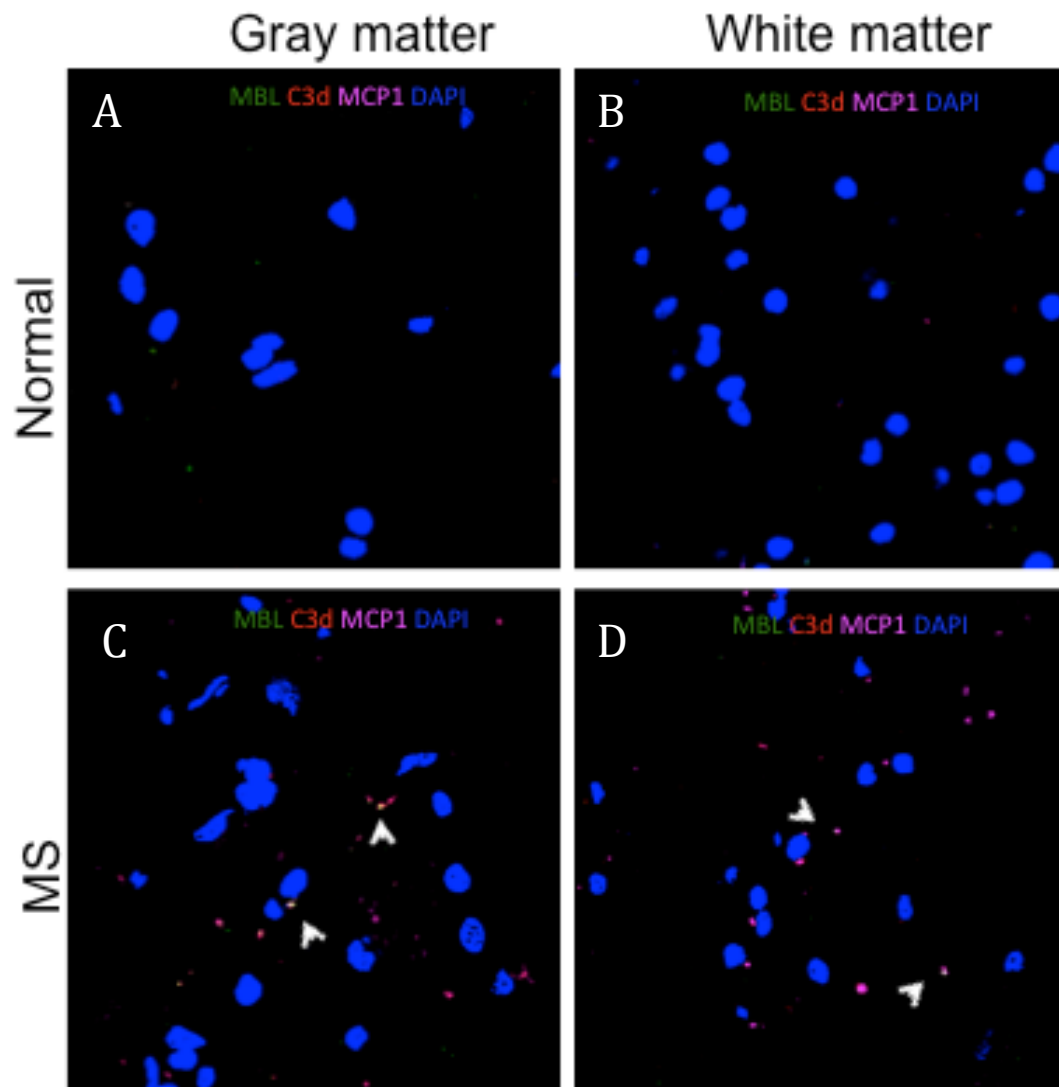


Figure 15a-d. Immunofluorescence analysis of MBL (green) and marker of immune complexes C3d (red) with a marker of neuroinflammation MCP-1 (magenta) expression in MS (C,D) and non-MS healthy (A,D) post-mortem brain tissue, along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MBL, C3d and MCP-1 is shown in white (white arrows). DAPI (blue), marker of nuclei, was used to ensure only intact cells were studied.

