Modulation of macrophage infiltration and inflammatory activity by the phosphatase SHP-1 in virus-induced demyelinating disease.

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ABSTRACT

The protein tyrosine phosphatase SHP-1 is a crucial negative regulator of cytokine signaling and inflammatory gene expression both in the immune and central nervous system (CNS). Mice genetically lacking SHP-1 (me/me) display severe inflammatory demyelinating disease following inoculation with the Theiler's murine encephalomyelitis virus (TMEV) compared to infected wild type mice. Therefore, it became essential to investigate the mechanisms of TMEV-induced inflammation in the CNS of SHP-1-deficient mice. Herein, we show that several genes relevant to inflammatory demyelination are elevated in the CNS of infected me/me mice compared to wild type mice. Furthermore, SHP-1 deficiency led to an abundant and exclusive increase in infiltrating CD45$^{hi}$CD11b$^{+}$Ly-6C$^{hi}$ macrophages into the CNS of me/me mice in concert with the development of paralysis. Histological analysis of spinal cords revealed localization of these macrophages to extensive inflammatory demyelinating lesions in infected SHP-1-deficient mice. Sorted populations of CNS infiltrating macrophages from infected me/me mice showed increased amounts of viral RNA and enhanced inflammatory profile compared to wild type macrophages. Importantly, application of clodronate liposomes effectively depleted splenic and CNS infiltrating macrophages and significantly delayed the onset of TMEV-induced paralysis. Furthermore, macrophage depletion resulted in lower viral loads, inflammatory gene expression, and demyelination in the spinal cords of me/me mice. Finally, me/me macrophages were more responsive to chemoattractive stimuli secreted by me/me glia serving as a mechanism for the increased numbers of infiltrating macrophages seen in the CNS of me/me mice. Taken together, infiltrating macrophages in SHP-1-deficient mice
play a crucial role in promoting viral replication by providing abundant viral targets and contribute to increased pro-inflammatory gene expression relevant to the effector mechanisms of macrophage-mediated demyelination.

**Key Words:**

Multiple Sclerosis

Demyelination

Chemokines

Inflammation

TMEV

Nuclear factor κ-B
INTRODUCTION:

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that remains a major cause of disability (76). Several studies demonstrated that MS lesions contain multiple leukocyte cell types including lymphocytes, macrophages, and dendritic cells all of which are believed to contribute to lesion formation by various distinct and interacting mechanisms (54, 61). Among these leukocyte subsets, infiltrating macrophages have been identified as major effectors of demyelination in both MS and animal models for MS (17, 44, 59, 102). In accord with these studies, it was recently described that the dominant mechanism of demyelination in MS is macrophage–mediated (15). In deed, in some models for MS, the requirement for lymphocytes is negligible and macrophages are sole mediators of demyelination (6, 72).

These findings have stimulated intense interest on the function of macrophages in lesion formation including signaling events that draw these cells into the CNS white matter and trigger the effector mechanisms by which these cells damage myelin. For instance, macrophages have been identified as the major responders to CNS chemokines and producers of a number of pro-inflammatory cytokines, chemokines, and toxic molecules known to promote demyelination (32, 44, 63, 85, 90, 91, 93, 103). Interestingly, both MS brains and brains of experimental animals with MS-like diseases contain activated transcription factors like NF-κB (34, 40), STAT1 (35, 37, 40) and STAT6 (18, 21, 109), which can lead to enhanced expression of these inflammatory molecules. Based on our previous work, we propose that modulation of inflammatory signaling via these transcriptional pathways may be deficient in leukocytes, including
macrophages of MS patients, and that this deficiency is responsible for susceptibility to inflammatory demyelinating processes within the CNS.

SHP-1 is a protein tyrosine phosphatase with two SH2 domains which acts as a negative regulator of both innate and acquired immune cytokine signaling via NF-κB (52, 67), STAT1 (29, 68), and STAT6 (13, 43, 45, 50). Mice genetically lacking SHP-1 (motheaten mice) display myelin deficiency, which may be mediated by increased innate inflammatory mediators in the CNS (66, 69). Furthermore, motheaten mice are highly susceptible to experimentally-induced demyelinating disease (30, 65). Taken together, these studies indicate that SHP-1 is a key regulator of inflammation in the CNS that may be relevant to the pathogenesis of MS. Indeed, we have recently reported that SHP-1 expression and function is deficient in leukocytes of MS patients compared to normal human subjects (21).

To further elucidate the function of SHP-1 in inflammatory demyelinating disease, we have been utilizing the Theiler’s murine encephalomyelitis virus (TMEV)-induced demyelinating disease animal model of MS (14, 20, 27, 33, 56). When susceptible strains of mice are inoculated intracerebrally with TMEV the mice develop immune-mediated demyelinating disease resembling human MS. TMEV infection leads to biphasic disease with an early gray matter infection followed with later progressive white matter disease involving a complex array of leukocytes and pro-inflammatory molecules that eventually cause demyelination. It is now well accepted that while lymphocytes are initiators/perpetuators of demyelination in this model, macrophages are the major effectors of demyelination (8, 24, 57, 87, 88, 94).
Previously, we have shown that SHP-1-deficient mice uniquely display an unusually rapid CNS demyelination associated with extensive white matter cellular infiltration and clinical paralysis within the first week of TMEV infection compared to their wild type littermates (65). These data suggested that SHP-1 modulates early events in TMEV infections of the CNS, which cause inflammatory demyelination. Moreover, the rapidity of demyelination suggests the involvement of innate inflammatory effectors of demyelination. Therefore it became essential to investigate the cellular and molecular mechanisms that contribute to the early-onset of severe CNS demyelination observed in me/me mice.

The present study demonstrates that a deficiency in SHP-1 leads to an augmented inflammatory gene profile and increased infiltration of peripheral macrophages into the CNS following TMEV infection. In addition, macrophage infiltration into spinal cords is concentrated to areas of demyelination in me/me mice. Accordingly, macrophage/monocyte depletion with clodronate liposomes results in a significant delay in the onset of TMEV-induced paralysis and decreased viral loads, inflammation, and demyelination in the spinal cords of me/me mice compared to wild type littermates. Furthermore, we show that SHP-1 controls chemokine production by CNS glia and responsiveness of macrophages to these chemokines, which play an important role in CNS macrophage-mediated disease. We therefore propose that SHP-1 is an important regulator of CNS inflammatory demyelination acting to control inflammatory gene expression and recruitment macrophages into the CNS in response to viral infection.

MATERIALS AND METHODS
**Animals:**

SHP-1-deficient motheaten (*me/me*) mice (C3HeB/FeJLe-a/a background) and their phenotypically normal wild type littermates were produced from heterozygous breeding pairs obtained from Jackson Laboratories (Bar Harbor, Maine). Strain designations for heterozygous breeders are C3FeLe.B6-a/a *Hcph*<sup>me/+</sup> (stock no. 000225) for motheaten mice.

**Paralysis Scores:**

Mice were scored daily for signs of paralysis. Mice were scored on a 5-point scale: 1 = incomplete hind limb paralysis (dragging hind limb but being able to move it), 2 = complete paralysis of one hind limb, 3 = complete paralysis of one hind limb and one forelimb or paralysis of both hind limbs, 4 = quadriplegia, 5 = death preceded by paralysis.

