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The control of reactive oxygen species production by SHP-1 in oligodendrocytes

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Abstract

We have previously described reduced myelination and corresponding myelin basic protein (MBP) expression in the CNS of Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) deficient motheaten (*me/me*) mice compared to normal littermate controls. Deficiency in myelin and MBP expression in both brains and spinal cords of motheaten mice correlated with reduced MBP mRNA expression levels *in vivo* and in purified oligodendrocytes *in vitro*.

Therefore, SHP-1 activity appears to be a critical regulator of oligodendrocyte gene expression and function. Consistent with this role, the present studies demonstrate that oligodendrocytes of motheaten mice and SHP-1 depleted N20.1 cells produce higher levels of reactive oxygen species (ROS) and exhibit corresponding markers of increased oxidative stress. In agreement with these findings, we demonstrate increased production of ROS coincides with ROS-induced signaling pathways known to affect myelin gene expression in oligodendrocytes. Antioxidant treatment of SHP-1-deficient oligodendrocytes reversed the pathological changes in these cells with increased myelin protein gene expression and decreased expression of nuclear factor (erythroid-2)-related factor 2 (Nrf2) responsive gene, heme oxygenase-1 (*HO-1*). Furthermore, we demonstrate that SHP-1 is expressed in human white matter oligodendrocytes and there is a subset of multiple sclerosis (MS) subjects that demonstrate a deficiency of SHP-1 in normal appearing white matter (NAWM). These studies reveal critical pathways controlled by SHP-1 in oligodendrocytes that

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relate to susceptibility of SHP-1-deficient mice to both developmental defects in myelination and to inflammatory demyelinating diseases.

Keywords

oxidative stress; inflammation; myelin; multiple sclerosis; normal appearing white matter

Introduction

The Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) is an important regulator of cytokine responses in hematopoietic, epithelial, and glial cells by controlling receptor-mediated phosphorylation of downstream signaling molecules and transcription factors. Loss of SHP-1 by null mutation, as in motheaten (*me/me*) mice, results in a number of hematopoietic abnormalities leading to increased inflammation, oxidative stress, and systemic autoimmune disease (Green and Shultz 1975; Lyons et al. 2003; Shultz and Green 1976; Sidman et al. 1978). We and others have demonstrated that oligodendrocytes of SHP-1-deficient mice express less mature myelin proteins and exhibit dysmyelination compared to their wild type littermates (Massa et al. 2000; Massa and Wu 1998; Massa et al. 2004; Wishcamper et al. 2001). However, the mechanisms by which oligodendrocyte pathology and aberrant myelin gene expression are affected by SHP-1 deficiency are not known.

As a regulator of cell signaling, SHP-1 controls production of reactive oxygen species (ROS) in endothelial (Krotz et al. 2005) and hematopoietic cells (Lyons et al. 2003). SHP-1 negatively regulates the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Krotz et al. 2005), NADPH oxidase is inducible in and toxic to oligodendrocytes (Johnstone et al. 2013). Moreover, SHP-1 negatively regulates signaling pathways known to promote the generation of ROS via nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Signal transducer and activator of transcription 3 (STAT3), and STAT6 activities (Mandal et al. 2010; Mariappan et al. 2010; Reich 2009). ROS can act as a signaling molecule to activate Janus activating kinase (JAK)/STAT and NFκB pathways (Gloire et al. 2006; Simon et al. 1998). NFκB signaling is altered in multiple sclerosis (MS) (Mc Guire et al. 2013) and JAK/STAT signaling pathways are dysregulated in normal appearing white matter (NAWM) of MS subjects (Cannella and Raine 2004; Zeis et al. 2008). In the CNS, ROS production and resultant oxidative stress has been implicated in many neurological disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis.

Oligodendrocytes are particularly susceptible to oxidative stress due to very high iron content, low glutathione stores (Juurlink et al. 1998; Thorburne and Juurlink 1996), and necessarily high mitochondrial activity required to produce and maintain myelin membranes (McTigue and Tripathi 2008). Oxidative stress can result in oligodendrocyte death, particularly that of immature oligodendrocytes (Back et al. 1998), and can disrupt oligodendrocyte differentiation by suppressing critical transcription factors (French et al. 2009). In addition, cytokine-mediated oxidative stress can down-regulate myelin protein

genes of purified human oligodendrocytes (Jana and Pahan 2005). As ROS have been shown to negatively impact the viability, differentiation and function of oligodendrocytes and SHP-1 has been shown to control production of ROS in cells, we examined whether SHP-1 may control the production of ROS and corresponding ROS-induced signaling pathways in oligodendrocytes. The data presented here show that oligodendrocytes lacking SHP-1 produce greater amounts of ROS which correlates with increased oxidative stress and oxidative alterations in their connected myelin sheaths *in vivo*. These findings are particularly relevant to the physiological function of SHP-1 in oligodendrocytes and the role of SHP-1 in inflammatory demyelinating disease where ROS production in oligodendrocytes may be particularly increased.

Materials and Methods

Animals

SHP-1-deficient motheaten (*me/me*) mice on a C3HeB/FeJLe-a/a background and their phenotypically normal littermates (wild type, WT) were produced from heterozygous breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA). The strain designation for heterozygous breeders for motheaten mice is C3FeLe.B6 a/a-Ptpn6^{me}/J (stock no. 000225). All animal procedures were approved by the Institutional Animal Care and Use Committee at SUNY Upstate Medical University in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Immunohistochemistry (IHC) and Immunocytochemistry (ICC)

Spinal cords from 14-day-old control and motheaten mice were used for immunohistochemical analyses coinciding with myelination in the murine CNS.

Myelin protein immunohistochemistry—Mice were anesthetized and intracardially perfused first with 10 ml of PBS and then with 20 ml of 4% paraformaldehyde in PBS. Spinal cords were dissected, postfixed in 4% paraformaldehyde in PBS for 1h, and then placed in a 30% sucrose solution in PBS overnight. Spinal cord samples were embedded in Tissue-Tek OCT compound (Ted Pella, Inc., Redding, CA), frozen on dry ice, and sectioned at 8µm with a cryostat. Cross-sections were used to visualize myelin gene expression within spinal cord myelinated tracts.

Immunohistochemistry of fresh frozen tissue—Mice were anesthetized, sacrificed and cooled on ice for 15 minutes, and unfixed spinal cords were dissected, placed in OCT and snap frozen in a 2-methylbutane (Sigma Aldrich, St. Louis, MO) slurry cooled with liquid nitrogen. Frozen tissue was cryostat sectioned at 8µm, dried on Superfrost Plus slides (Thermo Fisher Scientific Inc. Waltham, MA), and fixed using acetone at -20°C for 10 minutes. Endogenous peroxidase was quenched using 3% H₂O₂ in PBS for 15 minutes at room temperature (RT). The tissue was then blocked in PBS containing 10% horse serum for 2h before incubating with primary antibody overnight at 4°C. After rinsing, the tissue was incubated in the appropriate biotin-conjugated secondary antibody (Invitrogen) for 1 hour (h) at RT and then incubated in a streptavidin-alkaline phosphatase conjugate for 15 minutes. A blue alkaline phosphatase reaction product was produced using a BCIP (5-

bromo-4-chloro-3-indolylphosphate)/Nitro Blue Tetrazolium substrate kit (Zymed/Invitrogen). Alternatively, after biotin secondary, a streptavidin-HRP conjugate followed by AEC reaction was used to form a red precipitate.

Fluorescence microscopy—Cell lines were fixed with 4% paraformaldehyde in PBS, and stained as above, however secondary antibodies were fluorescently conjugated (Jackson ImmunoResearch, West Grove, PA). The following primary antibodies were used for immunohistochemistry and/or immunofluorescent staining: goat anti-MBP (Santa Cruz Biotechnology), mouse anti-PLP (Millipore, Billerica, MA), rabbit anti-Nrf2 (Santa Cruz Biotechnology), rat anti-HO-1 (R&D Systems, Minneapolis, MN), mouse anti-SHP-1 (R&D Systems), rabbit anti-Olig2 (Millipore) and goat-anti SOX10 (Santa Cruz Biotechnology).

Protein carbonylation derivatization for IHC

Protein carbonylation is a stable marker of oxidation. In order to examine whether oxidative changes had occurred in proteins of CNS tissue from P14 motheaten and WT mice, carbonyl groups were derivatized to DNP and were detected using IHC. Briefly, acetone fixed tissue sections of fresh frozen spinal cords or cultured cells on coverslips were equilibrated in TBS containing 20% methanol for 5 minutes, followed by a 5 minute incubation in 2N HCl. Tissue or coverslips were then immersed in a solution of 1 mg/ml 2,4-Dinitrophenylhydrazine (DNPH) in 2N HCl for 30 minutes. Tissue was rinsed as follows: 2N HCl 5 minutes, 50% ethanol for 2 minutes, 75% ethanol for 2 minutes, 100% ethanol for 5 minutes, 50% ethanol/50% ethyl acetate for 5 minutes, 75% ethanol for 1 minute, and 1× TBS for 1 minute. Tissue or cells were blocked as above and then incubated with primary rabbit anti-DNP (Cell Biolabs, San Diego, CA). The remaining steps were identical to the normal IHC protocol outlined above.