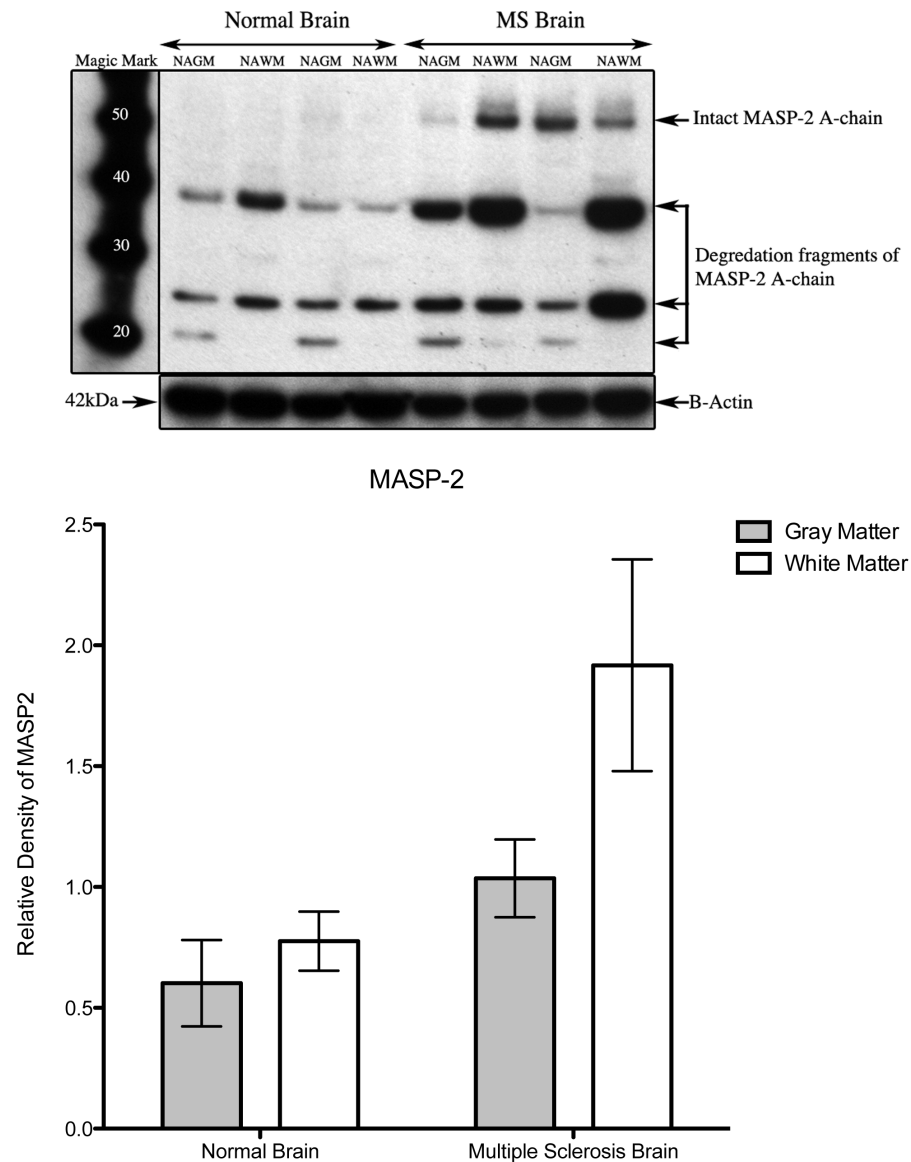


Figure 16. Western blot analysis (top) of MBL-associated serine protease 2 (MASP-2) shows an increase in MASP-2 expression in MS post-mortem brain tissue as compared to non-MS normal cases. Image J analysis (bottom) using reference protein beta-actin shows a 2.7-fold increase in the white matter of MS brain compared to white matter of non-MS healthy control brain ($p=0.057$, unpaired t test). Also there is a 1.9-fold increase in the gray matter of MS brain compared to the gray matter of non-MS healthy control brain ($p=0.047$, unpaired t test). $n=6$ for normal cases and $n=12$ for MS cases.