**Virus Inoculation:**

The attenuated strain of Theiler’s murine encephalomyelitis virus (TMEV), BeAn 8386, (ATCC, Manassas, VA) was propagated and titrated by plaque assay in BHK-21 cells. Whole glial cultures were inoculated with 1x10<sup>6</sup> PFU/mL at a multiplicity of infection of 1.0. Twelve-day-old mice were inoculated intracerebrally (i.c.) in the right hemisphere with 5x10<sup>5</sup> PFU of BeAn 8386 in a volume of 0.005 ml. Mice were observed daily for paralysis. Unless otherwise specified, four days post-infection mice were anesthetized and perfused and the right cerebral hemispheres and spinal cords were
suspended in RNA STAT-60 (TEL-TEST, Friendswood, TX) for RNA analysis. Also, brain and spinal cords were used to prepare single cell suspensions for flow cytometry analysis.

**In vivo depletion of macrophages:**

To deplete macrophages in wild type and me/me mice, liposome-encapsulated clodronate (clodronate liposomes) were used. Clodronate was purchased from Roche pharmaceuticals (Germany) and was encapsulated into liposomes by Encapsula Nanosciences (Nashville, TN). The final solution contained 5mg/mL of encapsulated clodronate drug. Control liposomes contained phosphatidylcholine and cholesterol without clodronate. Mice weighing approximately 5 grams were injected intraperitoneally (i.p.) with 0.2 mg clodronate at 2 days before infection with the TMEV virus. This injection route and the method was previously shown to specifically deplete CD11b^{+}Ly6C^{hi} monocytes/macrophages in multiple tissues and blood (10, 83, 110). As controls, mice were injected i.p. with the same volume of control liposomes 2 days before TMEV infection. Mice further received either control liposomes or 0.1mg clodronate liposomes at day 2 and 6 post-infection. Macrophage depletion in clodronate-liposome-treated mice was evaluated by staining for CD45^{hi}CD11b^{+}, CD11b^{+}Ly-6C^{hi}, and F4/80^{+} cells in the spleen and CNS of mice 4 or 6 days after the first clodronate injection and was compared to mice that received the control liposome injections.

**Real-Time RT-PCR:**
Total RNA was isolated using RNA STAT-60. RNA was quantified spectrophotometrically and 0.5 µg of total RNA was converted into cDNA. Briefly, total RNA and random primers (Invitrogen, Carlsbad, CA) were incubated at 72 degrees for 10 minutes. Reverse transcription was performed using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and followed the specification of the manufacturer. cDNA was diluted to 200µl with water and 4µl was used for quantitative real time PCR using SYBR Green kit (Abgene, Epson, UK). The PCR parameters were 15 minutes for 95 degrees and 35 cycles of 95 degrees for 15 seconds and 60 degrees for 1 minute in ABI prism 700 (Applied Biosystems, Foster city, CA). The primers were used at 10 nM. Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run to generate a standard curve relating copy number with threshold amplification cycle (22). Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed both by examination of melting peaks and by agarose gel electrophoresis. The primer pairs used in this study are shown in table 1.

Flow cytometry:

Characterization and quantification of immune cell infiltration in the CNS: The brain and the spinal cord were removed from mice and placed in 2mL of cold HBSS. The tissue was homogenized with a fine tip glass pipette and then filtered through a 40µm cell strainer cap as previously described (58) but without collagenase digestion. The single cell suspensions were washed twice with cold HBSS with 5% FBS. Aliquots of 1x10^6
cells were resuspended in a 100µL of HBSS and incubated with 10µL of CD3-FITC, CD11b-PE, CD19-FITC, CD5-PE, CD49b-PE, Gr1-FITC, Ly-6C-FITC, (Becton Dickinson, Mountain View, CA).

**Sorting infiltrating macrophages:** Aliquots of 2x10\(^6\) single cell suspensions of the brain, spinal cord, or spleen of TMEV infected mice were stained for CD11b-PE and Ly-6C-FITC (Becton Dickinson, Mountain View, CA). Two populations, double-positive cells and double-negative cells were sorted by a fluorescence-activated cell sorter (FACS Vantage S/E; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Aliquots of 5x10\(^4\) cells were collected and lysed in the RNA isolation reagent.

**Intracellular staining:** Single cell suspensions in 1.0 ml PBS received 100µL of 16% stock paraformaldehyde for fixation (1.5% paraformaldehyde) for 5 minutes and then incubated in 90% methanol at 4°C for half an hour to permeabilize cells for intracellular staining. Cells were washed twice with the staining media containing 0.5% BSA and 0.02% sodium azide in PBS. The levels of several intracellular antigens were concurrently analyzed with CD45-FITC. Fixed and permeabilized cells were incubated overnight at 4°C with 1µg of goat anti-ADAM8, goat anti-MMP3, goat anti MBP (Santa Cruz, CA) antibodies or goat polyclonal IgGs for isotype control. Then the cells were incubated for 3 hours in 1µg of swine anti-goat secondary antibody conjugated to PE (Invitrogen, Carlsbad, CA). Similarly, cells were single stained with rat anti-mouse LT-α (R and D systems), rat anti mouse F4/80 (Serotec), and mouse anti-arginase-I (BD Biosciences) overnight and then stained with swine anti-rat or goat anti-mouse secondary antibodies conjugated to PE (Invitrogen, Carlsbad, CA). Single cell suspensions were also double stained with CD45 and rabbit anti-TMEV Ab (gift from Howard L. Lipton,
Northwestern University) followed by goat anti-rabbit secondary antibody conjugated to PE (Invitrogen, Carlsbad, CA). Cells were analyzed on an LSRII analyzer (Becton Dickinson, Mountain View, CA) and the percent positively stained cells and the mean florescence intensity (MFI) was recorded. Data were analyzed with FlowJo software (Ashland, OR).

**Immunohistochemical analysis:**

Spinal cords from wild type and me/me mice before and after 4 days infection with TMEV were used for immunohistochemical analysis. Also spinal cords of mice that were pretreated with control liposomes or clodronate liposomes and then infected with TMEV were analyzed. Mice were anesthetized and intracardially perfused via the left ventricle first with 10 mL phosphate-buffered saline (PBS) followed by 20 mL of 4% paraformaldehyde in PBS. Spinal cords were further incubated in 4% paraformaldehyde in PBS for 1 hour and then dissected out and placed in 30% sucrose solution in PBS overnight. Spinal cords were embedded in OCT compound, frozen on dry ice, and sectioned with a cryostat at 8µm thickness at -16°C. To double stain with MBP and CD11b, sections were first stained for mouse CD11b mAb directly conjugated to biotin (R&D Systems) followed by incubation in a streptavidin-alkaline phosphatase conjugate. A blue alkaline phosphatase reaction product was produced using a BCIP/NBT substrate kit (Zymed/InVitrogen, Carlsbad, CA). The same sections were then stained with a goat anti-MBP Ab (sc-13914, Santa Cruz biotechnology), followed with anti-goat immunoglobulins-HRP (DAKO) and red color was developed using the HRP substrate.
AEC chromogen (Zymed). Also, spinal cords were stained with rat biotinylated ani-CD4 and anti-CD8 monoclonal antibodies (Biosource).

**Glial Cultures:**

Mixed glial cultures containing astrocytes, oligodendrocytes, and microglia were produced from brains of 8-day-old mice by a modified procedure as previously described (46, 66, 71). Briefly, brains were minced in Kreb’s buffer with curved scissors into fine pieces. The minced brain was centrifuged and resuspended in Kreb’s buffer containing 0.25% trypsin and incubated at 37°C for 1 minute. After addition of 5% fetal bovine serum and 40 microgram/ml DNase in Kreb’s buffer to stop trypsinization, the tissue was pelleted and resuspended in fresh Kreb’s/FBS/DNase buffer. The tissue was repeatedly triturated with a fire polished pipette to dissociate cells. The cells were centrifuged, then resuspended in complete culture medium containing Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 24.5mM KCl, and 100micrograms/ml insulin and plated onto polylysine-coated dishes. Every three days cells were fed with fresh medium consisting of DMEM with 10% heat-inactivated horse serum (heat-inactivated at 56°C for 1 hour), and used at 10 days after plating. By morphological criteria glial cultures appear to be composed of 10% microglia, 55% astrocytes, and 35% oligodendrocytes.