Brain-derived Mixed Glial Cultures

Oligodendrocytes were isolated from cultures produced from mouse brain tissue as previously described (Hudson et al. 2008). We found this technique was superior to typical postnatal day 1-2 (P1-2) glial cell cultures for generating motheaten oligodendrocytes. Briefly, cerebella were isolated from P8 mouse brains and minced in Kreb's buffer. The minced tissue was centrifuged and resuspended in Kreb's buffer containing 0.25% trypsin, and the suspension was incubated at 37°C for 1 minute. After the addition of 5% FBS and 40 µg/ml DNase in Kreb's buffer, the tissue was pelleted, resuspended in fresh Kreb's buffer-FBS-DNase, and gently triturated with a fire-polished pipette to dissociate cells. The resulting cells were centrifuged and then resuspended in complete culture medium containing Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) with 20% FBS (Gibco), 24.5 mM KCl, and 100 µg/ml of insulin and plated onto poly-L-lysine coated dishes. At 4 days post-plating and every 3 days thereafter, cells were fed with fresh medium consisting of DMEM without additional KCl/insulin and containing 10% heat-inactivated horse serum, and cells were used 10 days after plating when contaminating granule cell neurons are minimal. By morphological criteria, the remaining glia in these cultures were composed of 10% microglia, 55% astrocytes, and 35% oligodendrocytes. Oligodendrocytes were purified from 10-day mixed glial cultures as described previously (Massa et al. 2000) by differential detachment of oligodendrocytes, sieving at 15µm with sterilized Nitex nylon

fabric (SEFAR, Heiden, Switzerland), and negative selection of microglia by adherence to hydrophobic plastic. Purified oligodendrocytes were then plated onto culture chambers coated with 12.5 µg/ml poly-L-lysine. Resulting cultures were approximately 90-95% pure oligodendrocytes based on cellular morphology.

Real-time RT-PCR

Total RNA was isolated using RNA STAT-60 (Tel-test, Inc, Friendswood, TX). RNA was quantified spectrophotometrically, and 500 ng of total RNA was converted into cDNA as described (Christophi et al. 2008a). cDNA was diluted with water to a final volume of 200 µl, and 2µl was used for quantitative real-time PCR with a SYBR green kit (ABgene, Epsom, United Kingdom). The PCR parameters were 15 min for 95°C, and 40 cycles of 95°C for 15s, and 60°C for 1 min in a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). The primers were used at 10 nM. A threshold was set in the linear part of the amplification curve and the number of cycles needed to reach it was determined (Ct). Fold induction was calculated as 2^{-Ct} (Livak and Schmittgen 2001). GAPDH was used as an internal control for normalization. The amplification of each gene-specific fragment was confirmed by the examination of melting peaks. The primer pairs used in this study are shown in Table 1; myelin gene primers were generated using the Primer Bank database (Wang and Seed 2003).

Lipid Peroxidation (TBARS) Assay

To quantify the lipid peroxidation in the CNS and glial cultures, the TBARS assay was utilized according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). Briefly, for brain, 25mg of tissue was homogenized in 250µl RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at $1,600 \times g$ for 10 minutes at 4°C. For whole glia 2×10^7 cells were lysed in 1mL RIPA buffer. 100µl of sample or standard was combined with 100µl SDS solution, mixed, and then 4ml of color reagent was added and samples were boiled for 1h. The reaction was terminated by placing the samples on ice for 10 minutes; samples were quantified by measuring absorbance at 540nm (BioTek Synergy 2, Winooski, VT).

Hydrogen Peroxide Assay

The Amplex red assay was performed according to the manufacturer's specifications (Invitrogen). Briefly, control and motheaten glial cultures were incubated for 4h in fresh phenol-free DMEM containing the Amplex Red reagent and HRP. Hydrogen peroxide levels in supernatants were measured at 1, 2, and 4h by measuring absorbance at 585 nm. Significance was determined by Student's t-test for individual points, or Two-way ANOVA for the three time points combined.

NFκB DNA-binding activity assay

To quantify NFκB activity in motheaten and WT brain-derived whole glial cultures, NFκB DNA-binding activity was analyzed using the TransAM NFκB p65 transcription factor assay kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. Briefly, nuclear extracts were prepared (Massa and Wu 1998) from whole glial cultures of control

and motheaten mice that were treated with media alone or with 100 ng/mL of TNF- α . Protein levels of the nuclear extracts were quantified and 10 μ g were incubated in a 96-well plate coated with oligonucleotide containing the NF κ B consensus-binding sequence 5'-GGGACTTTCC-3'. Bound NF κ B was then detected by a p65-specific primary antibody as detailed (Christophi et al. 2009).

Chemokine and cytokine ELISA

The levels of the chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2) and the cytokines interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) in glial culture supernatants were measured using DuoSet enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems).

Live cell imaging of ROS generation

Intracellular ROS detection was determined using carboxy-H₂DCFDA according to the manufacturer's specifications (Invitrogen). Briefly, cells were plated on poly-L-lysine coated coverslips, loaded with 25 μ M carboxy-H₂DCFDA for 30 minutes at 37°C, rinsed, and treated as specified. Images were taken on a Nikon Eclipse TE300 with Spot camera and environmental chamber.

Quantitative ROS detection

Constitutive ROS production was measured by flow cytometry in cells isolated from the spinal cords of 11-day-old motheaten mice and normal littermate controls. Animals were sacrificed then perfused with 20mL ice cold PBS, and spinal cord was dissected and homogenized with a fire-polished glass pipet. Myelin debris was removed from the cell suspension using Myelin Removal Beads II and the QuadroMACS® separating system (Milenyi Biotec Inc). Following isolation, cells were labeled with the ROS indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Molecular Probes) at a concentration of 10 μ M for 30 minutes at 37°C, and then double labeled for the oligodendrocyte specific marker O4 (R&D Systems). To quantify ROS, the mean fluorescence of 10,000 – 40,000 cells per sample was measured on a BD LSR Fortessa cytometer (BD Biosciences) and the data were analyzed using FlowJo version 10.0.4. Data was standardized for normal variations in CM-H₂DCFDA intensity between experiments by dividing all samples by a WT sample in each experiment.

Electromobility Shift Assay

Specificity protein 1 (Sp1) DNA binding sequence (5'-GAGGACAACACCTTCAAAGAC-3') was annealed with the complementary strand and labeled with gamma-³²P ATP. Nuclear extracts were harvested from purified oligodendrocytes of each genotype (motheaten and normal littermates) and nuclear binding reactions with the probe were prepared. Antibodies directed against Sp1, Sp3, and Sp4 (Santa Cruz Biotechnology) were utilized to produce a supershift to determine the identity of the proteins binding to the radiolabeled probe.

N20.1 cell line

The immortalized mouse oligodendrocyte cell line, N20.1, generously provided by Dr. A.T. Campagnoni (UCLA), was maintained at 34°C, 5% CO₂, in Ham's F-12/DMEM (1:1) (Invitrogen) supplemented with 100 µg/L G418 (Invitrogen) and 10% FBS (Gibco). Cells were plated on 12.5 µg/ml poly-L-lysine coated cover slips and differentiated in 1% FBS at the permissive temperature of 39°C for 3 days in an atmosphere of 5% CO₂ before use (Verity et al. 1993).

siRNA treatment of N20.1 cell line

N20.1 cell lines were transfected using DharmaFECT-4 (Dharmacon, Lafayette, Colorado) transfection reagent and Ambion silencer select SHP-1 siRNA 5'-GCAGAAUACAAACUGCGAAtt-3' (Invitrogen) or control scrambled siRNA according to the manufacture's specifications.

Glutathione Assay in N20.1 cell line

Glutathione was measured using the GSH-Glo glutathione assay (Promega, Madison, WI) according to the manufacturer's specifications. Briefly, control or SHP-1 siRNA treated N20.1 cell lines were plated on a poly-L-lysine coated 96 well plates at a density of 1×10⁴ cells per well. 1× GSH-Glo reagent was added to each well followed by a 30 minute RT incubation. Next, luciferin detection reagent was added to each well and incubated for 15 minutes. Finally, luminescence was read on a BioTek Synergy 2 microplate reader (BioTek). A standard curve was generated by serial dilutions of a 10× glutathione solution.

Western immunoblot

Whole-cell extracts were prepared as previously described (Massa et al. 2004). Cells or tissues were homogenized in RIPA buffer. 25µg of protein per lane was electrophoresed through a 12.5% polyacrylamide resolving gel and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% nonfat dry milk for 1h, and then incubated with primary antibody followed by HRP-conjugated IgG secondary antibody (DAKO Corporation, Carpinteria, CA). Enhanced chemiluminescence (Amersham Life Sciences Inc., Cleveland, OH) was used to visualize reactive protein bands on X-ray film (Kodak, Rochester, NY). For human studies, membranes were incubated with LI-COR fluorescent secondary antibodies, imaged on a LI-COR Odyssey Fc (Lincoln, NE), and quantified using LI-COR's Image Studio Lite Version 3.1. Primary antibodies used: mouse anti-GAPDH (Thermo Fisher Scientific) mouse anti-SHP-1 (R&D Systems), and mouse anti-β-actin (MP Biomedicals, Solon, OH).

Butylated Hydroxyanisole (BHA) treatment

Brains and spinal cords of 7-day-old WT and motheaten mice were dissociated using a neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions, and oligodendrocytes were purified using O4 antibody conjugated to magnetic beads. The O4-positive oligodendrocytes were passed through a magnetic column on a QuadroMACS™ Separator (Miltenyi Biotec) to remove unlabeled glia and neurons. Following collection, O4-positive oligodendrocytes were placed in phenol-free, serum-free

DMEM. Enriched oligodendrocytes from each mouse were divided into two fractions; vehicle treated, or treated with 3 μ M BHA for 5h at 37°C, 10% CO₂, in a humidified incubator. After the 5h vehicle or BHA treatment the RNA was extracted and expression levels were quantified using real-time RT-PCR. BHA (Sigma-Aldrich) was solubilized in 100% ethanol as a 30mM stock solution in and diluted to 3 μ M in phenol-free, serum-free DMEM.

