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Inhibition of System $\text{X}_c^-$ Transporter Attenuates Autoimmune Inflammatory Demyelination

Kirsten S. Evonuk,*,†,*,‡ Brandi J. Baker,*,†,‡ Ryan E. Doyle,*,† Carson E. Moseley,‡ Christine M. Sester,§,† Bryce P. Johnston,*,†,‡ Patrizia De Sarno,‖ Andrew Tang,‡ Igor Gembitsky,§ Sandra J. Hewett,§ Casey T. Weaver,‡ Chander Raman,§ and Tara M. DeSilva*,†,**

T cell infiltration into the CNS is a significant underlying pathogenesis in autoimmune inflammatory demyelinating diseases. Several lines of evidence suggest that glutamate dysregulation in the CNS is an important consequence of immune cell infiltration in neuro-inflammatory demyelinating diseases; yet, the causal link between inflammation and glutamate dysregulation is not well understood. A major source of glutamate release during oxidative stress is the system $\text{X}_c^-$ transporter; however, this mechanism has not been tested in animal models of autoimmune inflammatory demyelination. We find that pharmacological and genetic inhibition of system $\text{X}_c^-$ attenuates chronic and relapsing-remitting experimental autoimmune encephalomyelitis (EAE). Remarkably, pharmacological blockade of system $\text{X}_c^-$ 7 d after induction of EAE attenuated T cell infiltration into the CNS, but not T cell activation in the periphery. Mice harboring a Slk7a11 (xCT) mutation that inactivated system $\text{X}_c^-$ were resistant to EAE, corroborating a central role for system $\text{X}_c^-$ in mediating immune cell infiltration. We next examined the role of the system $\text{X}_c^-$ transporter in the CNS after immune cell infiltration. Pharmacological inhibitors of the system $\text{X}_c^-$ transporter administered during the first relapse in a SJL animal model of relapsing-remitting EAE abrogated clinical disease, inflammation, and myelin loss. Primary coculture studies demonstrate that myelin-specific CD4$^+$ Th1 cells provoke microglia to release glutamate via the system $\text{X}_c^-$ transporter, causing excitotoxic death to mature myelin-producing oligodendrocytes. Taken together, these studies support a novel role for the system $\text{X}_c^-$ transporter in mediating T cell infiltration into the CNS as well as promoting myelin destruction after immune cell infiltration in EAE. * The Journal of Immunology, 2015, 195: 450–463.
The system \( X_c \) transporter imports \( L \)-cystine into the cell to be used in the metabolism of the cellular antioxidant glutathione in exchange for the export of glutamate. This mechanism may be important for the survival of reactive glia in oxidizing environments because cultured macrophages deficient in system \( X_c \) die in response to inflammatory stimuli unlike wild-type macrophages (18). Furthermore, gliomas (cancerous glial cells) upregulate the system \( X_c \) transporter, increasing their antioxidant defense at the expense of killing neurons by excitotoxicity (19). LPS-activated microglia in vitro release glutamate and increase their antioxidant defense at the expense of killing neurons (20); however, this mechanism has not been tested in vivo or in models of autoimmune inflammatory demyelination.

To explore the link between inflammation and glutamate dysregulation in autoimmune inflammatory demyelination, we used pharmacological inhibition as well as genetic alteration of system \( X_c \). Unexpectedly, we found that genetic deletion or pharmacological inhibition of the system \( X_c \) transporter reduced T cell infiltration in the CNS in experimental autoimmune encephalomyelitis (EAE). No reduction in T cell proliferation was found in spleens, suggesting that altering the function of system \( X_c \) did not affect T cell activation, but rather perturbed infiltration into the CNS. These data support a critical role for system \( X_c \) in immune cell infiltration into the CNS in chronic EAE. To examine the hypothesis that cytokine-mediated excitotoxic oligodendrocyte death is initiated by myelin OL glycoprotein (MOG)–specific Th cells, pharmacological inhibition of system \( X_c \) was performed after immune cell infiltration in a relapsing-remitting model of EAE. Blocking system \( X_c \) in this regard attenuated clinical scores, which was consistent with a reduction in both reactive gliosis and myelin damage. Furthermore, we demonstrated that myelin-specific CD4+ T cells coopt microglia to release glutamate via the system \( X_c \) transporter, resulting in mature OL death. These findings suggest that system \( X_c \) not only promotes excitotoxic damage to myelin, ultimately linking inflammation to excitotoxicity, but also plays an important role in peripheral immune cell infiltration in autoimmune inflammatory demyelinating diseases.