**Macrophage Culture:**

Spleens were removed from mice and ground between the frosted surfaces of two glass slides in Hanks Balance Salt Solution (HBSS). Freed cells were left for 2 minutes on ice, pelleted by centrifugation, and the supernatant discarded. The pellet was
resuspended in 4ml/spleen of red blood cell lysis buffer (155 mM NH$_3$Cl, 0.1 mM EDTA, 12mM NaHCO$_2$) and incubated on ice for one minute. Cells were washed twice with Hank’s balanced salt solution (HBSS) and were resuspended in RPMI medium with 15% FBS and supplemented with 10% (v/v) L929 cell (ATCC) culture supernatant (sL929-medium) (12). Cells were plated in at a density of 5x10$^5$cells/ml and were fed every three days. On day nine, viability was tested with trypan blue and the adherent cells were infected with the TMEV virus for 48 hours. Following culture, more than 97% of the cells were CD11b$^+$ Ly6C$^+$ assessed by flow cytometry.

**Chemokine/Cytokine ELISA**

The levels of the chemokine MCP-1 were measured in supernatants of glial cultures using the DuoSet ELISA kits (R&D Systems) following the manufacturer's protocol. The levels of the cytokines TNF-α and IL-6 were quantified in spinal cord homogenates. Spinal cords were homogenized in RIPA buffer, the amount of total protein was quantified, and the amount of TNF-α and IL-6 was quantified using the DuoSet ELISA kits (R&D Systems) following the manufacturer's protocol.

**Chemotaxis Assay:**

Cell migration of splenic macrophages was measured using the CytoSelect 96 well cell migration assay (Cell Biolabs, San Diego, CA). Briefly, either RPMI with 10% FBS containing 40ng of MCP-1 or the supernatants of 48hr glia cells infected or mock infected with TMEV virus served as test chemoattractants. Splenic macrophages were cultured as described above and 7.5 x 10$^5$ of either wild type or me/me cells were placed
in the 5µm polycarbonate membrane chambers. Cells were incubated in the different
chemoattractant solutions for 6 hours and cells that migrated were lysed and quantified
with CyQuant® GR dye by reading the fluorescence at 480 nm/520 nm. In the MCP-1
function-blocking experiments, glial supernatants were incubated with 10µg/mL of rat
anti-MCP-1 neutralizing antibody (MAB489, R and D systems) or 10µg/mL of rat anti-
IgG control antibody (6-001-A, R and D systems) at 37°C for one hour.

Statistical Analysis:

Histograms or tables represent the mean values with standard errors. The number
of samples/individual mice used in each assay is indicated in the figure legend. The p
values were generated using the unpaired Student’s t test and a p value of less than 0.05
was used for statistical significance between two samples.

RESULTS:

Elevated inflammatory gene expression in the brains and spinal cords of me/me
mice following TMEV infection

It was previously shown that cultured CNS glia of SHP-1-deficient me/me mice
display augmented signal transduction through STATs and NF-κB resulting in increased
inflammatory gene expression. Regulation of these pathways by SHP-1 may therefore
account for increased inflammatory demyelination in the CNS of me/me mice infected
with TMEV where these signaling molecules become activated (13, 52). Consequently,
we investigated whether me/me mice showed increased expression of STAT and NF-κB responsive genes in the brain or spinal cords of me/me and wild type littermates following TMEV infection (Table 2). Wild type and me/me mice were inoculated with the BeAn strain of TMEV and four days post infection (p.i.) the spinal cords were analyzed for gene expression using real time RT-PCR. At four days post infection, me/me mice exhibited complete hind limb paralysis compared to wild type mice that did not show signs of paralysis. The spinal cord of me/me mice showed a significant 12-fold increase in the amount of TMEV RNA compared to wild type cords.

The STAT6-responsive gene arginase-I (79), was elevated both constitutively and following TMEV infection in the brain and spinal cord of me/me mice (Table 2). Examination of genes that are both regulated by STAT6 and NF-κB such us the protease ADAM8 (53, 95), the chemokine eotaxin/CCL11 (70), and lymphotoxin-alpha (LT-α) (81, 106) revealed substantial increases in me/me mice following TMEV infection compared to wild type mice.

Because SHP-1 has been shown to control NF-κB signaling in CNS glia and hematopoietic cells, it was of interest to examine the expression of NF-κB inducible genes like the cytokines TNF-α and IL-6, the chemokines MCP-1 and MIP-1α, and the matrix metalloproteinase MMP3 (Table 2). TNF-α, MCP-1, and MIP-1α were constitutively higher in me/me spinal cords before infection with TMEV. Following viral infection, TNF-α, IL-6, MCP-1, and MIP-1α were upregulated in both wild type and me/me spinal cords following TMEV infection but were substantially more abundant in spinal cords of me/me compared to wild type mice. Similarly, higher expression of these genes was observed in me/me compared to wild type mouse brains (data not shown).
These findings point to the importance of SHP-1 in controlling CNS inflammatory gene expression that may play a role in progression of paralytic disease.

**Immune cell infiltration in the CNS:**

The observation that TMEV infection induced an exaggerated inflammatory profile with substantially increased chemokine expression in me/me CNS compared to wild type mice, led us to examine potential infiltrating inflammatory cells in these animals (Figure 1). Brains and spinal cords of wild type and me/me mice before and after 4 days of infection with TMEV were homogenized into single cell suspensions and the presence of blood-derived leukocytes was quantified using flow cytometry as previously described (58). First, double staining with CD45 and CD11b revealed that following TMEV infection, 10% of the cells in wild type brains were CD45$^\text{hi}$CD11b$^+$ while me/me brains contained 27% of CD45$^\text{hi}$CD11b$^+$ cells (Figure 1A & C). In spinal cords, wild type mice contained less than 2% CD45$^\text{hi}$CD11b$^+$ cells compared to 30% of CD45$^\text{hi}$CD11b$^+$ cells in me/me spinal cords following TMEV infection. Because me/me mice had a substantially higher infiltration of CD45$^\text{hi}$CD11b$^+$, these cells were further characterized based on recently developed criteria for blood-derived monocytes/macrophages. First, similar percentages of cells stained for CD45$^\text{hi}$Gr-1$^+$ and F4/80$^+$ (55, 88) indicating that a large majority of the cells were of monocyte/macrophage origin (Figure 1D & F). Furthermore, the majority of the CD11b$^+$ infiltrating cells were Ly-6C$^\text{hi}$ (Figure 1E), which was recently established as a reliable marker of blood-derived macrophages (1, 99, 100). Taken together, these data suggest that the CNS of me/me mice displays a dramatic increase in the number of infiltrating macrophages (7, 41, 58, 88).
Since the lack of SHP-1 has been associated with increased susceptibility to inflammation in the lung in which eosinophils play a role (50), we investigated the presence of eosinophils in the CNS of me/me mice by staining with eosinophil peroxidase (EPX) (19, 42, 89). Both in the brain and the spinal cord of me/me infected mice we found a small (3.5%), but significant increase in EPX+ cells (Figure 1G). We did not observe a significant amount of CD45\textsuperscript{hi}/CD11b\textsuperscript{−} single-positive cells in the CNS of either TMEV-infected wild type or me/me mice, indicating that most leukocytes infiltrating the CNS were of macrophage origin. However, to directly address whether other leukocyte subsets may infiltrate the CNS following infection, we stained tissue homogenates with antibodies to CD3 (Figure 1H), CD49b, CD19, and CD5 (data not shown) to examine the levels of infiltrating T cells, NK cells, B cells, and total lymphocytes respectively. Staining revealed that the levels of T, NK, and NK cells did not significantly increase in the CNS following TMEV infection in either wild type or me/me mice. Taken together these data indicate that macrophages and fewer numbers of eosinophils are the main leukocytes infiltrating the CNS of SHP-1-deficient motheaten mice at 4 days post-infection. Motheaten mice demonstrate a dramatic 3-fold increase in the brain and 15-fold increase of infiltrating macrophages into the spinal cord compared to their wild type littermates and thus these cells constitute the main inflammatory cellular infiltrate especially in spinal cords of TMEV-infected me/me mice.

