Loss of Myelin Basic Protein Function Triggers Myelin Breakdown in Models of Demyelinating Diseases

Marie-Theres Weil, Wiebke Möbius, Anne Winkler, Torben Ruhwedel, Claudia Wrzos, Elisa Romanelli, Jeffrey L. Bennett, Lukas Enz, Norbert Goebels, Klaus-Armin Nave, Martin Kerschensteiner, Nicole Schaeren-Wiemers, Christine Stadelmann, and Mikael Simons
**Supplemental Figures:**

Figure S1. Complement injection does not cause myelin damage; and QD9 recognizes MBP, related to Figure 1, 2, 3 and 4 (A) Quantification of myelin fragmentation upon complement or PBS injection (18 h p.i.) in the corpus callosum of Lewis rats. Bars represent mean with SEM (n=3 animals, >250 axons per animal, Student’s t-test). (B) QD9 detects full length monomeric MBP in Western blots of myelin preparations and MBP-GFP transfected PtK2 cells (C) (for all images: scale bar 25 µm).
Figure S2. Myelin pathology in cuprizone fed mice starts with vesiculation of myelin at the inner tongue and induces unmasking of the QD9 epitope, related to Figure 1 and 2.

(A) Representative images of cross-sections of C57/B6N mice fed with 0.2 % cuprizone (scale bar 500 nm). The white box displays magnification of split myelin sheath at 2 weeks of cuprizone treatment. (B) Quantification of the percentage of different myelin fragmentation profiles during cuprizone treatment. Bars shown mean with SEM (n=3 animals, >300 axons per animal, **=p<0.01, ***=p<0.001, ANOVA). (C) Representative images of sagittal brain sections of C57/B6N mice fed with 0.2 % cuprizone (scale bar 1 mm) with MBP staining depicted in green and QD9 staining in red. (D) Magnification of caudal corpus callosum marked by the white box in (C). Quantification of the absolute staining intensity with QD9 (E) and MBP (F) over cuprizone time course. (G) Quantification of the ratio of the staining intensities of MBP and QD9. All graphs show the mean with SEM (n=3 animals, 3-5 regions of same size per animal, *=p<0.05, **=p<0.01, ***=p<0.001, ANOVA).
Figure S3. Epitope unmasking after loss of membrane binding, related to Figure 4. (A) Treatment of primary mouse oligodendrocytes at DIV 5 with the calcium ionophore ionomycin (10 µM, 1 or 2 mins) and subsequent immunostaining for MBP in green and with QD9 in red. (B) Quantification of the MBP to QD9 ratio of integrated density (n=3, >80 cells per condition, **=p<0.01, one-way ANOVA). (C) Representative images of MBP staining in green and PIP2 stain in red of oligodendrocytes at DIV 5 after 10 µM ionomycin treatment for 2 mins. (D) Quantification of the MBP to PIP2 ratio of integrated density (n=3, >80 cells per condition, ***=p<0.001, Student’s t-test). (E) Images representative of oligodendrocytes at DIV 5 after treatment with 100 µM sphingosine for 5 mins stained for MBP in green and QD9 in red (zoom in in the white box, scale bar 25 µm) and in (F), the quantification of the MBP to QD9 ratio of the integrated density (n=3, >80 cells per condition, **=p<0.01, ***=p<0.001, Student’s t-test). All graphs show the mean with SEM. (G) Acute brain slices of mouse brains were treated with 50 µM ionomycin in aCSF and subsequently stained with MBP (green) and QD9 (red) (scale bar 200 µm). (H) Quantification of the MBP to QD9 integrated density ratio (n=3 animals, 3-5 regions of same size per animal, **=p<0.001, Student’s t-test). (I) Representative electron micrographs of high-pressure frozen samples after 30 mins ionomycin treatment (scale bar 500 nm). (J) Quantification of percentage of vesiculated myelin sheaths over ionomycin treatment time course (50 µM, 5 to 30 mins). Bars shown mean with SEM (n=3 animals, >200 axons per animal, ***=p<0.001, one way ANOVA).
Figure S4. QD9 stains border zone around chronic white matter lesions, related to Figure 4. Representative images of myelin stains at the interface of subcortical white to cortical grey matter in multiple sclerosis; both panels depict anti-MBP IHC, anti-QD9 IHC and LFB for myelin in (A) and CD68 counterstained with hemalaun (H) in (B). (A) Images demonstrate that MBP-positive fibers in normal appearing white matter (NAWM) are not stained by the QD9 antibody. (B) Lesion border of a chronic active white matter lesion at higher magnification of the same MS case in an adjacent gyrus. Single QD9-positive fibers can be detected in proximity to CD68-positive cells (arrows). Scale bars: in a= 500 µm; in b= 100 µm. Blood vessels are indicated by asterisks. Abbreviations: Luxol Fast Blue, LFB; NAWM, normal appearing white matter; NAGM, normal appearing grey matter.
Supplemental Experimental Procedures:

**Experimental autoimmune encephalomyelitis (EAE)**
Biozzi ABH mice were obtained from Harlan UK Ltd. (Bicester, UK). Mice were injected subcutaneously with 50 µg recombinant MOG emulsified in complete Freund’s adjuvants (CFA) on d0 and d7. Furthermore, the animals received an i.p. injection of 50 ng pertussis toxin (PTX) (List Biological Laboratories, Campbell, USA) on d0, d1, d7 and d8. The animals were scored daily for the development of paralytic disease and processed for electron microscopy at the peak of the first relapse.