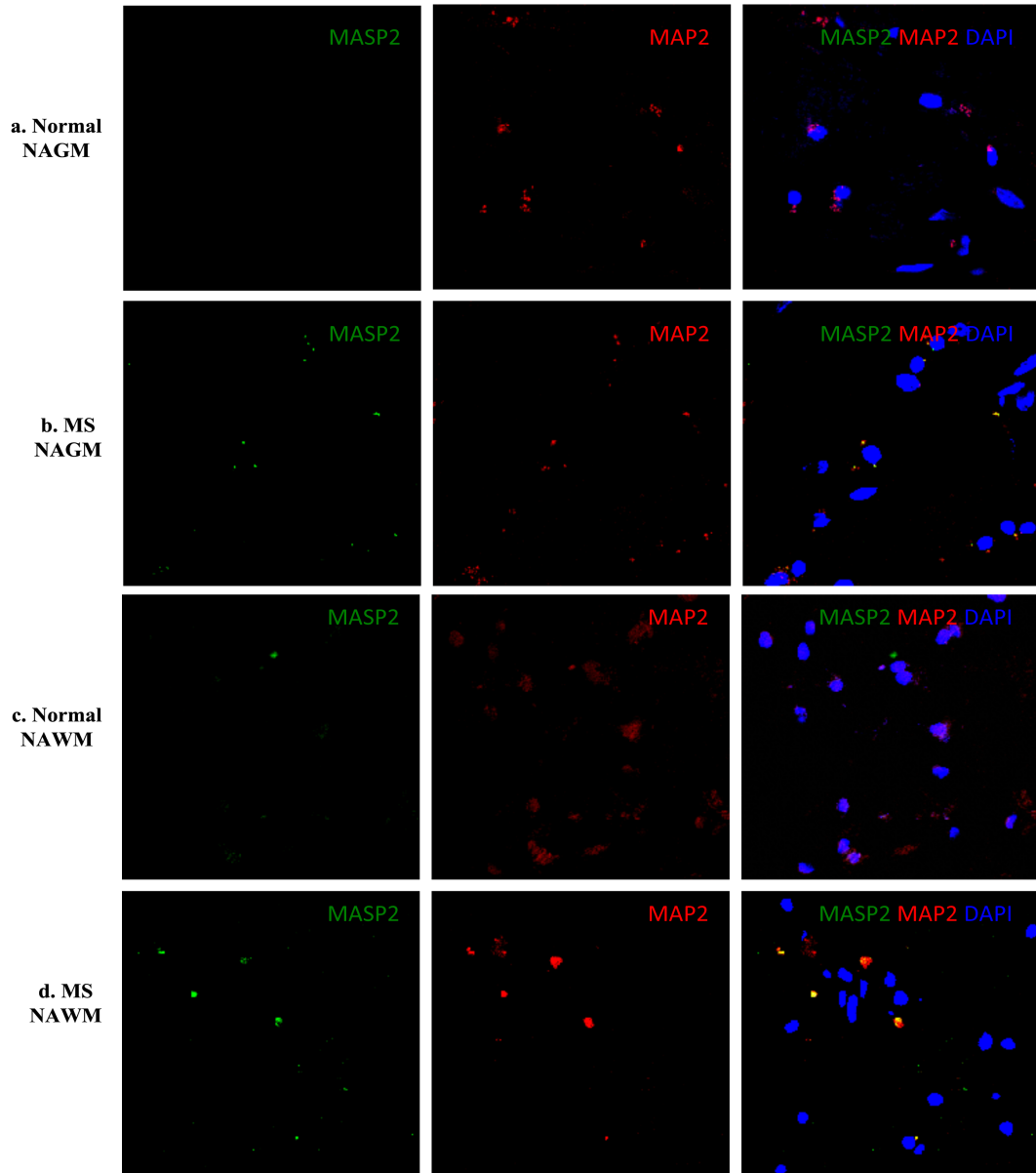


Figure 17a-d. Immunofluorescence analysis showing immunoreactivity between MASP2 and neurons. Immunofluorescence analysis of expression patterns of MASP-2 in gray (a,b) and white (c,d) matter of MS (b,d) and non-MS (a,c) healthy control post-mortem brain tissue. Antibodies against MAP2 were used as a cell marker for neurons, shown as red fluorescence. Antibodies against MASP-2 are shown in green fluorescence. Co-localization of MASP-2 and MAP2 shows immunoreactivity of MASP-2 with neurons in the brain, this is shown as yellow. There is an increased expression of MASP-2 in both normal appearing gray matter (NAGM) and normal appearing white matter (NAWM) of MS cases compared to normal cases ($p=1.7 \times 10^{-7}$, unpaired t test). $n=2$ for normal cases and $n=4$ for MS cases. DAPI (blue), marker for nuclei, was used to ensure that only intact cells were studied.