**Infected SHP-1-deficient me/me mice display massive macrophage infiltration into inflammatory spinal cord demyelinating lesions**
As shown above, macrophages are the main inflammatory cellular infiltrate seen in the CNS of paralyzed me/me mice infected with TMEV. Paralysis in TMEV-infected mice was previously reported to relate to the appearance of extensive spinal demyelinating lesions (65). However, the association between these two observations has not been established. To do so, we performed double-color immunohistochemistry of CD11b and myelin basic protein (MBP)(Figure 2). Spinal cords from wild type and me/me mice that either were uninfected or infected with TMEV for 4 days were fixed, frozen, and sectioned. The spinal cords of each group of animals were surveyed in several sections at different levels of each spinal cord. Such staining allowed an examination of the spatial distribution between macrophage infiltration (CD11b accumulation) and demyelination (MBP loss from specific white matter tracts). Wild type and me/me mice that were not infected (Figure 2A & 2B) did not show areas of focal demyelination and were free of CD11b\(^+\) cells. Similarly, TMEV-infected wild type mice did not show any focal demyelination or presence CD11b\(^+\) cells (Figure 2C). In contrast, infected me/me spinal cords displayed extensive areas of demyelination primarily in ventrolateral tracts that contained large numbers of CD11b+ cells concentrated within the lesions. In contrast, gray matter regions or normal appearing white matter containing relative fewer or no CD11b+ cells indicating a preferential localization of macrophages within inflammatory demyelinating lesions (Figure 2D). Focal demyelination associated with macrophage infiltration was seen throughout the white matter of spinal cords of infected me/me mice with more severe lesions observed in the lumbar section. Closer magnification revealed that the CD11b\(^+\) cells displayed a simple rounded non-ramified morphology consistent with blood-born derivation rather than microglial origin. In
addition spinal cords of infected wild type and me/me mice were stained with anti-CD4 and anti-CD8 antibodies and showed no immunoreactivity (data not shown).

**Quantifying viral loads and gene expression of sorted CNS infiltrating macrophages:**

Because macrophages were the major infiltrating leukocyte seen in the CNS of infected me/me mice and localized within areas of demyelination, we wanted to assess the contribution of these cells to inflammatory gene expression in infected mice. CD11b$^+$Ly-6C$^{hi}$ cells (macrophages) were sorted from 4-day TMEV infected mice and were compared to CD11b$^-$Ly-6C$^-$ cells (CNS resident cells) (Figure 3). Total RNA was isolated from $5 \times 10^4$ cells of each sample and the amount of virus or inflammatory gene expression was quantified per 1 ng of total RNA. In addition, CD11b$^+$Ly-6C$^{hi}$ splenic macrophages were analyzed for comparison. First, the amount of TMEV RNA was quantified (Figure 3B). Although the mean TMEV mRNA was slightly higher in me/me compared to wild type brains, viral load between the wild type and me/me infected brain or in macrophage-depleted brain cellular compartment was not significantly different. However, analysis of the sorted infiltrating macrophage compartment from these same specimens indicated that me/me brains contained 5-fold higher viral RNA compared to wild type macrophages. This observation suggests that SHP-1 may control selective tropism for these cells (25, 60, 64, 74) and/or increased TMEV replication in the macrophage compartment.

Because infected me/me mice have increased inflammatory gene expression compared to wild type mice, it was important to examine the expression of these genes in
wild type and me/me infiltrating macrophages. The protease ADAM8, which has been implicated in neurodegeneration (95) and was shown to directly degrade myelin basic protein (MBP) (2), was 25-fold higher in infected me/me brain compared to wild type brains (Figure 3C). Interestingly, ADAM8 mRNA transcripts were 1000-fold more abundant in infiltrating macrophages compared to CNS tissue depleted of macrophages indicating preferential expression of ADAM8 in infiltrating macrophages. Furthermore, there was a substantial upregulation of ADAM8 in CNS-infiltrating macrophages compared to splenic macrophages. Similarly, LT-α mRNA message was higher in me/me compared to wild type brain following TMEV infection. In contrast, LT-α mRNA was significantly 10-fold higher in CD11b Ly-6C− cells compared to infiltrating macrophages consistent with reports that LT-α is specifically expressed by astrocytes in a macrophage-mediated demyelinating mouse model (81). Furthermore, arginase I mRNA transcripts were significantly increased in the infected brains, infiltrating macrophages, and CNS resident cells of me/me mice compared to wild type littermates (Figure 3E). Finally, IL-6 and MCP-1 mRNA transcripts were substantially more abundant in me/me brains compared to wild type brains. Importantly, infiltrating CNS macrophages had more than $1 \times 10^4$ higher IL-6 and MCP-1 mRNA transcripts than splenic macrophages and had more than 10-fold higher expression than CNS resident cells. Taken together, these data demonstrate that both the increased number of infiltrating macrophages along with an augmented inflammatory profile may contribute to the demyelination seen in TMEV-infected me/me mice.
Depletion of infiltrating macrophages using clodronate liposomes:

The above observations implicated a role for infiltrating macrophages in the paralysis, demyelination, and increased CNS inflammatory profile seen in TMEV infected me/me mice compared to wild type mice. To directly demonstrate this role, macrophages were experimentally depleted in vivo by injecting mice with liposome-encapsulated clodronate. Clodronate liposomes cause transient and selective elimination of macrophages in the spleen, multiple peripheral tissues, and CD11b⁺/Ly6C⁺ monocytes in the blood (47, 98). Also, it has been shown that clodronate liposomes do not deplete microglia in the brain parenchyma (5, 82). Importantly, clodronate liposomes were successfully used to deplete blood-derived macrophages from participating as effectors in CNS inflammatory disease (38, 48, 101). Hence, wild type and me/me mice received either clodronate liposomes or PBS control liposomes two days before and two and six day after TMEV infection. Animals were sacrificed 4 days post-infection and the spleens, brains, and spinal cords were analyzed for the presence of CD45⁺CD11b⁺, F4/80⁺, and CD11b⁺Ly-6C⁺ cells (Figure 4). Clodronate liposome treatment effectively depleted macrophages from the spleens while there was no effect with control liposome treatment (Figure 4A). Furthermore, there was a 5-fold decrease, from 27% down to 5%, in the number of CD45⁺CD11b⁺ cells in the spinal cords of me/me mice that were treated with clodronate liposomes compared to control liposomes (Figure 4B). TMEV-infected brains of animals treated with clodronate liposomes displayed a 2-fold decrease in wild type and a 3-fold decrease in me/me mice of CD45⁺CD11b⁺ macrophages compared to brains of control liposome-treated animals (Figure 3C & D). Similarly, CD11b⁺Ly-6C⁺ cells that were shown to be specifically depleted by clodronate treatment in multiple tissues (99)
were significantly decreased by clodronate in wild type and me/me CNS tissue (Figure 4D). Taken together, these data indicate that clodronate liposomes were effective in substantially reducing infiltration of blood-derived macrophages into the CNS of TMEV-infected mice.