Human Tissue Collection

Fresh frozen tissue specimens were obtained from the Human Brain and Spinal Fluid Resource Center, VA West Los Angeles Healthcare Center, Los Angeles, CA 90073, which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, and the Department of Veteran Affairs. Additional frozen normal subject tissues were obtained from the Taub Institute, New York Brain Bank, Columbia University. Frozen tissue samples from normal controls and MS patients were screened by a neuropathologist to confirm the clinical diagnosis of MS and exclude confounding pathologies. Analyzed tissue samples included normal control white matter, and MS NAWM.

Statistical analysis

Histograms and tables show the mean values with standard error of the mean. The numbers of samples or individual mice used in each assay are indicated in the figure legends. For comparisons between two samples p-values were generated using the unpaired Student's t-test. Groups of three or more were analyzed using Two-way ANOVA. The particular test used for each experiment is indicated in the figure legend. A p-value of less than 0.05 was interpreted as a statistically significant difference. All statistical analysis were performed using Graphpad Prism (version 6.02).

Results

Increased oxidative stress and decreased myelin in SHP-1-deficient mouse spinal cords

We have previously shown that SHP-1-deficient mice have a defect in myelination (Massa et al. 2000). We examined spinal cord cross sections from WT and motheaten mice using IHC for myelin markers to clearly demonstrate a generalized decrease in myelin protein expression in spinal cord myelinated tracts. In agreement with previous studies, we found that two markers of myelination, MBP and proteolipid protein (PLP), are reduced in motheaten spinal cords compared to age matched WT spinal cords, and this is consistent with mRNA isolated from brain (Figure 1A, Table 2 † denotes $p < 0.05$). As a possible explanation for myelin deficiency in motheaten mice, we note previous reports that SHP-1 controls production of ROS in various cell types (Krotz et al. 2005; Lyons et al. 2003) which would affect oligodendrocyte physiology and myelin gene expression (Druzhyna et al. 2003; Smith et al. 1999; Ziabreva et al. 2010). To examine this possibility, spinal cords of SHP-1-deficient and control mice were analyzed for protein carbonylation, a stable footprint of increased ROS and oxidative stress in tissues (Dasgupta et al. 2013). In DNP-derivatized tissue sections, we found particularly strong staining in the white matter regions of the spinal cord of motheaten compared to control mice (Figure 1B). This result prompted us to analyze expression of anti-oxidant response genes nuclear factor

(erythroid-2)-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which are considered signature markers of tissue oxidative stress (Kohen and Nyska 2002). We examined longitudinal spinal cord sections to observe staining specifically within the white matter, particularly of interfascicular oligodendrocytes. Nrf2 immunohistochemistry demonstrated very strong nuclear staining throughout spinal cord sections of SHP-1 deficient mice (Figure 1B). HO-1, a detoxifying enzyme activated by Nrf2 was also highly upregulated in spinal cord sections and mRNA from brains of motheaten mice (Figure 1B, Table 2) relative to control mice. Thus, motheaten CNS tissues show signatures of oxidative stress as well as oxidative changes to white matter regions with antioxidant gene responses and reduced myelin gene expression.

Lipid peroxidation and hydrogen peroxide production in SHP-1-deficient CNS

Increased carbonylation and increased Nrf2 and HO-1 staining in motheaten spinal cord sections relative to controls indicated that SHP-1-deficient mice experience oxidative stress within the CNS. To confirm this observation, we performed the thiobarbituric acid-reactive substances (TBARS) assay, a measure of lipid peroxidation products; TBARS were significantly higher in motheaten brains compared to control animals, $p=0.02$ (Figure 2A). To address the possible cell source of lipid peroxides in motheaten CNS, we analyzed TBARS expression in mixed glial cultures. We found a significant increase ($p<0.05$) in TBARS in SHP-1-deficient glial cultures compared to control glia (Figure 2B). In addition, analysis of H_2O_2 production using the Amplex Red assay in mixed glial cultures showed a higher rate of H_2O_2 accumulation over time ($p=0.02$) and significantly higher levels of H_2O_2 after 4 hours of accumulation ($p=0.048$) in motheaten compared to WT mouse glial cultures (Figure 2C). This indicated that SHP-1 deficient glia account at least in part for production of oxidative stress and resulting lipid peroxidation in the CNS of motheaten mice.

Increased inflammatory signaling in SHP-1-deficient mice

The ability of oxidative stress to increase inflammatory signaling in tissues and the established role of oxidative stress in promoting inflammation is well-documented (Kamata et al. 2005; Reuter et al. 2010). Therefore, we analyzed the expression of genes known to participate in this type of inflammation. We found that the brain and cultured brain-derived glia of motheaten mice demonstrate a constitutive increase in numerous proinflammatory cytokines, chemokines, and metalloproteinases. The proinflammatory cytokines TNF- α and IFN- γ were induced ten-fold and six-fold, respectively, in motheaten brain (Table 2, † denotes $p<0.05$). These cytokines activate intracellular pathways that are negatively regulated by SHP-1 (Christophi et al. 2008b) and are known to increase ROS in neural cells (Mir et al. 2009). SHP-1 loss leads to over-activation of these pathways and an induction of genes containing responsive elements. Indeed, we found that STAT1 and NF κ B regulated genes are over-expressed constitutively at the mRNA level in motheaten compared to WT mouse brain and glia.

Heightened NF κ B signaling and responsive inflammatory mediators in SHP-1 deficient brain-derived glia

Numerous studies describe NF κ B activation by ROS (Schreck et al. 1991; Takada et al. 2003). Accordingly, increased NF κ B-mediated proinflammatory mRNAs were present in motheaten brains and cultured glia (Table 2). Based on this observation, we examined constitutive and induced NF κ B activity in motheaten and WT mouse brain-derived whole glial cell cultures. We observed a constitutive increase in NF- κ B binding in nuclear extracts isolated from SHP-1-deficient compared to control glia, $p=0.01$ (Figure 3A). Further, TNF- α (10 ng/mL) treatment significantly increased NF κ B binding in motheaten relative to WT glial extracts, indicating that SHP-1 is a negative regulator of TNF- α -induced inflammatory signals in glial cells (Figure 3A). Finally, NF κ B responsive proteins, IL-1 β , IL-6, and MCP-1 were significantly elevated in motheaten glial cultures relative to controls after the addition of TNF- α (Figure 3B).

Increased oxidative stress and ROS generation in CNS glia of motheaten mice

We have confirmed that motheaten mice have a reduction in myelin proteins, increased measures of oxidative stress, and elevated expression of inflammatory pathways associated with oxidative stress. To determine if oligodendrocytes in SHP-1-deficient mice are specifically affected by increased oxidative stress, we stained DNP-derivitized brain-derived glial cultures of motheaten and WT mice for carbonylated proteins using IHC (Figure 4A). By light microscopy, compared to controls, carbonylation was localized primarily to cells in motheaten cultures with morphologically distinct features of oligodendrocytes, astrocytes, and microglia (Figure 4A). Moreover, after a two-hour treatment with TNF- α , which increased ROS production in oligodendrocytes (Jana and Pahan 2005), we found an increase in carbonylation staining in both control and motheaten glia including astrocytes with flattened morphology, ramified microglia, and oligodendrocytes (Figure 4A). These results indicate that SHP-1 plays a role in mitigating constitutive oxidative stress in CNS glia. The localization of intense protein carbonylation staining to oligodendrocytes suggests that these cells are a substantial source of ROS. To confirm ROS generation specifically in oligodendrocytes, we incubated oligodendrocytes with carboxy-H₂DCFDA, an intracellular fluorescent ROS indicator dye that localizes ROS to individual cells. Via fluorescence microscopy we found that oligodendrocyte-enriched cultures from motheaten brain-derived whole glia expressed conspicuously high levels of ROS-mediated fluorescence compared to control oligodendrocytes (Figure 4B). After a 2-hour treatment with TNF- α , both SHP-1-deficient and WT oligodendrocytes induced high levels of ROS that appeared qualitatively higher in motheaten cells (Figure 4B).

To quantitatively demonstrate that oligodendrocytes were a primary source of ROS generation, we performed flow cytometry on O4⁺ oligodendrocytes freshly isolated from postnatal day 11 motheaten and control mouse CNS. Figure 4C and 4D demonstrate that O4⁺ oligodendrocytes isolated from motheaten mice produced significantly more constitutive ROS than oligodendrocytes isolated from control mice as measured by carboxy-H₂DCFDA flow cytometry (Figure 4C,D). Therefore, SHP-1 regulates constitutive production of ROS in oligodendrocytes identifying novel roles for SHP-1 within oligodendrocytes.

Oxidative stress relates to decreased Sp1 binding activity in SHP-1-deficient oligodendrocytes

Previous studies have demonstrated a role of reactive oxygen species in oligodendrocyte pathology and reduced myelin gene expression (French et al. 2009; Jana and Pahan 2005). The transcription of multiple oligodendrocyte-specific genes is regulated through transcription factors interacting at both positive and negative regulatory elements (Carson et al. 1983; Goujet-Zalc et al. 1993; Tamura et al. 1989). In particular, Sp1 and Sox10 transcription factors have been found to be essential for the high levels of tissue-specific expression of *MBP*, *PLP*, and *connexin 32* gene promoters (Peirano and Wegner 2000; Wei et al. 2004). Sp1 belongs to the Sp/KLF family of zinc-finger transcription factors and binds to GC- or GT-rich DNA sequences and activates gene transcription including the *MBP* and *PLP* genes (Janz and Stoffel 1993; Tretiakova et al. 1999), and its DNA binding activity is sensitive to ROS (Chou et al. 2005). As Sp1 is critical for myelin gene expression and is susceptible to oxidation, we examined Sp1 binding in motheaten oligodendrocytes purified from brain-derived whole-glial cultures. We confirmed that Sp1 was indeed sensitive to oxidation in a dose-dependent manner as evidenced by less binding to an Sp1 probe after a 2-hour treatment *in vitro* with H₂O₂ (data not shown). Importantly, purified motheaten oligodendrocytes demonstrated significantly less nuclear Sp1 binding activity than WT oligodendrocytes (Figure 5), consistent with increased ROS in motheaten cells.