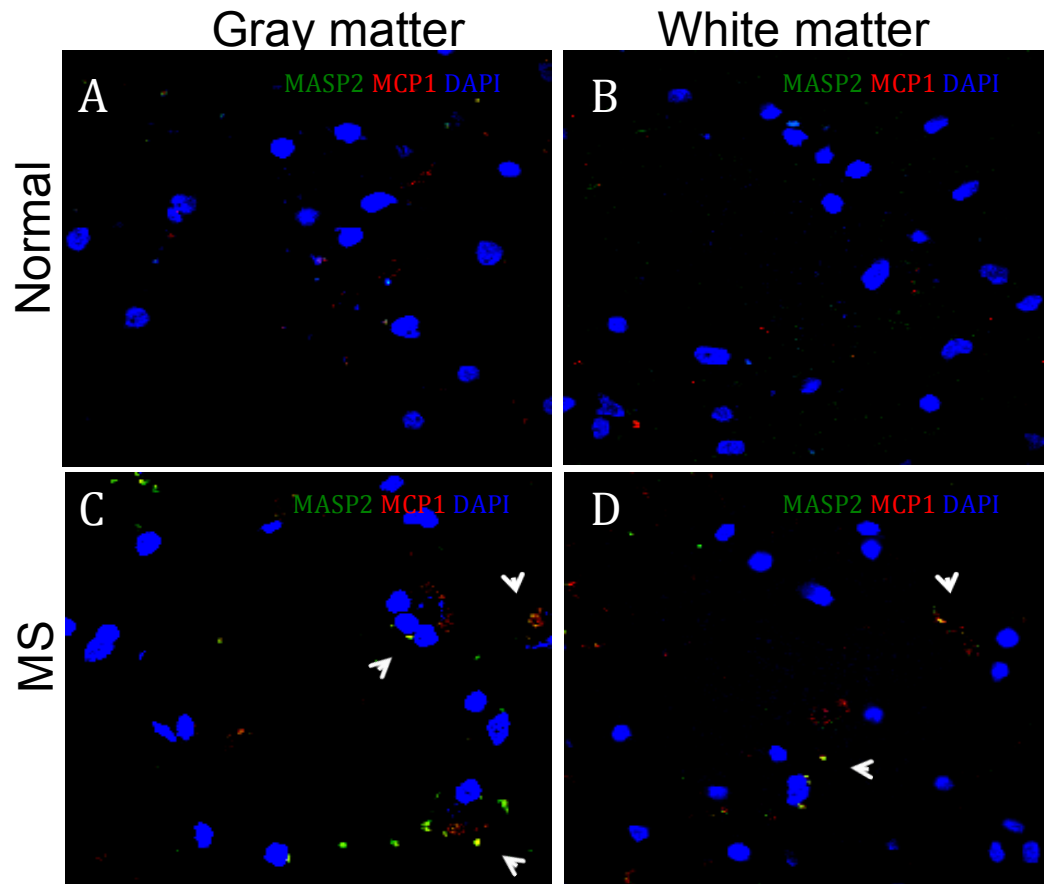


Figure 18a-d. Immunofluorescence analysis of MASP-2 (green) and MCP-1 (red) expression in MS (C,D) and non-MS healthy (A,D) post-mortem brain tissue, along with analysis of expression patterns in normal appearing gray matter (A,C) and normal appearing white matter (B,D). Co-localization of MASP-2 and MCP-1 is shown in yellow (white arrows). DAPI (blue), marker of nuclei, was used to ensure only intact cells were studied.