In addition, to assess the effect of macrophage depletion on demyelination in the spinal cords of TMEV-infected me/me mice following treatment with clodronate liposomes, double-color immunohistochemistry for MBP and CD11b was performed (Figure 2). Spinal cords of TMEV infected wild type mice that were either treated with control or clodronate liposomes were free of CD11b+ macrophages and showed no signs of demyelination (data not shown). In contrast, spinal cords of infected SHP-1 deficient me/me mice that were treated with control liposomes showed the typical outcome of TMEV infection with substantial white matter macrophage infiltration associated with extensive demyelination (Figure 2F). Importantly, macrophage depletion in infected me/me mice with clodronate liposomes resulted in both a disappearance of CD11b+ macrophages and focal white matter demyelination in stained sections of the spinal cord (Figure 2E).

Paralysis scores of wild type and me/me mice following infection with TMEV:

Concomitantly with assessment of CNS macrophage depletion, the effects of clodronate liposomes on the paralysis and mortality of TMEV-induced disease was determined. TMEV-induced paralysis scores were recorded in 8 groups of mice that had received either clodronate or control liposomes (Figure 5A). No groups of wild type mice regardless of liposome treatment showed early signs of paralysis following TMEV
infection. Of the wild type mice observed daily for paralysis up to 3 months p.i., only 7/25 (25%) showed paralysis with a much latter onset than the me/me littermates (4-5 weeks p.i.), consistent with the typical TMEV-induced disease of wild type mice. In sharp contrast, TMEV-infected me/me mice that received either no treatment or control liposomes showed initial signs of paralysis at day 3 p.i. and by day 5 p.i. almost all the mice displayed terminal morbidity similar to that previously described (65) and were therefore sacrificed (scored as death preceded by paralysis). Importantly, infected me/me mice that were pretreated with clodronate liposomes had significantly delayed signs of paralysis appearing at day 6 p.i. and remained alive until day 12 post-infection. Therefore, depleting macrophages in me/me mice resulted in a delayed onset and less severe disease following TMEV infection.

In addition, the quantity of CD45<sup>hi</sup>CD11b<sup>+</sup> cells present in the CNS at the time of sacrifice was correlated with the severity of clinical disease. The spinal cords of TMEV infected me/me mice at different stages of clinical disease between day 3 and day 5 p.i. were analyzed for the presence of CD45<sup>hi</sup>CD11b<sup>+</sup> cells (Figure 5B). The paralysis scores showed a direct correlation with the amount of infiltrating macrophages detected in the spinal cords in TMEV-infected me/me mice. More specifically, when the amount of infiltrating macrophages was below 10%, no signs of disease were observed. When the amount of spinal cord infiltrating macrophages rose above 20% and up to 35%, then severe paralysis was observed in infected SHP-1-deficient me/me mice. Taken together, these data indicate that the number of infiltrating macrophages isolated from spinal cords of TMEV-infected me/me mice correlated with severity of paralytic disease and that
pharmacological depletion of these macrophages resulted in delayed onset and less severe
disease following TMEV infection.

**CD45\textsuperscript{hi} infiltrating cells contribute to both increased viral loads and inflammation in**

**SHP-1 deficient mice:**

CNS infiltrating macrophages are known targets for TMEV infection and
significantly contribute to both virus burden and the process of inflammatory
demyelination in spinal cords of mice (25, 60, 64, 74). Therefore, the contribution of
infiltrating macrophages to the viral burden seen in the CNS of TMEV infected mice was
determined. The amount of viral antigen (Figure 6A) and viral RNA (Figure 7A) were
quantified in the spinal cords of TMEV-infected mice that had been pretreated with either
control or clodronate liposomes. Both the TMEV antigens and RNA were more than 10-
fold higher in the spinal cords of me/me mice 4 days p.i. compared to wild type mouse
spinal cords. In addition, double-immunofluorescence staining of CD45 and TMEV
antigens revealed that CD45\textsuperscript{hi} cells in me/me spinal cords contained abundant TMEV
antigens (Figure 6A). Importantly, clodronate treatment resulted in a significant decrease
both in the levels of TMEV\textsuperscript{+} CD45\textsuperscript{hi} cells and TMEV RNA in me/me spinal cords
(Figure 6A & 7A). In sum, TMEV antigens and RNA are dramatically higher in spinal
cords of TMEV-infected me/me mice and depletion of macrophages effectively decreases
these levels.

CNS-infiltrating macrophages mediate CNS inflammation and demyelination via
the secretion of multiple inflammatory mediators (9, 39, 44, 75, 92). Therefore, the
contribution of infiltrating macrophages in the local production of these mediators was
assessed in the CNS of TMEV-infected me/me mice. Recent evidence indicates that the matrix metalloproteinase ADAM8 plays an important role in both trans-endothelial migration and proteolysis of myelin proteins by macrophages in inflammatory demyelinating diseases (2, 84, 108). By flow cytometry, ADAM8 was very highly expressed in CD45+ cells in TMEV infected me/me CNS (Figure 6B) and these cells were specifically depleted by clodronate liposomes. Spinal cords of wild type mice did not show an increase in ADAM8 expression compared to an 8-fold increase in me/me spinal cords following infection. Macrophage depletion resulted in a significant decrease in ADAM8 expression in infected me/me spinal cords (Figures 6B & 7D). In parallel with ADAM8 expression, clodronate depletion of macrophages produced a significant reduction in another macrophage effector molecule, TNF-α (Figure 6C). Taken together, clodronate-mediated depletion of macrophages may ultimately act by reducing macrophage effector molecules that cause spinal cord demyelination.

To corroborate and expand the above macrophage effector analysis, we examined the expression levels of two inflammatory cytokines, TNF-α (80, 96) and IL-6 (23, 78) by ELISA in the spinal cords of TMEV-infected mice. Similar to FACS analysis, TNF-α as well as IL-6 were not significantly upregulated in wild type spinal cords. In sharp contrast, we observed a 5-fold upregulation of both TNF-α and IL-6 in me/me spinal cords following TMEV infection (Figure 7C & 7D). Notably, macrophage depletion with clodronate significantly decreased both TNF-α and IL-6 expression in me/me infected spinal cords. Similar changes in ADAM8, TNF-α, IL-6, arginase I, and LT-α were observed after these treatments when quantified with real-time RT-PCR (data not shown).

In agreement with previous observations, the chemokine MCP-1 (CCL2) expression in
the CNS was increased following TMEV infection in wild type mice but was substantially higher in SHP-1-deficient me/me mice (Figure 7B). Importantly, macrophage depletion before TMEV infection resulted in a 50-fold decrease in the expression of MCP-1 in me/me spinal cords implicating macrophages as a principle source or stimulus for MCP-1 production in the CNS of TMEV-infected me/me mice.