Oxidative damage in the N20.1 oligodendrocyte cell line

To ascertain the role of SHP-1 in oligodendrocyte ROS production without the complications of other cell types or potential developmental abnormalities in an SHP-1-deficient environment, we utilized a well-characterized oligodendrocyte cell line, N20.1 (Verity et al. 1993). Carboxy-H₂DCFDA staining demonstrated that N20.1 cells expressed low levels of ROS, which were increased by TNF- α (Figure 6). To test the function of SHP-1 in ROS generation, we depleted SHP-1 in these cells using siRNA (Figure 6A,B). Following SHP-1 knockdown, expression of both constitutive and inducible ROS increased relative to N20.1 cells treated with scrambled control siRNA. Thus, SHP-1 controlled ROS production in an autonomous manner within N20.1 oligodendrocytes (Figure 6C). Moreover, nuclear localization of Nrf2 was constitutively increased within SHP-1-deficient N20.1 cells indicating constitutive oxidative stress and a concomitant anti-oxidative response (Figure 6D) as was seen in oligodendrocytes of motheaten mice (Figure 1). TNF- α further increased nuclear localization of Nrf2 within control N20.1 cell lines, but not to either constitutive or induced levels seen in SHP-1-depleted oligodendrocytes (Figure 6D). In addition, we observed glutathione depletion in SHP-1-deficient N20.1 cells indicating constitutive oxidative stress (Figure 6E). Taken together, data from N20.1 oligodendrocytes indicate that the role of SHP-1 in controlling ROS production and oxidative stress in oligodendrocytes is likely to be a direct effect of SHP-1 loss within oligodendrocytes rather than an indirect effect from other SHP-1-deficient cells in the CNS. Thus, these data support the hypothesis that oligodendrocytes are a distinct source for ROS production in the CNS and that SHP-1 is a key regulatory molecule in this production.

Abnormal gene expression in SHP-1-deficient primary oligodendrocytes is directly attributed to increased ROS

Mothaten mouse oligodendrocytes express abnormally low levels of the of the mature myelin protein genes, *MBP*, *MAG* and *CNPase*, and significantly higher levels of the Nrf2 target gene *HO-1* (Figure 7). Elevated *HO-1* is expected to result, in part, from unusually high levels of ROS compared to oligodendrocytes of WT mice. To test this hypothesis, we treated purified O4+ oligodendrocytes from both genotypes with the antioxidant BHA *ex vivo* immediately following isolation from mothaten or WT mouse, combined brain and spinal cord. As expected, BHA treatment restored mothaten oligodendrocyte *MBP*, *MAG*, *CNPase* and *HO-1* mRNA expression, and levels were no longer statistically different from WT oligodendrocytes indicating that abnormal gene expression in mothaten oligodendrocytes is directly attributed to increased ROS expressed within these cells (Figure 7). Although not statistically significant, *PLP* demonstrated a trend similar to the other mature myelin protein genes in mothaten mice. Importantly, *Sox10* and *Olig2*, transcription factors responsible for oligodendrocyte differentiation, were unchanged in both genotypes and treatments indicating that SHP-1 and ROS may control myelin gene expression independently from general mechanisms of differentiation.

Abnormal SHP-1 expression in normal appearing white matter (NAWM) of multiple sclerosis subjects

We and others have previously found SHP-1 deficiency in PBMCs and macrophages from individuals with MS (Christophi et al. 2008b; Christophi et al. 2009; Feng et al. 2002), and in this report we demonstrate that SHP-1 functions, in part, to regulate ROS in the murine CNS and oligodendrocytes. Therefore, it was of interest to examine the expression of SHP-1 in white matter from control and MS subjects. SHP-1 is expressed in control NAWM but is decreased in five MS patients (Figure 8A,B). In figure 8B, six control subjects expressed similar amounts of SHP-1 (1 ± 0.080) when normalized to GAPDH. In MS NAWM, SHP-1 expression segregates into two groups that when averaged appear similar to controls (1.167 ± 0.317) ($p=0.61$). However, when separated, three MS NAWM samples have a 2.23 ± 0.102 fold increase in SHP-1 relative to controls ($p<0.0001$) and a group of five MS NAWM samples have an approximately 50% reduction in SHP-1 relative to controls (0.522 ± 0.077 , $p=0.0021$). Of the patients with low SHP-1, three were female and two were male. Additionally, of the five SHP-1-low patients, 3 were classified as secondary progressive MS (SPMS), one as primary progressive MS (PPMS), and one as relapsing remitting MS (RRMS). All three SHP-1-high MS patients were classified as SPMS and consisted of two females and one male. Similar bimodal expression of SHP-1 in MS subjects was found when normalizing to an additional housekeeping gene, β -actin (data not shown).

Importantly, SHP-1 (blue) was expressed in SOX10 (red) (Figure 8C(c)) and Olig2 positive oligodendrocytes (red) (figure 8C(d)) demonstrating that SHP-1 is specifically enriched in oligodendrocytes of normal control subjects. These data, showing both the expression of SHP-1 in human oligodendrocytes and a bimodal expression of SHP-1 in MS NAWM, provide clinical relevance to our studies on the function of SHP-1 in CNS glia and oligodendrocytes.

Discussion

We have previously reported dysmyelination in the CNS and reduced myelin gene expression in cultured oligodendrocytes of motheaten mice (Massa et al. 2000; Massa et al. 2004). Additionally, when SHP-1 is silenced in purified oligodendrocytes they cannot differentiate properly (Kuo et al. 2010). The observed dysmyelination in SHP-1-deficient mice is likely the result of reduced myelin gene expression and subsequent suppression of proper myelin formation. While reduced myelin gene expression relates to the myelin deficit seen in motheaten mice, the signaling pathways that mediate this outcome are unknown. We propose that increased constitutive production of ROS by oligodendrocytes lacking SHP-1 regulatory activity may be a key alteration in oligodendrocyte physiology that leads to aberrant myelin gene expression. Indeed, numerous studies have implicated ROS in oligodendrocyte pathology and reduced myelin gene expression (Back et al. 1998; French et al. 2009; Jana and Pahan 2005).

Oligodendrocytes are particularly vulnerable to oxidative stress due to their high metabolic rate and high iron content (Thorburne and Juurlink 1996). Iron is a required co-factor in many myelin-synthesizing enzymes in oligodendrocytes (Connor and Menzies 1996), however it can be deleterious to the cell in Fenton reactions that form reactive hydroxyl radicals from hydrogen peroxide (Liu et al. 2004). The resultant hydroxyl radicals can react with polyunsaturated lipids in cell membranes to generate toxic lipid aldehydes, and can cause DNA damage and protein carbonylation (Welch et al. 2002). Hydroxyl radicals irreversibly modify lipids to highly reactive mono- and di-functional aldehydes including malonaldehyde and acrolein (Berlett and Stadtman 1997; Uchida 1999) which may contribute to myelin damage in experimental autoimmune encephalomyelitis (EAE) (Tully and Shi 2013). Accordingly, local increases in oxidative molecules can shift the normal homeostatic balance between ROS and endogenous antioxidants toward extreme oxidative stress in oligodendrocytes and myelin carbonylation in EAE (Dasgupta et al. 2013). Evidence that a similar shift away from ROS homeostasis occurs in SHP-1-deficient mice is shown by the induction of endogenous anti-oxidant mechanisms, particularly Nrf2 nuclear localization and an associated increase in HO-1 expression. A consequence of this imbalance is irreversible protein damage, which is indicated by increased carbonylation both *in vivo* (white matter regions of the spinal cord) and *in vitro* (predominantly in oligodendrocytes) as shown in these studies.

Treatment with TNF- α and/or IL-1 β is known to increase oxidative stress and decrease myelin gene and protein expression in oligodendrocytes (Cammer and Zhang 1999; Jana and Pahan 2005). Repression of myelin gene expression by inflammatory cytokines can be inhibited via anti-oxidants thereby demonstrating that ROS can specifically inhibit myelin gene expression (Jana and Pahan 2005). We and others have shown that MBP and PLP expression in SHP-1-deficient oligodendrocytes is reduced at the level of protein (Figure 1) and RNA (Table 2) (Kuo et al. 2010; Massa et al. 2000; Massa et al. 2004; Wishcamper et al. 2001). In agreement with the above studies, increased oxidative stress in the motheaten mice could explain this reduction in myelin genes/proteins in part by directly affecting the binding of oxidation-sensitive transcription factors such as Sp1, critical to *MBP* expression. SHP-1 may also play a role in oligodendrocyte maturation by acting as a regulator of growth

factors that affect oligodendrocyte proliferation and differentiation. However, as we observe that *SOX10* and *Olig2* transcription factors appear to be unaffected by SHP-1 loss, we believe there is a more acute and reversible effect of ROS on mature myelin gene expression, rather than epigenetic alterations responsible for stable differentiation of oligodendrocytes. We will examine this hypothesis in future studies by analyzing SHP-1-deficient oligodendrocytes in the context of a low oxidative stress environment achieved pharmacologically with antioxidants.