### Materials and Methods

#### Animals

Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME), and female C57BL/6 mice were purchased from National Cancer Institute–Frederick Cancer Research (Frederick, MD). Timed pregnant female rats were obtained from Charles River Laboratories. All animals were housed and treated in accordance with National Institutes of Health and University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines. Female wild-type C3H/HeSnJ and C3H/HeSnJ-Slk7a1+/- mice for these studies were derived from hemizygous C3H/HeSnJ-Slk7a1+/- mice (The Jackson Laboratory 001310) breeding units maintained at Syracuse University’s laboratory animal resource facility in accordance with their institutional animal care and use guidelines. Genotyping was previously described (21).

#### Oligodendrocyte and microglia cultures

OLs and microglia were obtained from postnatal day 2 or 3 Long–Evans rats using previously described methods (22). Mixed glia were grown on poly-

#### Transmission electron microscopy

Male C57BL/6 mice (8–10 wk old) were induced with EAE using 50 \( \mu g \) MOGp and 125 \( \mu g \) desiccated Mycobacterium tuberculosis H37Rv (DIFCO Laboratories, Detroit, MI) emulsified in IFA, as well as i.p. injection of 200 \( \mu g \) P. aeruginosa (List Biological Laboratories, Campbell, CA) in PBS on days 0 and 2, as described previously (24). Mice were monitored and scored daily for signs of EAE induction.

#### Generation and characterization of MOG35–55–specific Th and Th17 cells

MOG35–55–specific (MOGp) Th1 and Th17 cells were generated as described previously (23, 24). Briefly, splenocytes and draining lymph node cells were obtained from male C57BL/6 mice 12 d postimmunization with 150 \( \mu g \) MOGp (CPC Scientific, San Jose, CA) in CFA. The monoclonal cell preparation was depleted of B cells and CD8+ T cells by magnetic bead chromatography using Dynabeads Biotin Binder (Invitrogen, Grand Island, NY). The CD4+ enriched monoclonal cells were differentiated to Th1 or Th17 and characterized as described previously (24). All Abs were obtained from BioLegend (San Diego, CA).
with 50 ng/ml PMA and 750 ng/ml ionomycin in the presence of Golgi Plug (BD Biosciences, San Jose, CA) for 4 h. Single-cell suspensions from spinal cord or spleen cells were incubated with Fc Block (2.4G2), and the following Abs were used, as appropriate: anti-CD4 (RM4-5; BD Biosciences); anti-Foxp3 (FJK-16s), anti–IFN-γ (XMG1.2), and anti–IL-17A (eBio17B7), and anti–Ki-67 (SolA15) all from eBioscience (San Diego, CA). Cell viability was determined by staining with Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies, Grand Island, NY). To evaluate system Xc\textsuperscript{−} expression on immune cells, cells were incubated with Fc Block (2.4G2), and then surface stained using anti-CD11b (M1/70) and CD45 (30-F11) (all from eBioscience), followed by intracellular staining for system Xc\textsuperscript{−} (Abcam, Cambridge, MA; ab37185). Cell viability was again determined by staining with Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies). Stained cells were run on a LSR II Flow Cytometer (BD Biosciences) and analyzed by FlowJo (Tree Star).

**Evaluation of splenocytes**

Single-cell suspensions of splenocytes isolated from immunized mice were unstimulated or restimulated with either anti-CD3 (145-2C11; 1.0 µg/ml) or MOG35-55 peptide (1.0 or 10.0 µg/ml) for 3 d. The frequency of splenocytes in cycle was measured by pulse-labeling cells with 5-ethyl-2′-deoxyuridine (EdU; 10 µM) for 60 min before analyzing EdU incorporation using the Click-IT EdU Flow Cytometry Assay Kit (Invitrogen Molecular Probes) and live/dead staining, as previously described (24).

**NO assay**

Conditioned media from each experimental condition was collected and analyzed for NO production using the Nitrate/Nitrite Fluorometric Assay Kit, per the manufacturer’s instructions (780051; Cayman Chemical, Ann Arbor, MI).

**Glutamate assay**

Conditioned media from each experimental condition was collected and analyzed for glutamate concentration using the Glutamate Assay Kit per the manufacturer’s instructions (ab83389; Abcam).