To corroborate the immunohistochemical analysis of demyelination (Figure 2), we quantified the expression of myelin basic protein (MBP) by flow cytometry (Figure 7E), which we previously showed was substantially reduced in the spinal cords in me/me mice following TMEV inoculation (65, 66, 104). Consistent with previous observations (65), the spinal cords of TMEV infected me/me mice showed significantly lower MBP content compared to their infected wild type littermates. In contrast, when macrophage depleted me/me mice were infected with TMEV, MBP expression in the spinal cord was not significantly reduced compared to spinal cords of uninfected me/me mice, suggesting that macrophages are important in mediating the reduction of MBP in the spinal cord. Taken together, these data suggest that CNS infiltrating macrophages in me/me mice play a crucial role in promoting viral replication by serving as a viral pool, contributing to increased gene expression relevant to the mechanisms of inflammatory demyelination, and reducing MBP in the spinal cords of SHP-1-deficient animals.

**SHP-1 deficient glia express more chemokines and SHP-1-deficient macrophages are more sensitive to chemoattractive stimuli.**

Considering the essential role that infiltrating macrophages play in mediating CNS inflammation in SHP-1-deficient mice, it became important to investigate the
specific mechanisms of increased macrophage infiltration in the CNS of me/me mice following TMEV infection. It was previously shown that me/me glial cells have exaggerated NF-κB signaling (52, 67) and we have shown that the CNS of infected me/me mice has increased amounts of the NF-κB-inducible chemokine MCP-1 (Table 2; Figures 3G & 7B). In order to further investigate whether SHP-1 controls MCP-1 secretion in CNS resident cells, primary glial cultures from wild type and me/me mice were infected with TMEV for 48hr and the secretion of MCP-1 was quantified with ELISA (Figure 8A). MCP-1 was highly inducible following TMEV infection, in glia of both wild type and me/me mice; however, SHP-1 deficient me/me glia secreted three times the amount of MCP-1 (40ng/10⁶ cells) compared to wild type glia following TMEV infection.

To test whether MCP-1 and perhaps other virus-induced chemokines from CNS glia were biologically active, supernatants from wild type and me/me mouse glial cultures before and after TMEV infection were used to chemoattract cultured splenic macrophages in a dual chamber assay system (Figure 8B). Cell migration was reported as the percent of cells migrated to the lower chamber out of the total number of cells seeded in the upper chamber. Figure 8B shows that both exogenous MCP-1 and supernatants of TMEV-infected glia contained significant chemotactic activity towards splenic macrophages compared to supernatants of uninfected cultures. Further, chemotactic activity was induced to higher levels in infected me/me glia compared to wild type mouse glia. Supernatants from infected wild type glia induced 23% migration of me/me macrophages compared to supernatants from infected me/me glia that induced 40% migration of me/me macrophages (Figure 8B).
Further, we wanted to examine whether the splenic macrophages of SHP-1-deficient me/me mice were more or less responsive to a set chemokine concentration compared to wild type macrophages. Indeed, media containing exogenous MCP-1, used as a standard positive control, induced a 27% migration of wild type macrophages but a significantly higher 43% migration of me/me macrophages. Second, the same supernatant from infected me/me glia stimulated a significantly increased cell migration of me/me macrophages compared to wild type macrophages. Therefore, not only do me/me glial cells show increased production of chemokines/chemoattractant stimuli, but also me/me macrophages are more sensitive to a particular concentration of a chemoattractive stimulus.

Because MCP-1 has repeatedly been shown to have a central role in chemoattracting macrophages and modulating the severity of disease both in the TMEV (7, 51, 62) and EAE (4, 49) mouse models of MS, we wanted to assess the contribution of glial-derived MCP-1 in the chemoattraction of macrophages. Supernatants from wild type and me/me glial cultures before and after TMEV infection were incubated at 37°C for 1 hour with either control antibody or function-blocking anti-MCP-1 antibody. Then, supernatants were used to chemoattract cultured splenic macrophages from wild type and SHP-1-deficient mice (Figure 8C). Incubation of medium containing the MCP-1 standard with the anti-MCP-1 antibody significantly decreased migration of wild type and me/me macrophages by 6-fold. Furthermore, anti-MCP-1 antibody added to media from infected me/me glia effectively decreased migration of wild type and SHP-1-deficient macrophages. Altogether, these data indicated that MCP-1 is a predominant chemokine induced in CNS glia by TMEV in agreement with previous studies, that SHP-1 modulates...
the production of MCP-1 in CNS glia, and that SHP-1 controls responsiveness of macrophages towards MCP-1-induced chemotaxis.

DISCUSSION

This study aimed to characterize the immunological and molecular mechanisms governing the increased susceptibility of SHP-1-deficient mice to virus-induced CNS inflammatory demyelinating disease (30, 65). Herein, we showed that the spinal cords of SHP-1 deficient mice displayed an augmented inflammatory profile and higher viral loads following TMEV infection compared to their wild type littermates. Importantly, the CNS of infected me/me mice contained a significantly higher number of infiltrating macrophages compared to wild type mice and the number of infiltrating macrophages directly correlated with disease severity. Moreover, infiltrating macrophages localized within the white matter and were concentrated within areas of focal demyelination in infected SHP-1-deficient mice. In turn, macrophage depletion resulted in a significant delay in the onset and severity of TMEV-induced paralytic disease seen in SHP-1-deficient mice. Furthermore, reduction of CNS macrophage infiltration results in an attenuated inflammatory profile, reduced viral burden, and demyelination. Taken together, this study points to the essential role of SHP-1 on controlling virus infection, macrophage infiltration, and macrophage effector functions in virus-induced CNS inflammatory demyelination.

As previously reported, SHP-1-deficient mice do not display the typical biphasic TMEV-induced disease characterized by an acute gray matter disease followed by a latter chronic white matter demyelination (77). In contrast, infection of SHP-1-deficient mice
results in an acute white matter disease in which inflammatory demyelination occurs very early (with in the 4 days p.i.). Moreover, in distinction to TMEV-induced demyelinating disease in wild type mice in which both acquired and innate immunity including lymphocytes and macrophages play an important role, TMEV-induced demyelination in SHP-1-deficient mice involves primarily innate immune effectors that we identified presently as blood-borne macrophages. The overwhelming presence of macrophages within the demyelinated areas of the white matter of the spinal cords of infected me/me mice underscores the importance of SHP-1 in controlling macrophage trafficking into the CNS and controlling the effector functions of macrophages in mediating demyelination. As such, the present studies are relevant to CNS inflammatory diseases in which either adaptive or innate immune responses play an important role but in which macrophages constitute the main effectors of tissue destruction. Indeed, infiltrating macrophages have been previously demonstrated to be essential effectors of demyelination in both the TMEV (7, 25, 86, 88) and the EAE (26, 48, 49, 101) mouse models of MS. Also, while the role of T cells in disease pathogenesis may or may not be a predominant feature of demyelinating lesion in both humans with MS and in animal models, the importance of macrophages as essential effectors of demyelination and paralysis is now well established. Previous TMEV studies have shown that the extent of CNS macrophage infiltration correlates with disease severity (58, 88). The present studies have characterized a relatively simple and rapid model for analyzing macrophage effector functions in a virus-induced inflammatory demyelination in the CNS.