Oligodendrocytes have a lower antioxidant capacity relative to other glial cells resulting from reduced levels of GSH, glutathione peroxidase, and mitochondrial manganese superoxide dismutase (Juurink et al. 1998). Thus, oligodendrocytes are subject to intracellular GSH depletion (Back et al. 1998; Cammer 2002). We demonstrated that the N20.1 oligodendrocyte cell line exhibits glutathione depletion after experimentally silencing SHP-1. We believe this is a result of the constitutive ROS generated within the N20.1 cells when SHP-1 is removed. This agrees with data obtained from brain, spinal cord, and mixed glial cultures of motheaten mice, specifically exhibiting increased markers of oxidative stress relative to WT controls. Additionally, in acutely isolated purified CNS oligodendrocytes treated *ex vivo* with the antioxidant BHA, we showed the importance of oligodendrocyte-mediated ROS in the expression of the antioxidant gene *HO-1*, and in the expression of the myelin genes *MBP*, *MAG*, and *CNPase* that encode proteins critical for normal myelination. Increased *HO-1* expression is observed in motheaten oligodendrocytes along with reduced *MBP*, *MAG*, and *CNPase*. Importantly, these trends are reversed with the antioxidant BHA to levels seen in WT oligodendrocytes. These studies are important for understanding SHP-1-regulated signaling mechanisms vis-à-vis ROS production in oligodendrocytes, the potential role of ROS in oligodendrocyte pathology and how we might approach future *in vivo* rescue experiments by treating with antioxidants.

We propose a possible mechanism of increased ROS production, reduced myelin gene and protein expression, lipid oxidation, and resulting reduced myelin sheath elaboration in oligodendrocytes from mice deficient in SHP-1 (Figure 9, schematic); loss of SHP-1 can cause dysregulation of NFκB and STAT signaling. The resultant aberrant signaling may generate oxidative stress either through mitochondria or by cytoplasmic oxidases. This in turn can initiate a cycle of ROS generation and NFκB activation, as described in the hierarchical oxidative stress model (Gloire et al. 2006) and/or trigger JAK/STAT signaling (Park et al. 2012; Simon et al. 1998). This is illustrated by the increased constitutive and TNF-α-induced NFκB activity, and upregulated inflammatory cytokines and chemokines (Table 2). As an additional source of CNS damage, the innate immune system, especially microglia and macrophages, may be stimulated by oxidative damage to myelin. For instance, during conditions of oxidative stress, hydrogen peroxide and hydroxyl radicals may react directly with the lipid rich myelin and trigger phagocytosis of myelin (van der Goes et al. 1998). Finally, protein tyrosine phosphatases are highly sensitive to oxidative inactivation leading to a potential positive regulatory loop of inflammatory signaling (Meng et al. 2002; Tonks 2005).

We have previously demonstrated SHP-1 deficiency in approximately 2/3s of MS patient PBMCs and cultured macrophages that may be in part due to promoter methylation

(Christophi et al. 2008b; Christophi et al. 2009; Kumagai et al. 2012). We found that SHP-1 is reduced in a similar proportion of MS NAWM (5/8s) samples relative to MS hematopoietic cells (2/3s), and that SHP-1 is expressed in human CNS white matter oligodendrocytes. SHP-1-deficient MS monocyte derived macrophages demonstrate enhanced STAT1, STAT6 and NF κ B signaling (Christophi et al. 2009), STAT signaling is altered in multiple sclerosis NAWM (Cannella and Raine 2004; Zeis et al. 2008) and is critical to oligodendrocyte viability (Zhang et al. 2011). Another group of MS NAWM (3/8) demonstrate increased SHP-1 levels; As SHP-1 is highly expressed in hematopoietic cells, it is therefore possible that MS subjects with higher levels of SHP-1 in NAWM could have a small degree of peripheral immune cell infiltration that was undetected by pathological screening. Conversely, increased SHP-1 could result from an upregulation in resident cells of the NAWM. These data on SHP-1 expression illustrate that conditions may be present in a proportion of MS brains that would allow for hyperactivation of cytokine signaling in the CNS and could affect ROS production in oligodendrocytes. This concurs with mouse studies that demonstrate SHP-1 deficiency enhances susceptibility to both virus- and EAE-induced demyelination in rodent models of the human demyelinating disease multiple sclerosis (Deng et al. 2002; Massa et al. 2002). Thus, the bimodal expression of SHP-1 in NAWM of MS subjects, and the expression of SHP-1 in oligodendrocytes provide a possible clinical relevance to our research using *in vitro* and *in vivo* mouse models and justify further study of SHP-1 in the CNS.

The source or sources of ROS in motheaten mice and how SHP-1 regulates its production in oligodendrocytes is presently unclear. However, a number of likely possibilities are being considered including cytoplasmic oxidases and mitochondria. Mitochondrial ROS are attractive candidates as cytokines and signaling molecules controlled by SHP-1 are known to directly stimulate ROS generation from these sources. For instance, the absence of SHP-1 increases the activity of several transcription factors, including NF κ B, STAT3, and STAT6, which are known to individually or in combination induce ROS production via mitochondrial and NADPH oxidases (Anrather et al. 2006; Sarafian et al. 2010; Sharma et al. 2008; Shulga and Pastorino 2012; Wegrzyn et al. 2009). Future studies will characterize the mechanisms by which SHP-1 controls ROS production in oligodendrocytes and the role for this regulation in CNS homeostasis and inflammatory demyelinating disease.

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References

- Anrather J, Racchumi G, Iadecola C. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J Biol Chem.* 2006; 281:5657–67. [PubMed: 16407283]
- Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J Neurosci.* 1998; 18:6241–53. [PubMed: 9698317]

- Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem.* 1997; 272:20313–6. [PubMed: 9252331]
- Cammer W. Protection of cultured oligodendrocytes against tumor necrosis factor-alpha by the antioxidants coenzyme Q(10) and N-acetyl cysteine. *Brain Res Bull.* 2002; 58:587–92. [PubMed: 12372563]
- Cammer W, Zhang H. Maturation of oligodendrocytes is more sensitive to TNF alpha than is survival of precursors and immature oligodendrocytes. *J Neuroimmunol.* 1999; 97:37–42. [PubMed: 10408976]
- Cannella B, Raine CS. Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation. *Ann Neurol.* 2004; 55:46–57. [PubMed: 14705111]
- Carson JH, Nielson ML, Barbaresi E. Developmental regulation of myelin basic protein expression in mouse brain. *Dev Biol.* 1983; 96:485–92. [PubMed: 6187613]
- Chou WC, Chen HY, Yu SL, Cheng L, Yang PC, Dang CV. Arsenic suppresses gene expression in promyelocytic leukemia cells partly through Sp1 oxidation. *Blood.* 2005; 106:304–10. [PubMed: 15761015]
- Christophi GP, Hudson CA, Gruber R, Christophi CP, Massa PT. Promoter-specific induction of the phosphatase SHP-1 by viral infection and cytokines in CNS glia. *J Neurochem.* 2008a; 105:2511–23. [PubMed: 18331586]
- Christophi GP, Hudson CA, Gruber RC, Christophi CP, Mihai C, Mejico LJ, Jubelt B, Massa PT. SHP-1 deficiency and increased inflammatory gene expression in PBMCs of multiple sclerosis patients. *Lab Invest.* 2008b; 88:243–55. [PubMed: 18209728]
- Christophi GP, Panos M, Hudson CA, Christophi RL, Gruber RC, Mersich AT, Blystone SD, Jubelt B, Massa PT. Macrophages of multiple sclerosis patients display deficient SHP-1 expression and enhanced inflammatory phenotype. *Lab Invest.* 2009; 89:742–59. [PubMed: 19398961]
- Connor JR, Menzies SL. Relationship of iron to oligodendrocytes and myelination. *Glia.* 1996; 17:83–93. [PubMed: 8776576]
- Dasgupta A, Zheng J, Perrone-Bizzozero NI, Bizzozero OA. Increased carbonylation, protein aggregation and apoptosis in the spinal cord of mice with experimental autoimmune encephalomyelitis. *ASN Neuro.* 2013; 5:e00111. [PubMed: 23489322]
- Deng C, Minguela A, Hussain RZ, Lovett-Racke AE, Radu C, Ward ES, Racke MK. Expression of the tyrosine phosphatase SRC homology 2 domain-containing protein tyrosine phosphatase 1 determines T cell activation threshold and severity of experimental autoimmune encephalomyelitis. *J Immunol.* 2002; 168:4511–8. [PubMed: 11970996]
- Druzhyna NM, Hollensworth SB, Kelley MR, Wilson GL, Ledoux SP. Targeting human 8-oxoguanine glycosylase to mitochondria of oligodendrocytes protects against menadione-induced oxidative stress. *Glia.* 2003; 42:370–8. [PubMed: 12730957]
- Feng X, Petraglia AL, Chen M, Byskosh PV, Boos MD, Reder AT. Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1. *J Neuroimmunol.* 2002; 129:205–15. [PubMed: 12161037]
- French HM, Reid M, Mamontov P, Simmons RA, Grinspan JB. Oxidative stress disrupts oligodendrocyte maturation. *J Neurosci Res.* 2009; 87:3076–87. [PubMed: 19479983]
- Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol.* 2006; 72:1493–505. [PubMed: 16723122]
- Goujet-Zalc C, Babinet C, Monge M, Timsit S, Cabon F, Gansmuller A, Miura M, Sanchez M, Pournin S, Mikoshiba K. The proximal region of the MBP gene promoter is sufficient to induce oligodendroglial-specific expression in transgenic mice. *Eur J Neurosci.* 1993; 5:624–32. others. [PubMed: 7505168]
- Green MC, Shultz LD. Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. *J Hered.* 1975; 66:250–8. [PubMed: 1184950]
- Hudson CA, Christophi GP, Gruber RC, Wilmore JR, Lawrence DA, Massa PT. Induction of IL-33 expression and activity in central nervous system glia. *J Leukoc Biol.* 2008; 84:631–43. [PubMed: 18552204]
- Jana M, Pahan K. Redox regulation of cytokine-mediated inhibition of myelin gene expression in human primary oligodendrocytes. *Free Radic Biol Med.* 2005; 39:823–31. [PubMed: 16109311]