**Immunoblotting**

Cells or tissue from whole spinal cord were lysed in a 0.1 M NaPO4 buffer containing 10% SDS, a Mini Protease Inhibitor Cocktails (Roche, Indianapolis, IN), and 1% Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich). A dendritic cell protein assay (Bio-Rad, Hercules, CA) was used to determine the extent of demyelination according to the proportion of white matter affected: 0, 1, 2, 3, or 4 for none; ~25%, ~50%, ~75%, or ~100%, respectively. For C57BL/6 spinal cord assessments, seven sections from thoracic, lumbar, and sacral regions of the spinal cord from each animal were analyzed. For SJL brain assessments, four to six sections of the corpus callosum at the level of the anterior hippocampus were assessed.

**Immunohistochemistry of brain and spinal cord sections**

Ag retrieval was performed using 10 mM citrate buffer (pH 3 or 6), with boiling for 20 min. For diaminobenzidine (DAB) staining, slides were incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were blocked in 5% serum corresponding to the host of the secondary Ab with 0.3% Triton X-100 for 1 h at room temperature. Primary Abs were diluted in blocking buffer and incubated on sections overnight at 4°C. Secondary Abs were diluted in blocking buffer and incubated on sections for 1 h at room temperature. For DAB staining, a Tyramide Signal Amplification Kit (Perkin Elmer, Waltham, MA) was used with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), and a Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Slides used for DAB staining were dehydrated and mounted with Permount (Fisher Scientific, Waltham, MA). For fluorescent staining, slides were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). Mouse anti–glial fibrillary acidic protein (GFAP; 1:1000; Sigma-Aldrich, SAB2500462) was used to evaluate expression of astrocytes. Rabbit anti-iBa1 (1:1000; Wako, 019-19741) or goat anti–Iba1 (Abcam, ab107159) was used to evaluate expression of microglia and macrophages. Rabbit anti–c–kit (1:100; Abcam, ab37185) was used to evaluate expression of the system Xc\textsuperscript{−}.

**Imaging**

Confocal images were captured using a Leica TCS SPE laser-scanning confocal microscope. The Leica LASAF software and Adobe Photoshop (for contrast, brightness, and color adjustments) were used to create figures and process images. Phase-contrast images for cell cultures were captured using a Nikon Ti-U inverted microscope using NIS-Elements software (Melville, NY).

**Quantification of GFAP, Iba-1, and system Xc\textsuperscript{−} staining in spinal cord and brain sections**

For quantification of GFAP, Iba-1, and system Xc\textsuperscript{−} staining in spinal cord sections, fluorescent images were acquired using an Olympus BX51 mi-
croscope (Olympus America, Melville, NY) with 4×, 0.13 NA lens, and Stereo Investigator software (version 11.04; MBF Bioscience, Williston, VT). Cellular staining in thoracic and lumbar sections was analyzed (Image J software; 1.48v) using the area fraction technique (29). Briefly, images were de-noised using rolling-ball background subtraction. The threshold was set to the same baseline across experimental groups for each Ab to measure area of cellular staining. All assessments were made from six sections from thoracic and lumbar regions of the spinal cord from each animal. For quantitative analyses of GFAP- and Iba-1–immunopositive cells in SJL mouse brains, DAB-stained bright field images were acquired using an Olympus BX51 microscope (Olympus America) with 20×, 0.50 NA lens, and Stereo Investigator software (version 11.04; MBF Bioscience). Three adjacent sections, each in three regions of the brain (approximately bregma −0.10 mm, −1.70 mm, and −2.30 mm), were used for a total of nine sections per animal. Cell bodies in a 214 µm × 142 µm region of the corpus callosum and cingulum bundle were counted in the left and right hemispheres, and these numbers averaged to obtain a count of cells per mm² tissue.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism software version 5.03 (GraphPad Software, La Jolla, CA). Specific analyses performed including p values are reported where indicated.

**Results**

**System Xc\textsuperscript{−} transporter inhibitors attenuate experimental autoimmune encephalomyelitis**

System Xc\textsuperscript{−} is an important glutamate release mechanism in oxidizing environments that may contribute to excitotoxic mecha-
nisms in autoimmune demyelination. System \( \text{Xc}^- \) transporter expression is upregulated in spinal cords of C57BL/6 mice with EAE (Supplemental Fig. 1) (30). We tested the effect of treating EAE in C57BL/6 mice with the system \( \text{Xc}^- \) transporter inhibitor SAS. Treatment with SAS was initiated 7 d after immunization with MOGp. EAE was significantly less severe in C57BL/6 mice treated with SAS versus mice treated with PBS (Fig. 1A). Luxol fast blue staining of spinal cords demonstrated reduced demyelination in EAE mice treated with SAS compared with EAE mice treated with PBS (Fig. 1B). Additionally, mice treated with S-4-CPG, an inhibitor of the system \( \text{Xc}^- \) transporter with no known effect on NF-\( \kappa \)B (19), also demonstrated significantly reduced clinical disease (Fig. 1C) that was associated with decreased demyelination (Fig. 1D). These data provide evidence that inhibiting the system \( \text{Xc}^- \) transporter attenuates clinical disease consistent with a reduction in demyelination.