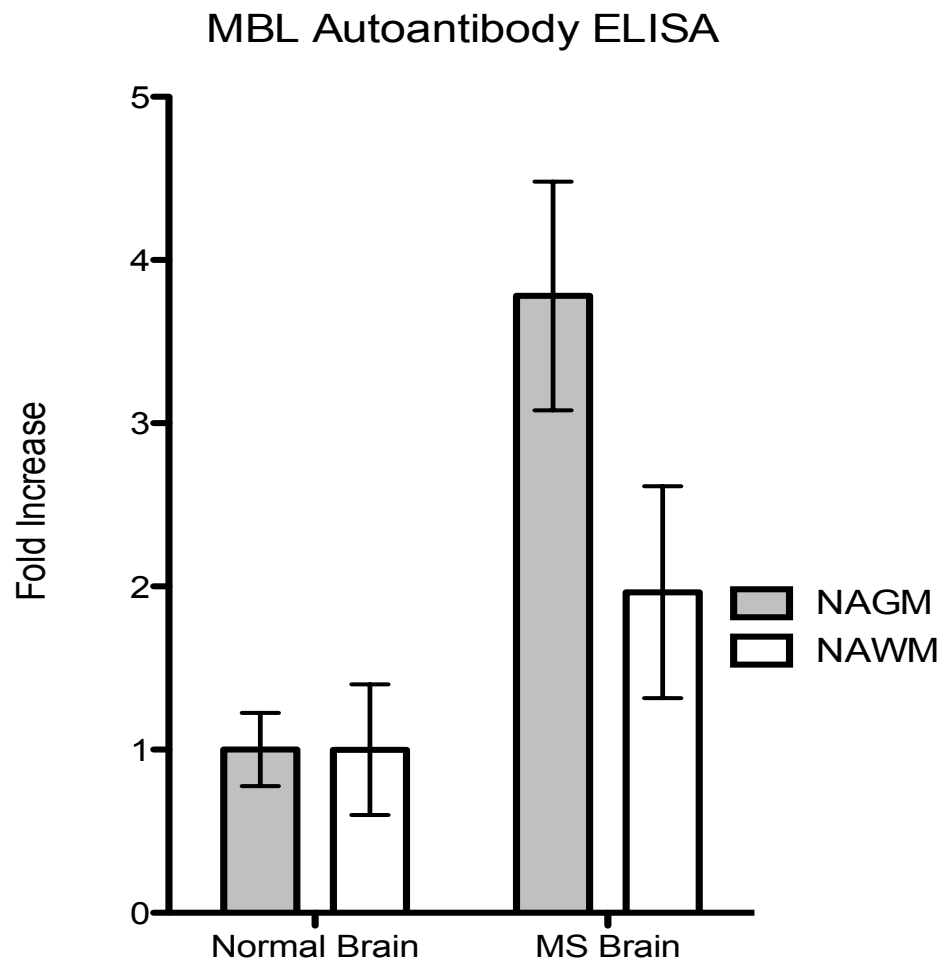


Figure 19. Detection of MBL autoantibodies in MS brain by ELISA. Anti-MBL autoantibodies were detected in the MS brain (p value 0.04, unpaired t test, n=4 for normal cases and n=15 for MS cases).