- Janz R, Stoffel W. Characterization of a brain-specific Sp1-like activity interacting with an unusual binding site within the myelin proteolipid protein promoter. *Biol Chem Hoppe Seyler*. 1993; 374:507–17. [PubMed: 7692880]
- Johnstone JT, Morton PD, Jayakumar AR, Johnstone AL, Gao H, Bracchi-Ricard V, Pearse DD, Norenberg MD, Bethea JR. Inhibition of NADPH oxidase activation in oligodendrocytes reduces cytotoxicity following trauma. *PLoS One*. 2013; 8:e80975. [PubMed: 24260524]
- Juurink BH, Thorburne SK, Hertz L. Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress. *Glia*. 1998; 22:371–8. [PubMed: 9517569]
- Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. 2005; 120:649–61. [PubMed: 15766528]
- Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol*. 2002; 30:620–50. [PubMed: 12512863]
- Krotz F, Engelbrecht B, Buerkle MA, Bassermann F, Bridell H, Gloe T, Duyster J, Pohl U, Sohn HY. The tyrosine phosphatase, SHP-1, is a negative regulator of endothelial superoxide formation. *J Am Coll Cardiol*. 2005; 45:1700–6. [PubMed: 15893190]
- Kumagai C, Kalman B, Middleton FA, Vyshkina T, Massa PT. Increased promoter methylation of the immune regulatory gene SHP-1 in leukocytes of multiple sclerosis subjects. *J Neuroimmunol*. 2012; 246:51–7. [PubMed: 22458980]
- Kuo E, Park DK, Tzvetanova ID, Leiton CV, Cho BS, Colognato H. Tyrosine phosphatases Shp1 and Shp2 have unique and opposing roles in oligodendrocyte development. *J Neurochem*. 2010; 113:200–12. [PubMed: 20132481]
- Liu Q, Berchner-Pfannschmidt U, Moller U, Brecht M, Wotzlaw C, Acker H, Jungermann K, Kietzmann T. A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. *Proc Natl Acad Sci U S A*. 2004; 101:4302–7. [PubMed: 15010533]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
- Lyons BL, Lynes MA, Burzenski L, Joliat MJ, Hadjout N, Shultz LD. Mechanisms of anemia in SHP-1 protein tyrosine phosphatase-deficient “viable motheaten” mice. *Exp Hematol*. 2003; 31:234–43. [PubMed: 12644021]
- Mandal D, Fu P, Levine AD. REDOX regulation of IL-13 signaling in intestinal epithelial cells: usage of alternate pathways mediates distinct gene expression patterns. *Cell Signal*. 2010; 22:1485–94. [PubMed: 20570727]
- Mariappan N, Elks CM, Sriramula S, Guggilam A, Liu Z, Borkhsenius O, Francis J. NF-kappaB-induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes. *Cardiovasc Res*. 2010; 85:473–83. [PubMed: 19729361]
- Massa PT, Ropka SL, Saha S, Fecenko KL, Beuler KL. Critical role for protein tyrosine phosphatase SHP-1 in controlling infection of central nervous system glia and demyelination by Theiler’s murine encephalomyelitis virus. *J Virol*. 2002; 76:8335–46. [PubMed: 12134038]
- Massa PT, Saha S, Wu C, Jarosinski KW. Expression and function of the protein tyrosine phosphatase SHP-1 in oligodendrocytes. *Glia*. 2000; 29:376–85. [PubMed: 10652447]
- Massa PT, Wu C. Increased inducible activation of NF-kappaB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. *J Interferon Cytokine Res*. 1998; 18:499–507. [PubMed: 9712366]
- Massa PT, Wu C, Fecenko-Tacka K. Dysmyelination and reduced myelin basic protein gene expression by oligodendrocytes of SHP-1-deficient mice. *J Neurosci Res*. 2004; 77:15–25. [PubMed: 15197735]
- Mc Guire C, Prinz M, Beyaert R, van Loo G. Nuclear factor kappa B (NF-kappaB) in multiple sclerosis pathology. *Trends Mol Med*. 2013; 19:604–13. [PubMed: 24007818]
- McTigue DM, Tripathi RB. The life, death, and replacement of oligodendrocytes in the adult CNS. *J Neurochem*. 2008; 107:1–19. [PubMed: 18643793]

- Meng TC, Fukada T, Tonks NK. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell*. 2002; 9:387–99. [PubMed: 11864611]
- Mir M, Asensio VJ, Tolosa L, Gou-Fabregas M, Soler RM, Llado J, Olmos G. Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants. *Neuroscience*. 2009; 162:959–71. [PubMed: 19477238]
- Park SJ, Lee JH, Kim HY, Choi YH, Park JS, Suh YH, Park SM, Joe EH, Jou I. Astrocytes, but not microglia, rapidly sense H₂O₂ via STAT6 phosphorylation, resulting in cyclooxygenase-2 expression and prostaglandin release. *J Immunol*. 2012; 188:5132–41. [PubMed: 22504638]
- Peirano RI, Wegner M. The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences. *Nucleic Acids Res*. 2000; 28:3047–55. [PubMed: 10931919]
- Reich NC. STAT3 revs up the powerhouse. *Sci Signal*. 2009; 2:pe61. [PubMed: 19797267]
- Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*. 2010; 49:1603–16. [PubMed: 20840865]
- Sarafian TA, Montes C, Imura T, Qi J, Coppola G, Geschwind DH, Sofroniew MV. Disruption of astrocyte STAT3 signaling decreases mitochondrial function and increases oxidative stress in vitro. *PLoS One*. 2010; 5:e9532. [PubMed: 20224768]
- Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J*. 1991; 10:2247–58. [PubMed: 2065663]
- Sharma P, Chakraborty R, Wang L, Min B, Tremblay ML, Kawahara T, Lambeth JD, Haque SJ. Redox regulation of interleukin-4 signaling. *Immunity*. 2008; 29:551–64. [PubMed: 18957266]
- Shulga N, Pastorino JG. GRIM-19-mediated translocation of STAT3 to mitochondria is necessary for TNF-induced necroptosis. *J Cell Sci*. 2012; 125:2995–3003. [PubMed: 22393233]
- Shultz LD, Green MC. Motheaten, an immunodeficient mutant of the mouse. II. Depressed immune competence and elevated serum immunoglobulins. *J Immunol*. 1976; 116:936–43. [PubMed: 56406]
- Sidman CL, Shultz LD, Unanue ER. The mouse mutant “motheaten.” II. Functional studies of the immune system. *J Immunol*. 1978; 121:2399–404. [PubMed: 363947]
- Simon AR, Rai U, Fanburg BL, Cochran BH. Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol*. 1998; 275:C1640–52. [PubMed: 9843726]
- Smith KJ, Kapoor R, Felts PA. Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol*. 1999; 9:69–92. [PubMed: 9989453]
- Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. *J Biol Chem*. 2003; 278:24233–41. [PubMed: 12711606]
- Tamura T, Aoyama A, Inoue T, Miura M, Okano H, Mikoshiba K. Tissue-specific in vitro transcription from the mouse myelin basic protein promoter. *Mol Cell Biol*. 1989; 9:3122–6. [PubMed: 2476662]
- Thorburne SK, Juurlink BH. Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. *J Neurochem*. 1996; 67:1014–22. [PubMed: 8752107]
- Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell*. 2005; 121:667–70. [PubMed: 15935753]
- Tretiakova A, Steplewski A, Johnson EM, Khalili K, Amini S. Regulation of myelin basic protein gene transcription by Sp1 and Puralpha: evidence for association of Sp1 and Puralpha in brain. *J Cell Physiol*. 1999; 181:160–8. [PubMed: 10457364]
- Tully M, Shi R. New insights in the pathogenesis of multiple sclerosis-role of acrolein in neuronal and myelin damage. *Int J Mol Sci*. 2013; 14:20037–47. [PubMed: 24113583]
- Uchida K. Current status of acrolein as a lipid peroxidation product. *Trends Cardiovasc Med*. 1999; 9:109–13. [PubMed: 10639724]

- van der Goes A, Brouwer J, Hoekstra K, Roos D, van den Berg TK, Dijkstra CD. Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *J Neuroimmunol.* 1998; 92:67–75. [PubMed: 9916881]
- Verity AN, Bredesen D, Vonderscher C, Handley VW, Campagnoni AT. Expression of myelin protein genes and other myelin components in an oligodendrocytic cell line conditionally immortalized with a temperature-sensitive retrovirus. *J Neurochem.* 1993; 60:577–87. [PubMed: 7678286]
- Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res.* 2003; 31:e154. [PubMed: 14654707]
- Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A. Function of mitochondrial Stat3 in cellular respiration. *Science.* 2009; 323:793–7. others. [PubMed: 19131594]
- Wei Q, Miskimins WK, Miskimins R. Sox10 acts as a tissue-specific transcription factor enhancing activation of the myelin basic protein gene promoter by p27Kip1 and Sp1. *J Neurosci Res.* 2004; 78:796–802. [PubMed: 15523643]
- Welch KD, Davis TZ, Van Eden ME, Aust SD. Deleterious iron-mediated oxidation of biomolecules. *Free Radic Biol Med.* 2002; 32:577–83. [PubMed: 11909692]
- Wishcamper CA, Coffin JD, Lurie DI. Lack of the protein tyrosine phosphatase SHP-1 results in decreased numbers of glia within the motheaten (me/me) mouse brain. *J Comp Neurol.* 2001; 441:118–33. [PubMed: 11745639]
- Zeis T, Graumann U, Reynolds R, Schaeren-Wiemers N. Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. *Brain.* 2008; 131:288–303. [PubMed: 18056737]
- Zhang J, Zhang Y, Dutta DJ, Argaw AT, Bonnamain V, Seto J, Braun DA, Zameer A, Hayot F, Lopez CB. Proapoptotic and antiapoptotic actions of Stat1 versus Stat3 underlie neuroprotective and immunoregulatory functions of IL-11. *J Immunol.* 2011; 187:1129–41. others. [PubMed: 21709156]
- Ziabreva I, Campbell G, Rist J, Zamboni J, Rorbach J, Wydro MM, Lassmann H, Franklin RJ, Mahad D. Injury and differentiation following inhibition of mitochondrial respiratory chain complex IV in rat oligodendrocytes. *Glia.* 2010; 58:1827–37. [PubMed: 20665559]

Main Points

SHP-1 deficiency results in increased CNS inflammation and oxidative stress. SHP-1 deficient oligodendrocytes produce more ROS resulting in oxidative stress, oxidized myelin and a deficiency in myelination. SHP-1 is deficient in a proportion of MS NAWM.