Evaluation of markers for specific inflammatory cell types by immunofluorescent staining demonstrated an increase in reactive astrocytes (GFAP) and activated microglia (Iba-1) in EAE (Fig. 2A, middle panels) compared with control (unimmunized) mice (Fig. 2A, left panels). In SAS-treated mice, the expression of GFAP and Iba-1 (Fig. 2A, right panels) was reduced to levels equivalent to control mice (Fig. 2A, left panel). The expression of system \( \text{Xc}^- \) was also elevated in PBS-treated EAE spinal cords (Fig. 2A, middle panel) compared with control mice (Fig. 2A, left panel). In SAS-treated mice, the expression of system \( \text{Xc}^- \) (Fig. 2A, right panel) was reduced to levels equivalent to control mice (Fig. 2A, left panel). Quantification of system \( \text{Xc}^- \) expression is reported in Fig. 2B, right panel. The expression of system \( \text{Xc}^- \) transporter colocalized with reactive microglia (Fig. 3) in gray and white matter from spinal cords subjected to EAE. These data indicate that the increase in system \( \text{Xc}^- \) expression in EAE spinal cords is due in part to an increase in system \( \text{Xc}^- \) transporter expression in reactive microglia.

**Reduced immune cell infiltration into the spinal cords of SAS-treated EAE mice**

To determine whether the CNS protection observed in SAS-treated EAE mice is a result of reduced peripheral immune cell migration into the CNS, spinal cords were analyzed for altered inflammatory cell populations 7 d after immunization (i.e., peak of disease). Representative dot plots (Fig. 4A) demonstrate a reduction in overall number of CD4$^+$ infiltrating T cells in spinal cords from SAS-treated mice compared with spinal cords from PBS-treated mice (numbers in upper right quadrants). IFN-\( \gamma \)$, IL-17$, and Foxp3$^+$ were also reduced (Fig. 4B). Although there is a statistically significant reduction in CD4$^+$, IFN-\( \gamma \)$, IL-17$, and Foxp3$^+$ T cells in the spinal cord (Fig. 4B), the proportion of infiltrating IFN-\( \gamma $, $\gamma $, IL-17$, IL-17$, IFN-\( \gamma $, and T regulatory$^+$ cells remained unchanged between PBS- and SAS-treated mice (Fig. 4C).

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**FIGURE 1.** Pharmacological blockade of the system \( \text{Xc}^- \) transporter attenuates EAE. (A) EAE clinical scores (mean ± SEM) of C57BL/6 mice treated with PBS (\( n = 20 \)) or SAS (\( n = 19 \)) from day 7 postimmunization with MOGp. Data are from three pooled independent experiments. (B) Luxol fast blue immunohistochemistry of spinal cords from PBS- and SAS-treated mice. (C) Quantitation of demyelination (mean ± SEM) of C57BL/6 mice treated with PBS (\( n = 8 \)) or SAS (\( n = 8 \)) from day 7 postimmunization with MOGp. (D) Luxol fast blue immunohistochemistry of spinal cords from EAE mice treated with PBS and S-4-CPG. Representative areas of demyelination are in boxed or bracketed regions. Quantification of demyelination (mean ± SEM, \( n = 5 \)) were determined using a nonparametric two-tailed Mann–Whitney \( U \) test; top line represents values used to determine statistical measures. Two-tailed \( t \) test was used for all bar graphs. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
SAS also inhibits NF-κB (31), and therefore low numbers of inflammatory cells in the spinal cord may be a result of poor activation of T cells in the periphery. To test for this, the proportion of CD4+ T cells, as well as IFN-γ+, IL-17A+, and Foxp3+ cells, was assessed in spleens from PBS- and SAS-treated mice, and no statistical difference was observed (Fig. 5A). Additionally, the percentage of proliferating cells in spleens from PBS- and SAS-treated mice detected by labeling with Ki-67 showed no statistically significant change (Fig. 5B, left panel; representative dot plots, right panels). Ki-67+ staining in naive mouse spleens demonstrated that 10% of CD4+ cells were proliferating, similar to published results (32). This suggests that there is a 2- to 3-fold increase in proliferation as a consequence of EAE. To further evaluate T cell activation in the periphery, spleen cells from SAS- and PBS-treated mice were restimulated with 1 μg/ml MOGp or anti-CD3 and 10 μg/ml MOGp. Proliferation was assessed by the EdU pulse incorporation assay (24). Representative dot plots (Fig. 5C, left panel) demonstrate little change in the proportion of proliferating cells (numbers in lower right quadrants), which is not statistically significant (Fig. 5C, bar graphs). These results show that the precursor frequency of peripheral MOGp-reactive T cells and ability to enter into the cell cycle were not affected by SAS treatment. We therefore conclude that low numbers of infiltrating inflammatory cells in the CNS of SAS-treated mice are not due to compromised activation of MOGp T cells in the periphery.