TMEV antigen (28), virions (11), and viral RNA (3) have been detected in macrophages in demyelinating lesions in mice, indicating that macrophages further
promote disease progression by providing an essential reservoir of antigenic and innate inflammatory stimulation in the CNS (25, 59, 60, 64, 74). This study demonstrates that the spinal cords of SHP-1-deficient mice have higher viral antigen and RNA following TMEV infection, which correlates with a substantially higher number of infiltrating macrophages. Further, macrophage depletion resulted in significantly lower viral load in the spinal cord, indicating that the increased number of infiltrating macrophages is important in contributing to the viral levels in SHP-1-deficient mice. In agreement with this observation, macrophages isolated from TMEV infected brains of me/me mice contain significantly higher TMEV transcripts per cell compared to wild type macrophages. We have previously shown that SHP-1 deficiency in CNS glia results in higher viral replication both in vivo and in vitro (13, 65) correlating with increased arginase I and decreased nitric oxide (NO) production. Similarly, cultured splenic macrophages of SHP-1-deficient mice display increased viral antigen and RNA compared to wild type macrophages after infection in vitro with TMEV (personal unpublished observations). Taken together, SHP-1 controls both the number of macrophages entering the CNS and viral replication within macrophages, which in turn both contribute to the increased virus seen in the spinal cords of me/me mice.

As macrophages are essential effectors of inflammatory demyelinating disease it was important to characterize the inflammatory profile of infiltrating macrophages between wild type and SHP-1-deficient mice. We found that several inflammatory genes including TNF-α, IL-6, MCP-1, arginase I, ADAM8, eotaxin, LT-α were significantly upregulated in the CNS of infected me/me mice. Importantly, reducing the number of macrophages entering the CNS by depleting peripheral macrophages resulted in a
significant decrease in the expression of several inflammatory genes, suggesting that the increased number of macrophages in the CNS of infected me/me significantly contributed to the inflammatory profile. Nevertheless, although infiltrating macrophages are major sources of inflammatory molecules in the CNS, infected resident glia also contribute to the increased levels of these inflammatory molecules in TMEV-infected CNS (Figure 3). These data are in accordance with previous studies showing that SHP-1 controls the activation of several transcription factors including STATs and NF-κB and corresponding pro-inflammatory genes in resident CNS glia and macrophages in response to either TMEV infection or microbial materials that act via Toll-like receptors (TLRs) (13, 29, 52, 67). Therefore, both the increased number of CNS infiltrating macrophages and increased inflammatory profile are likely to contribute to the severe TMEV-induced inflammatory demyelination seen in SHP-1-deficient mice.

Although macrophage depletion was able to delay paralysis in infected me/me mice, it was not sufficient to completely abolish disease. There was a substantial amount of macrophage infiltration in the spinal cords of clodronate-treated me/me mice at 8 days p.i. (data not shown). Therefore, the increased levels of macrophages in infected clodronate-treated animals at latter time points (8 days p.i.) could certainly account for the clinical paralysis seen in those mice. That said, we cannot rule out a possible role for parallel mechanisms independent of macrophage activation that may additionally affect increased demyelination and clinical signs at later time points after infection. The eventual progression of disease in macrophage depleted me/me mice can also be explained by multiple roles for SHP-1 in the CNS that are independent of macrophage
functions including control of virus replication in CNS glia (13, 65) and regulation of oligodendrocyte pathology (66).

We considered the specific mechanisms of increased macrophage recruitment in the CNS of me/me mice following TMEV infection. It was previously shown that the CNS of me/me mice maintains an intact blood-brain barrier and does not contain increased numbers of inflammatory cells (105). However, previous studies have shown that in response to inflammatory stimuli, cells of me/me mice secrete increased levels of chemokines compared to wild type animals that may promote increased monocyte infiltration and inflammation under certain conditions such as microbial invasion (36, 107). In accord with these studies, we found increased expression of the chemokines RANTES, eotaxin-1, MCP-1, and MIP-1α in the CNS of TMEV-infected me/me mice compared to their wild type littermates. Furthermore, mixed glial cultures lacking SHP-1 showed increased chemokine secretion following TMEV infection, which could promote enhanced macrophage chemotaxis. In addition, we showed that SHP-1 controlled MCP-1 expression, an important mediator of CNS inflammatory disease (4, 7, 8, 31, 36, 49) and blocking MCP-1 in vitro significantly diminishes macrophage migration in response to mixed glial supernants. Importantly, we show that SHP-1 deficient cultured macrophages show enhanced migration to the chemokine MCP-1 or to supernatants from TMEV infected glial cultures. The increased sensitivity of me/me macrophages could be in part to increased expression of chemokine receptors including CCR2 on SHP-1 deficient macrophages (51, 80) or enhanced signaling potential of individual chemokine receptors.

In multiple sclerosis, intense macrophage infiltration is present in active demyelinating lesions and both their numbers and differentiation/activation correlates
with disease severity (16, 17, 61, 102, 104). Macrophages can mediate myelin degradation, oligodendrogliopathy, and axonal loss both through cell-mediated processes and the secretion of inflammatory mediators (44, 76, 104). A key area of interest is the stimulus responsible for macrophage trafficking/infiltration into CNS demyelinating lesions. For instance, the chemokine MCP-1 is elevated in MS plaques and in the cerebrospinal fluid (CSF) of MS patients (62, 73, 97). An essential role for MCP-1 in infiltration of macrophages in CNS demyelinating diseases is supported observations in various animal models for MS. These findings point out that CNS-derived chemokines and peripherally derived macrophages have a prominent role in the initiation and progression of demyelination in multiple sclerosis.

We have recently shown that PBMCs of MS patients have a deficiency in the expression of the phosphatase SHP-1 that leads to an augmented inflammatory profile compared to PBMCs from normal subjects (21). Since numerous studies have shown that several inflammatory mediators/effectors are upregulated in MS lesions and it has been shown that SHP-1 is a master modulator of inflammatory signaling, deficiency of SHP-1 in leukocytes of MS patients may significantly contribute to MS disease processes. In relation to the present study, preliminary data indicate that macrophages of MS patients exhibit significantly lower levels of SHP-1 expression and function compared to those in normal subjects (personal, unpublished observations). Therefore, further studies on the extent and level of control that SHP-1 exerts on macrophage differentiation, activation, and chemotaxis will be important. This study demonstrates that the protein tyrosine phosphatase SHP-1 controls macrophage migration/trafficking into the CNS and the
expression of several inflammatory mediators within the CNS, making SHP-1 an attractive target in the treatment of MS.
ACKNOWLEDGEMENTS

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References:


Figure Legends:

Table 1: Sequences of primers used for real time RT-PCR analysis.

Table 2: Gene expression in spinal cords of wild type and me/me mice before and after 4-day infection with TMEV. Total RNA from wt uninfected (n=14), wt infected (n=13), me/me uninfected (n=11), and me/me infected (n=12) was analyzed with real time RT-PCR. The actual transcript copy number of each gene is reported in relation to the uninfected wild type mice, which is designated as 1. Gene expression was normalized to the housekeeping gene GAPDH. (* indicates significance of P<0.05 before and after virus infection and † indicates significance of P<0.05 between wt and me/me mice).

Figure 1: Characterization and quantification of leukocytes in the brain and spinal cord of wild type and me/me mice before and after 4-day infection with the TMEV virus. Single cell suspensions of the brain and the spinal cord were prepared from wt uninfected (n=8), wt infected (n=8), me/me uninfected (n=6), and me/me infected (n=7) mice. Cells were double-stained with CD45 and CD11b (Mac-1), CD45 and Gr-1, and Ly-6C and CD11b. Cells were single stained with F4/80 and EPX (Eosinophil peroxidase), and CD3. Histograms represent the mean percentage of positive cells in total cells analyzed.