Figure 1.

Immunohistochemical analysis of oxidative stress and reduced myelination in SHP-1 deficient spinal cords.

A. Wild type mouse spinal cord cross-sections demonstrate strong staining of myelin proteins (a,c). In contrast, motheaten spinal cords demonstrate reduced myelin protein staining (b,d). B. Higher DNP staining in motheaten spinal cord cross sections (b) indicates higher constitutive oxidative stress in naïve motheaten mice relative to control (a). Nrf2 and HO-1 staining indicate increased expression in longitudinal sections of motheaten spinal cords (d,f) relative to WT (c,e).

Figure 2.

Increased markers of ROS in motheaten brains and glia.

A. Thiobarbituric Acid Reactive Substances (TBARS) were used as a measure of lipid peroxidation. Lipid peroxidation is significantly higher ($p < 0.01$) in motheaten ($n=5$) brain lysates than in WT ($n=7$) brain lysates. B. Lipid peroxidation is significantly ($p < 0.05$) higher in motheaten ($n=4$) whole glial brain-derived lysates than in WT ($n=4$) whole glial lysates. C. Hydrogen peroxide production in glial cultures. WT ($n=4$) and motheaten ($n=4$) brain-derived glial cultures were incubated for 4 hours in fresh phenol free DMEM. Hydrogen

peroxide levels in supernatants were measured at 1, 2, and 4 hours using a hydrogen peroxide assay kit (Amplex Red, Invitrogen). Over the 4 hour time course, motheaten cultures produced significantly more hydrogen peroxide than WT cultures $p=0.02$ by two-way ANOVA: at 4 hour time point, $p=0.048$ by Student's t-test).

Figure 3.

NF-κB activation and cytokine upregulation in wild type and motheaten brain-derived glia.

A. Nuclear lysates from whole WT (n=6) and motheaten glia (n=6) in either media alone or with one hour TNF-α treatment (10ng/mL) were isolated and allowed to bind to an NF-κB consensus binding sequence. Bound NF-κB was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF-κB binding activity is reported as nanograms of bound p65 protein per 10μg of glial nuclear protein. A constitutive increase in NF-κB activation is seen in motheaten glia relative to WT. After TNF-α treatment, NF-κB binding increases in both WT and motheaten glia, however motheaten glia continue to demonstrate significantly higher NF-κB binding. B. NF-κB inducible proteins, IL-1β, IL-6, and MCP-1 were measured in glial supernatants after 18h treatments with TNF-α using ELISA. After TNF-α treatment SHP-1 deficient glia (n=6) demonstrate a significant increase in secreted NF-κB inducible proteins relative to WT (n=6), p value is shown, Student's t-test.

Figure 4. Increased oxidative stress in motheaten oligodendrocytes. A. Stable oxidative modifications to proteins in SHP-1 deficient mice. Immunoreactivity is markedly increased constitutively in motheaten (b) brain-derived oligodendrocytes relative to control (a), indicating increased protein carbonylation. A 2 hour treatment with TNF- α (100ng/ μ L) appeared to increase protein carbonylation in WT brain-derived oligodendrocytes (c). Motheaten oligodendrocytes (d) appeared to have similar levels of DNP staining. B. Increased reactive oxygen species in motheaten oligodendrocytes.

Untreated brain-derived oligodendrocytes from motheaten mice (b) demonstrate a higher level of constitutive ROS than WT oligodendrocytes (arrows) (a). After a 2 hour treatment with TNF- α (100ng/mL), oligodendrocytes from either WT mice (c), or motheaten mice (d) express a high level of reactive oxidative species. Cells were pre-loaded with Carboxy-H₂DCFDA (Invitrogen) for 15 minutes, rinsed, then treated with culture media or TNF- α , and then imaged live. C. Cell specific constitutive ROS production was quantified by flow cytometry in O4⁺ oligodendrocytes directly isolated from spinal cords motheaten mice and littermate controls. WT (n=7) and motheaten (n=4) cells were incubated with 10 μ M CM-H₂DCFDA and then labeled with O4 antibody. 10,000-40,000 events per sample were collected and analyzed using FlowJo (Tree Star), representative mean fluorescence curves for motheaten vs WT mice are shown. D. Average MFI for motheaten and controls are shown, data is standardized to WT MFI (Student's t-test, *p=0.042).

Figure 5.

Oxidation can directly affect binding of Sp1 to the myelin basic protein promoter region. A. Analysis of Sp1, Sp3, and Sp4 binding activities in purified brain-derived oligodendrocyte nuclei of WT and motheaten mice. Oligodendrocyte nuclear extracts were incubated with a ^{32}P -labeled Sp1 probe. In some reactions antibodies to either Sp1, Sp3, or Sp4 either singly or in pairs were added to supershift/delineate individual components of the overlapping Sp factors in the upper binding activity as indicated. B. Quantification of the upper binding activity containing Sp1, Sp3, and Sp4 in WT and motheaten oligodendrocytes. Pixel density of the upper band (Sp1) was determined using ImageJ and a histogram was created representing the fold difference in radiolabeled probe binding between nuclear extracts harvested from purified oligodendrocytes of WT and motheaten cultures. Statistical significance was determined by Student's t-test (* $p < 0.05$).

Figure 6.

Increased ROS generation in SHP-1 deficient N20.1 cells: A. Cells were transfected with scrambled siRNA control or SHP-1 siRNA and analyzed for SHP-1 expression by Western immunoblot, positive and negative control protein homogenates are isolated from motheaten and WT spleen. B. Cells were then double stained for Olig2 (green) or SHP-1 (red). C. Cells were loaded with 25 μ M carboxy-H₂DCFDA for 30 minutes at 37°C. Increased fluorescence is observed in media-treated SHP-1 deficient N20.1 cell lines (control). Following treatments with H₂O₂ or TNF- α (100ng/mL), SHP-1 deficient cells demonstrate a large

increase in fluorescence whereas only a nominal increase is seen in control cells. D. Nrf2 staining in cells transfected with control or SHP-1 siRNA. Nuclear localization can be seen in untreated SHP-1 deficient cells (arrowhead), and in both control and SHP-1 cells after a 2 hour treatment with TNF- α . E. Decreased glutathione content in SHP-1 deficient N20.1 cell line. The GSH-Glo glutathione assay was performed using control (n=5) or SHP-1 (n=5) deficient N20.1 cell lines. Untreated cell lines exhibited a significant constitutive reduction in glutathione content ($p < 0.01$) when SHP-1 was reduced using siRNA. A 2-hour treatment with TNF- α (100ng/mL) appeared to increase glutathione levels in control cells and to have the opposite effect on SHP-1 deficient cells, however neither of these were significant, $p > 0.05$. Statistical differences were determined by Two-Way ANOVA with Bonferroni's multiple comparison test, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Figure 7.

Treatment with the antioxidant butylated hydroxyanisole (BHA) restores mature myelin gene expression and reduces mRNA expression of a marker of oxidative stress, *HO-1*, in motheaten mice to WT levels. Oligodendrocytes from WT (n=7) and motheaten (n=5) brain and spinal cord were isolated by magnetic bead separation, analyzed by real time qRT-PCR. mRNA expression is represented as fold change from motheaten. Treatment with the antioxidant BHA for 5 hr *in vitro* at 37°C, 10% CO₂, increases mRNA expression of *MBP*, *MAG* and *CNPase*, and reduces *HO-1* to levels in WT mouse oligodendrocytes. Motheaten *HO-1* expression decreases 22.8% from 1.0±0.17 to 0.77±0.08 after BHA treatment. Treatment of isolated motheaten spinal cord and brain oligodendrocytes with BHA increases *MBP* expression by 23% from 1.0±0.04 to 1.23±0.07. After BHA treatment, *MAG* expression increases 47% from 1.0±0.08 to 1.47±0.28, and *CNPase* expression increased 36% from 1.0±0.08 to 1.36±0.19. The mRNA level of two pan-oligodendrocyte genes, *SOX10* and *Olig2*, are unchanged in both genotypes and following both treatments.

Statistical differences were determined by Two-Way ANOVA with Bonferroni's multiple comparison test, *p 0.05 **p 0.01 ***p 0.001.

Figure 8.

SHP-1 is expressed in human oligodendrocytes and is deficient in a population of MS patients. A. Western immunoblot for SHP-1, ~68kDa (top) and GAPDH (bottom). SHP-1 is expressed in control NAWM but is visibly decreased in five MS patients. The first lane of the SHP-1 Immunoblot is the 64kDa band of the molecular weight marker (MWM). B. Quantified SHP-1/GAPDH expressed as fold difference from control subjects. Control subject SHP-1 expression clusters in a tight grouping when SHP-1 is normalized to GAPDH (1 ± 0.080). However, MS NAWM SHP-1 expression segregates into two groups. When these groups are combined they appear similar in SHP-1 expression (1.167 ± 0.317) ($p=0.61$, Student's t-test). However when separated, three MS NAWM samples have a 2.23 ± 0.102 fold increase in SHP-1 ($p<0.0001$, Student's t-test) and a group of five MS NAWM samples have a deficiency 0.522 ± 0.077 ($p=0.0021$, Student's t-test). C. SHP-1 is expressed in human oligodendrocytes. Human control white matter was stained for SHP-1 (a) or IgG control (b). Arrows in figure 8C(a) indicate interfascicular oligodendrocyte chains. Oligodendrocyte staining was confirmed by staining for SHP-1 blue (c,d) and SOX10 red (c) or Olig2 red (d).