**Cuprizone treatment**
For cuprizone time course studies, eight week old, male wild type (WT) C57B6N mice were fed with 0.2 % cuprizone (Sigma-Aldrich, Munich, Germany) in powered chow18. The animals were weighed once per week and if the body weight dropped below 20 % of the starting weight, the animal was euthanized. The animals were treated for a maximum of 5 weeks, and every week, one set of animals was perfused for histological studies, whereas the other set was processed by high pressure freezing for electron microscopy.

**Acute slices of lesion site of AQP4 antibody injected Lewis rats**
After injection of Lewis rats with AQP4 antibody and complement, rats were decapitated. Coronal slices were cut using a Leica VT1200S Microtome (Leica) at 200 μm thickness in ice-cold cutting solution containing the following: 130 mM NaCl, 3.5 mM KCl, 10 mM MgSO4, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 24 mM NaHCO3, and 10 mM glucose with pH 7.4. Acute slices were allowed to recover in artificial CSF (aCSF) containing 148.2 mM NaCl, 3.0 mM KCl, 1.4 mM CaCl2, 0.8 mM MgCl2, 0.8 mM Na2HPO4 and 0.2 mM NaH2PO4 at 35°C for 1h. The slices were then treated with 25 mM EGTA in aCSF or aCSF alone for 2h at RT. ACSF was continuously bubbled with carbogen (95% O2 and 5% CO2) gas. The acute slices were then fixed with 4% PFA and subsequently stained with guinea pig GFAP (Synaptic Systems) to identify the lesion site, mouse QD9 (Abcam) and rabbit MBP (Dako). For treatment with ionomycin, the recovered slices were incubated in 50 µM ionomycin (dissolved in aCSF) at 35°C for 5 to 30 mins. Afterwards, the sections were processed for immunohistochemistry or for electron microscopy.

**Immunoelectron microscopy**
Immunoelectron microscopy was carried out as described previously (Werner et al., 2007). The brain sections were immersion fixed in 4% PFA with 0.25% glutaraldehyde, after infiltration with 2.3 M sucrose overnight, mounted on aluminium pins and snap-frozen in liquid nitrogen. The ultrathin cryosections were cut with a Leica UC6 cryoultramicrotome (Leica, Vienna, Austria) using a cryoimmuno diamond knife (Diatome, Biel, Switzerland).

The immunolabeling was performed as described previously (Peters and Pierson, 2008) using anti-MBP (Dako, rabbit, 1:300) or anti-PLP A431 (Sigma-Aldrich, rabbit, 1:250). The rabbit antibody was detected using protein A gold (10 nm, CMC, Utrecht, Netherlands) at a dilution of 1:20 for 20 min. The sections were imaged with a LEO912AB electron microscope (Zeiss, Oberkochen, Germany) equipped with a 2k CCD camera (TRS, Moorweis, Germany). The quantification of the labels was on >70 axons, where the gold particles for the compact myelin were counted and divided by the label obtained in vesiculated myelin.

**Antibodies, plasmids and other reagents**
The following antibodies were used in this study: mouse monoclonal IgG anti-MBP (Sternberger, Lutherville, USA), mouse monoclonal IgG QD9 (Abcam, Cambridge, UK), rabbit polyclonal IgG anti-MBP (Dako, Carpinteria, USA), rabbit polyclonal IgG anti-myc tag (Sigma-Aldrich, Munich, Germany), mouse monoclonal IgG anti-CD68 (Abcam, Cambridge, UK). Secondary antibodies conjugated with fluorophores or horseradish peroxidase were purchased from Dianova (Hamburg, Germany). Biotinylated secondary antibodies were purchased from Jackson Immuno Research Europe ltd. (Suffolk, UK).

Ionomycin was purchased from Calbiochem (Darmstadt, Germany).