Figure 2: Immunohistochemical analysis of MBP and CD11b in spinal cord sections of TMEV-infected wild type and SHP-1-deficient mice. Mice were anesthetized and perfused with paraformaldehyde 4 days after TMEV infection or sham infection. Frozen
spinal cords were sectioned at 8µm thickness and double-stained with anti-MBP Ab that appears red (AEC product) and anti-CD11b Ab that appears blue (BCIP/NBT product). The sections were photographed at 12X magnification (upper right corner of each panel appears a scale bar of 70µm). Representative spinal cord sections of A. wild type uninfected, B. me/me uninfected, C. wild type infected, D. me/me infected, E. clodronate treated me/me infected, and F. control liposome me/me infected are shown.

Figure 3: Quantifying gene expression by real time RT-PCR of sorted CNS infiltrating macrophages. Single cell suspensions of brains or spleens of TMEV-infected wt (n=10) and me/me (n=8) mice at 4 days p.i. were stained with CD11b and Ly-6C. Double positive cells (macrophages) and double negative cells (CNS resident cells/macrophage depleted brain) were sorted apart by FACS. A. Single cell suspension of the brain of an infected me/me mouse showing the gates used to sort out the Ly-6ChiCD11b+ and Ly-6C-CD11b- cells. The efficiency of the sorting was examined by analyzing the post-sort aliquot of the double positive or the double negative cells. B-H. 5x10^4 cells per aliquot were used to extract total RNA and perform real time RT-PCR. The expression of TMEV RNA and the mRNA transcripts of the protease ADAM8, Lymphotoxin alpha (LTα), Arginase I, IL-6, the chemokine MCP-1, and the housekeeping gene GAPDH were quantified per 1 ng of total RNA.

Figure 4: In vivo depletion of CNS infiltrating monocytes/macrophages using clodronate liposomes. Wild type and SHP-1-deficient mice were treated with either control
liposomes or liposomes encapsulating clodronate. Two days after liposome treatment the mice were infected intracerebrally with TMEV and sacrificed at day 4 post infection. Single cell suspensions of the brain and the spinal cord were prepared from control liposome-treated wild type (wt-C, n=8), clodronate liposome-treated wt (wt, CLD, n=11), control liposome-treated me/me (me-C, n=8), and clodronate liposome-treated me/me (me-CLD, n=9) mice. A. Splenocytes were stained with PE and FITC isotype Abs or with CD11b and CD45. The double positive cells representing the macrophage population were quantified in uninfected, or infected mice either pretreated with control or clodronate liposomes. B. CD11b and CD45 antibodies were used to stain single cell suspensions of spinal cords from wt or me/me mice. C. CD45^{hi}CD11b^{+} and D. and CD11b^{+}Ly-6C^{hi} cells were quantified in the brains and spinal cords of TMEV infected mice.

Figure 5: Paralysis scores of wild type and motheaten mice following infection with TMEV. Wild type (wt) and SHP-1-deficient mice (me) were intracerebrally infected with 5x10^5 PFU of BeAn TMEV. A. Wild type or me/me mice received either control liposomes (Lip) or clodronate liposomes (CLD) two days before TMEV infection. In total there were 8 groups of mice: wt uninfected (n=20), me uninfected (n=15), wt infected (n=35), me infected (n=16), wt control liposome infected (n=14), me control liposome infected (n=13), wt clodronate liposomes infected (n=19), and me clodronate liposomes infected (n=14). Mice were scored on a 5-point scale (1 = incomplete hind limb paralysis, 2 = complete hind limb paralysis, 3 = complete hind limb and partial forelimb paralysis, 4 = quadriplegic, 5= death preceded by paralysis). B. The
CD45^{hi}\text{CD11b}^+ cells in the spinal cords of TMEV-infected SHP-1 deficient mice were quantified between day 3 and day 5 p.i and were correlated to the paralysis scores exhibited by individual mice.

**Figure 6:** Levels of the TMEV antigen, the protease ADAM8, and TNF-\(\alpha\) in CD45+ cells in spinal cords of wild type and SHP-1 deficient mice (me/me). Mice were infected with the TMEV virus and two days before infection they received either control liposomes or clodronate liposomes. Spinal cords were removed 4 days p.i and single cell suspensions were stained and analyzed by flow cytometry. Cells from the spinal cord were double-stained for CD45 and either **A.** TMEV antigen, **B.** the protease ADAM8, or **C.** TNF-\(\alpha\). The left lower quadrant of the uninfected mouse in panel B & C displays the isotype control shown in gray color.

**Figure 7:** Quantification of TMEV RNA and inflammatory gene expression in the spinal cords of TMEV infected mice. Wild type and SHP-1 deficient (me) mice were treated with either control liposomes or liposomes encapsulating clodronate. Two days after liposome treatment the mice were infected with TMEV and sacrificed at day 4 post infection. Spinal cords from uninfected wt (wt, n=7), control liposomes wt (wt-V, n=9), clodronate liposomes wt (wt-C CLD, n=8), uninfected me/me (me, n=7), control liposomes me/me (me-V, n=9), and clodronate liposomes me/me (me-CLD, n=10) mice were analyzed. **A.** The amount of TMEV RNA and **B.** MCP-1 mRNA per ng of isolated total RNA was quantified using real time RT-PCR. The presence of **C.** TNF-\(\alpha\) and **D.** IL-6 in homogenized spinal cord tissue was quantified using ELISA. The expression of the
protease ADAM8 (E.), and myelin basic protein (MBP) (F.) was quantified in by
fluorescence cytometry from single cells suspension of spinal cords and the MFI was
normalized to uninfected wt spinal cord.

**Figure 8:** **A.** Mixed glial primary cultures from wild type (wt) and SHP-1-deficient mice
(me) were infected with the TMEV virus for 48 hours (MOI=1) and the supernatants used
to quantify the levels of the chemokine MCP-1 by ELISA. **B.** Chemotaxis assay of wild
type and SHP-1-deficient macrophages. Supernatants from wt uninfected (wt-C), wt
TMEV infected (wt-V), me/me uninfected (me-C), and me/me TMEV infected (me-V)
primary glial cultures were used as chemoattractants of cultured splenic macrophages
isolated from either wild type or me/me mice. In addition, media containing 40ng/mL of
MCP-1 served as a positive control to chemoattract wild type (white bars) and me/me
(grey bars) macrophages. Cell migration is reported as the number of cells migrated
divided by the total number of cells placed per well multiplied by 100 (percent
migration). **C.** MCP-1 neutralization assay. Supernatants from wt uninfected (wt-C), wt
TMEV infected (wt-V), me/me uninfected (me-C), and me/me TMEV infected (me-V)
were first incubated at 37°C for 1 hour with either control antibody or function-blocking
anti-MCP-1 antibody. Antibody-treated supernatants were used to chemoattract cultured
splenic macrophages from wild type and SHP-1-deficient mice.
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<td>*1.6 ± 0.3</td>
</tr>
<tr>
<td>MMP3</td>
<td>1 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td>*5.3 ± 3</td>
</tr>
<tr>
<td>ADAM8</td>
<td>1 ± 0.1</td>
<td>1.2 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Arg1</td>
<td>1 ± 0.1</td>
<td>3.5 ± 1.1 †</td>
<td>*1.8 ± 0.5</td>
</tr>
<tr>
<td>RANTES</td>
<td>1 ± 0.1</td>
<td>3.4 ± 0.8 †</td>
<td>*9.2 ± 1.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1 ± 0.3</td>
<td>8.1 ± 1.0 †</td>
<td>*1.1e ± 6²</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>1 ± 0.1</td>
<td>5.1 ± 1.1 †</td>
<td>*6.7 ± 1.3</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>1 ± 0.4</td>
<td>2.5 ± 0.6 †</td>
<td>*2.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table 2