Figure 9.

Schematic representation of dysmyelination by SHP-1 deficiency: SHP-1 acts as a constitutive inhibitor of cytokine, chemokine and growth factor signaling. In the absence of SHP-1, oligodendrocytes experience functional impairment partially due to increased constitutive ROS production. Increased ROS can activate NFκB and vice versa resulting in a self-perpetuating cycle of oxidative stress. Additionally, oxidized myelin and increased chemokine production by glia may attract SHP-1-deficient microglia, (or during active demyelination, infiltrating monocytes/macrophages) which in turn produce increased inflammation, resulting in a feed-forward loop of enhanced myelin degradation and inflammation.

Table 1
qRT-PCR primers

Product of target gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>TNF-α</i>	TGAACTTCGGGGTGATCGGTC	AGCCTTGTCCTTGAAGAGAAC
<i>IL-6</i>	CAGAGGATACCACTACCAACAG	TCTCATTCCACCACGATTCC
<i>LT-α</i>	TTCCTCCCAATACCCCTTC	AGGCTCCAAAGAATACACTGC
<i>IL-4</i>	GGTCTCAACCCCAAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
<i>IL-13</i>	CCTGGCTCTTGCTTGCCTT	GGTCTTGTTGATGTTGCTCA
<i>TARC</i>	TACCATGAGGTCACTTCAGATGC	GCACTCTCGGCCTACATTGG
<i>IFN-γ</i>	TCAAGTGGCATAGATGTGAAGAA	TGGCTCTGCAGGATTTTCATG
<i>IP-10</i>	CCAAGTGCTGCCGTCATTTC	GGCTCGCAGGGATGATTTCAA
<i>MMP3</i>	TTGACGTGAACGTAGG	GGCTGAGTGGTAGAGTCCCAG
<i>ADAM8</i>	GCAGGACCATTCCTCTAC	GACCCAACCGGAAAAAGC
<i>Arginase I</i>	TTTCTCAAAAGGACAGCCTC	GTGAGCATCCACCCAAATG
<i>RANTES</i>	AGCTGCCCTACCATCATC	CTCTGGGTGGCACACACTT
<i>MCP-1</i>	GTATGTCTGGACCATTCCTTC	GCTGTAGTTTTTGTACCAAGC
<i>MIP-1α</i>	CAGCCAGGTGTCATTTCCT	AGGCATTAGTTCCAGGTCA
<i>Eotaxin</i>	TGCTCACGGTCACTTCCTTC	TTTGCCCAACCTGGTCTTG
<i>MBP</i>	GGCGGTGACAGACTCCAA	GAAGCTCGTCGGACTCTGAG
<i>PLP</i>	TGAGCGCAACGGTAACAGG	GGGAGAACACCATAACATTCTGG
<i>CNPase</i>	GACTTCTCCGAGGCGTACAAG	CCGCTCGTGGTTGGTATCA
<i>MAG</i>	CTGCCGCTGTTTTGGATAATGA	CATCGGGGAAGTCGAAACGG
<i>Olig2</i>	TCCCAGAACCCGATGATCTT	CGTGGACGAGGACACAGTC
<i>SOX10</i>	GAAGCCCCACATCGACTTCG	GGCAGGTATTGGTCCAGCTC
<i>HO-1</i>	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
<i>NOX2</i>	ACTCCTGGGTGAGCACTGG	GTTCTGTCCAGTTGTCTTCG
<i>GAPDH</i>	ACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA

Table 2
Increased inflammatory and decreased myelin gene expression in brains of motheaten, glial cultures compared to control mice

Total RNA from WT mouse (n=14) and motheaten total brain homogenates (n=11) and brain-derived mixed glia (n=8 and n=8 respectively) were analyzed using qRT-PCR. Fold change is in relation to wild-type mice, which is designated at 1. Gene expression was normalized to the housekeeping gene GAPDH.

	Gene	CNS (brain)		Mixed Glia	
		WT	me/me	WT	me/me
STAT1 Inducible	<i>IFN-γ</i>	1 $\pm$ 0.3	6 $\pm$ 2 [†]	ND	ND
	<i>IP-10 (CXCL10)</i>	1 $\pm$ 0.2	9.3 $\pm$ 2 [†]	1 $\pm$ 0.3	1.1 $\pm$ 0.2
	<i>RANTES (CCL5)</i>	1 $\pm$ 0.1	5.3 $\pm$ 1.3 [†]	1 $\pm$ 0.3	0.5 $\pm$ 0.2
STAT6 Inducible	<i>IL-4</i>	1 $\pm$ 0.2	0.8 $\pm$ 0.3	1 $\pm$ 0.2	2.1 $\pm$ 0.8
	<i>IL-13</i>	1 $\pm$ 0.4	1.2 $\pm$ 0.3	1 $\pm$ 0.1	1.5 $\pm$ 0.5
	<i>TARC (CCL17)</i>	1 $\pm$ 0.1	1 $\pm$ 0.1	1 $\pm$ 0.2	1.4 $\pm$ 0.3
	<i>Arg1</i>	1 $\pm$ 0.1	3.2 $\pm$ 0.4 [†]	1 $\pm$ 0.2	3.6 $\pm$ 1.2 [†]
	<i>ADAM8</i>	1 $\pm$ 0.1	0.9 $\pm$ 0.3	1 $\pm$ 0.1	1.9 $\pm$ 0.2 [†]
	<i>Eotaxin (CCL11)</i>	1 $\pm$ 0.1	2.6 $\pm$ 0.8 [†]	1 $\pm$ 0.6	1.2 $\pm$ 0.2
NF κ B Inducible	<i>LT-α</i>	1 $\pm$ 0.1	2.9 $\pm$ 0.2 [†]	1 $\pm$ 0.1	0.9 $\pm$ 0.2
	<i>TNF-α</i>	1 $\pm$ 0.2	10 $\pm$ 2 [†]	1 $\pm$ 0.1	2.9 $\pm$ 0.1 [†]
	<i>IL-6</i>	1 $\pm$ 0.1	0.8 $\pm$ 0.2	1 $\pm$ 0.2	3.1 $\pm$ 0.2 [†]
	<i>MCP-1 (CCL2)</i>	1 $\pm$ 0.1	11 $\pm$ 3 [†]	1 $\pm$ 0.2	2.3 $\pm$ 0.1 [†]
	<i>MIP-1α (CCL3)</i>	1 $\pm$ 0.1	30 $\pm$ 5 [†]	1 $\pm$ 0.3	2.5 $\pm$ 1.1 [†]
	<i>MMP3</i>	1 $\pm$ 0.3	7 $\pm$ 2 [†]	1 $\pm$ 0.1	1.2 $\pm$ 0.5
Myelin Genes	<i>MBP</i>	1 $\pm$ 0.14	0.27 $\pm$ 0.1 [†]	1 $\pm$ 0.15	0.4 $\pm$ 0.14 [†]
	<i>PLP</i>	1 $\pm$ 0.23	0.13 $\pm$ 0.07 [†]	1 $\pm$ 0.15	0.02 $\pm$ 0.01 [†]
Oxidative Stress	<i>HO-1</i>	1 $\pm$ 0.1	2.6 $\pm$ 0.6 [†]	1 $\pm$ 0.52	3.8 $\pm$ 1.7
	<i>Gp91phox (Nox2)</i>	1 $\pm$ 1.02	10.7 $\pm$ 0.9 [†]	1 $\pm$ 0.25 [†]	3.2 $\pm$ 1.4 [†]
Control	<i>GAPDH</i>	1 $\pm$ 0.1	1.1 $\pm$ 0.2	1 $\pm$ 0.2	1.2 $\pm$ 0.2

[†] Indicates a significance of p<0.05. IFN- γ was not detected (ND) in glial cultures.

Table 3
Normal and Multiple Sclerosis (MS) subjects used in study

The mean age of normal subjects is 68.5 ± 6.2 years and that of MS subjects is 60.88 ± 4.7 years ($p=0.28$, Student's t-test). The mean autolysis time is 13.8 ± 2.3 h in normal subjects and 19.3 ± 2.0 h in MS subjects ($p=0.08$, Student's t-test).

Source	Case #	Age	Gender	Diagnosis	Neuropathology Diagnosis	Autolysis (hr)
UCLA	3912	80	M	Lung cancer	Normal	10.5
UCLA	3861	81	F	COPD	Normal	14.5
UCLA	3992	73	F	Heart attack	Normal	15
UCLA	3997	77	M	Lung cancer	Normal	12
NYBB	T-164	41	M	Non-neurologic	Normal	7
NYBB	T-274	65	F	Non-neurologic	Normal	23.5
UCLA	3831	82	F	SPMS	MS NAWM	21
UCLA	3163	57	F	SPMS	MS NAWM	20
UCLA	3185	50	M	PPMS	MS NAWM	13
UCLA	3025	71	M	SPMS	MS NAWM	23
UCLA	3289	54	F	RRMS	MS NAWM	29
UCLA	3161	51	F	SPMS	MS NAWM	19.5
UCLA	3250	75	M	SPMS	MS NAWM	17.5
UCLA	3260	47	F	SPMS	MS NAWM	11.5

Chronic Obstructive Pulmonary Disease (COPD), Normal Appearing White Matter (NAWM), Primary Progressive Multiple Sclerosis (PPMS) and Relapsing Remitting MS (RRMS), Secondary Progressive MS (SPMS).