**Cell culture, transfections and treatments**
Primary cultures of mouse oligodendrocytes were prepared from postnatal day zero (P0) mice as described previously (Trajkovic et al., 2006). In brief, the oligodendroglial progenitor cells growing on top of a monolayer of astrocytes were obtained via differential shaking. After their harvest, the cells were cultured on PLL-coated dishes or coverslips with minimum essential media containing B27 supplement, 1% horse serum, L-thyroxine, glucose, glutamine, penicillin, streptomycin, pyruvate and bicarbonate (SuperSato-B27). Treatment of the oligodendrocytes DIV 5 was carried out with 10 µM ionomycin in Krebs-Ringer solution (120 mM NaCl, 4.7 mM KCl, 10 mM glucose, 20 mM HEPES, 1.2 mM CaCl2, 0.7 mM MgSO4, pH 7.4) by first
Immunohistochemistry of human brain

25% glycerol, 25% ethylene glycol in PBS) at 0°C in blocking solution overnight. The tissues were washed thrice in PBS for 5 minutes at RT (for all washes, unless stated otherwise). For the MBP and QD9 slides were thawed for 15 mins at 4°C and then fixed in 4% PFA and 1% PFA prior to cryoprotection in 30% sucrose. After embedding, the tissues were cut using the Leica cryostat CM1850 and stored in cryoprotective solution (25% glycerol, 25% ethylene glycol in PBS) at -20°C. Sections for immunofluorescence were either processed free-floating after washing with PBS (thrice for 30 min) or processed in 100 in PBS for 1 h at room temperature (RT) and then, blocked in 5% horse serum, 5% goat serum and 5% fetal calf serum in 0.5% Triton X-100 (blocking solution) for 1 h at RT. For tissue injected with an human antibody, blocking with 1:100 dilution of goat anti-human IgG and IgM (Dianova, Hamburg, Germany) in blocking solution was carried out for 1 h at RT. The sections were incubated with the desired primary antibodies in appropriate dilution in blocking solution overnight. Primary antibodies used were polyclonal rabbit anti-MBP (Dako, Carpinteria, USA) and mouse monoclonal QD9 antibody (Abcam, Cambridge, UK) or mouse monoclonal MAG antibody (Millipore, Billerica, USA). The sections were washed and incubated with the secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 and the nuclear counterstain DAPI for 1 h at RT. After washing the sections, they were mounted using mowiol and dried.

Immunohistochemistry of human tissue

MS and control brain tissue samples were provided by the UK Multiple Sclerosis Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495). All brain tissues were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude other confounding pathologies. Immunohistochemistry of human brain tissues was performed on tissue sections from 6 tissue blocks from 4 MS cases and on 5 tissue blocks from 4 control cases. CNS tissue from 4 patients with serologically confirmed NMO or NMO spectrum disease obtained at biopsy was examined. Brain or spinal cord biopsy was performed for diagnostic purposes to exclude tumor, lymphoma or infection. The study was approved by the local ethics committee. All tissue blocks were fresh frozen within a post mortem-time of maximum 18 h and an average post-mortem time of 12 h. Cryostat sections of 12µm were mounted on Superfrost Plus glass slides, dried at room temperature (RT) and stored at -80°C. The slides were thawed for 15 mins at 4°C and then fixed in 4% fresh PFA for 5 minutes at RT. The sections were washed thrice in PBS for 5 minutes at RT (for all washes, unless stated otherwise). For the MBP and QD9 staining, a permeabilization was performed in 70% Ethanol in PBS overnight at RT. After washing, endogenous peroxidase activity was blocked with 0.06% hydrogen peroxide in PBS for 30 mins at RT (for CD68: 0.06% hydrogen peroxide and 80% methanol in PBS). The tissue sections were washed again and blocked in buffer containing 5% normal donkey serum and 0.05% Triton X-100 in PBS for 1h at RT (for CD68: 1% normal donkey serum, 0.1% Triton X-100 and 0.05% Tween-20 in PBS). The primary antibody was incubated overnight at 4°C in the following dilutions: MBP diluted 1:300, QD9 diluted 1:2000 and CD68 diluted 1:250 in blocking buffer. After washing the sections, the secondary antibody was added for 1 h at RT. The tissue sections were washed and the avidin biotin complex (PK6100-Standard, Vector Laboratories, Inc., California, USA) was added.
to the sections as described by the manufacturer and incubated for 30 mins at RT. The sections were washed and incubated in a solution containing 3-Amino-9-ethylcarbazol (AEC), 0.125% glacial acetic acid and 0.03% hydrogen peroxide for 6 mins at RT. The sections were washed as above, then washed twice in distilled water for 2 mins, mounted with Aquatex and dried overnight. The tissue stained for CD68 was stained with hemalaun prior to washing in distilled water by bathing the slides for 15 secs (Papanicolaou Solution 1a Harris Hematoxylin solution, Merck Millipore) and then rinsing them under flowing tap water for 8 mins.

**Statistical analysis**
All data were expressed as means ± standard errors of the mean (SEM). Statistical analysis was conducted with Prism software (GraphPad Software Inc., La Jolla, CA). We used the Student t-test, one-way or two-way ANOVA as indicated and with post-tests as appropriate. The level of significance was set at p< 0.05. Single, double, and triple symbols denote statistical significance at the 0.05, 0.01, and 0.001 levels, respectively.

**Supplemental References:**
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