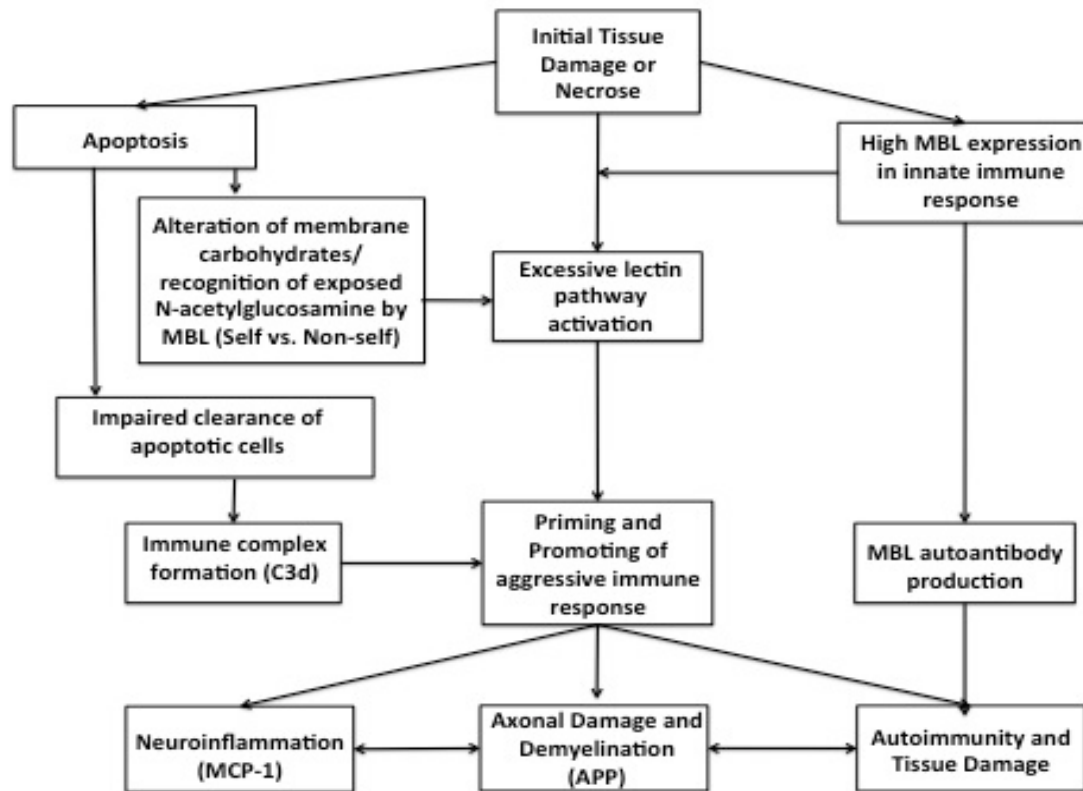


Figure 20. Working model for the role of the lectin complement activation pathway in neuroinflammation, axonal damage, and demyelination in multiple sclerosis. Initial tissue damage leads to three major consequences: apoptosis, high MBL expression due to innate response, and excessive lectin pathway activation. This leads to priming and promoting aggressive cytokine immune response that can result in neuroinflammation, axonal damage, and autoimmunity.

Table 1a. *MBL2* exon1 region genotyping of A/O polymorphism. Chi square test and fisher exact test for A/O alleles demonstrated insignificant p-values of 0.53 and 0.84, respectively. n=10 for normal cases and n=20 for MS cases. NF=not found.

	Genotype Frequency (n)					Allele Frequency		
	A/A	A/B	A/C	A/D	O/O	pB	pC	pD
Normal	8	1	1	0	0	0.05	0.05	0
Multiple Sclerosis	13	3	NF	2	2	0.175	0	0.05

Table 1b. *MBL2* promoter region genotyping of H/L polymorphism. Chi square test and fisher exact test for HL alleles demonstrated insignificant p-values of 0.30 and 0.34, respectively. n=10 for normal cases and n=20 for MS cases.

	Genotype Frequency (n)			Allele Frequency	
	L/L	L/H	H/H	pL	pH
Normal	5	2	3	0.6	0.4
Multiple Sclerosis	10	8	2	0.7	0.3

Table 1c. *MBL2* un-translated region genotyping of P/Q polymorphism. Chi square and fisher exact test for P/Q alleles demonstrated insignificant p-values of 0.81 and 0.99, respectively. n=10 for normal cases and n=20 for MS cases.

	Genotype Frequency (n)			Allele Frequency	
	P/P	P/Q	Q/Q	pP	pQ
Normal	6	3	1	0.75	0.25
Multiple Sclerosis	14	5	1	0.825	0.175

Table 1d. *MBL2* promoter region genotyping of X/Y polymorphism. Chi square and fisher exact test for the X/Y alleles demonstrated insignificant p-values of 0.69 and 0.72, respectively. n=10 for normal cases and n=20 for MS cases.

	Genotype Frequency (n)			Allele Frequency	
	Y/Y	Y/X	X/X	pY	pX
Normal	6	3	1	0.75	0.25
Multiple Sclerosis	10	9	1	0.725	0.275

Table 2. *MASP-2* exon 3 region genotyping of A/G polymorphism. Chi square and fisher exact test demonstrated insignificant p-values of 1.00 and 1.00, respectively. n= 10 for normal cases and n=20 for MS cases. (NF = not found)

	Genotype Frequency (n)			Allele Frequency	
	A/A	A/G	G/G	pA	pG
Normal	10	0	0	1	NF
Multiple Sclerosis	19	1	0	0.975	0.025

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