Mice lacking functional system xc− are resistant to EAE

To further corroborate the role of system xc− in modulating autoimmune encephalomyelitis, we evaluated the development of EAE in mice deficient in system xc− function (C3H/HeSnJ-Slc7a11<sup>sut/sut</sup>) and wild-type littermate controls (C3H/HeSnJ<sup>+/+</sup>). C3H/HeSnJ-Slc7a11<sup>sut/sut</sup> mice harbor a loss of function mutation
in the Slc7a11 gene that encodes for the system Xc<sup>−</sup> L chain in the C3H/HeSn<sup>+</sup> background (33). EAE in C3H/HeSn<sup>+</sup> littermates was significantly attenuated and delayed compared with C3H/HeSn<sup>sut/sut</sup> mice (Fig. 6A). The incidence of disease was 60% in the C3H/HeSn<sup>+</sup> compared with 20% in C3H/HeSn<sup>sut/sut</sup> mice (Fig. 6B). The average incidence of EAE in C3H/HeJ mice immunized with PLP190–209 is 50% (27). CD4<sup>+</sup> T cells responding to PLP190–209 in a recall assay were similar between C3H/HeSn<sup>+</sup> and C3H/HeSn<sup>sut/sut</sup> mice (data not shown). These data support the pharmacological evidence that the system Xc<sup>−</sup> transporter plays an important role in the pathogenesis of EAE.

**SAS attenuates relapsing-remitting EAE**

The SJL mouse immunized with the PLP<sub>139–151</sub> peptide develops a relapsing-remitting form of EAE that clinically reflects human relapsing-remitting MS (34). To test whether a system Xc<sup>−</sup> inhibitor could ameliorate relapsing-remitting EAE, SAS treatment in SJL mice with EAE was initiated on day 24 postimmunization during the first relapse after T cell infiltration into the CNS has occurred. We observed that clinical symptoms associated with EAE were significantly diminished in SAS-treated mice compared with PBS-treated controls (Fig. 7A). The recovery from EAE in SAS-treated mice was associated with reduced demyelination in the spinal cord (Fig. 7B). Brain pathology as well as spinal cord pathology occurs in SJL mice induced with EAE (34). As such, inflammation and myelin degradation in the corpus callosum and overlying cortex were also assessed in SJL mice when SAS treatment was initiated during an EAE relapse on day 24 postimmunization. The brains of SAS-treated mice had fewer activated microglia (Iba-1; Fig. 8, *top row*) and reactive astrocytes.
GFAP; Fig. 8, middle row), which was consistent with a reduction in myelin damage (luxol fast blue; Fig. 8, bottom row) compared with PBS-treated mice. These data provide evidence that SAS modulates disease pathology in the forebrain of SJL mice after immune cell infiltration into the CNS.

SJL mice treated with SAS before onset of clinical symptoms (from day 7 postimmunization) developed no clinical symptoms of EAE (data not shown). Overall, these results show that inhibition of the system Xc transporter attenuates established relapsing-remitting EAE.

Encephalitogenic Th1 but not Th17 cells promote microglia-induced death of mature OLs

Th1 and Th17 cells contribute to the pathogenesis of MS, and animal models that represent MS, by different modes of action

(Fig. 8, middle row), which was consistent with a reduction in myelin damage (luxol fast blue; Fig. 8, bottom row) compared with PBS-treated mice. These data provide evidence that SAS modulates disease pathology in the forebrain of SJL mice after immune cell infiltration into the CNS.

SJL mice treated with SAS before onset of clinical symptoms (from day 7 postimmunization) developed no clinical symptoms of EAE (data not shown). Overall, these results show that inhibition of the system Xc transporter attenuates established relapsing-remitting EAE.

FIGURE 5. MOG-responding T cells in the periphery are equivalent in PBS- and SAS-treated EAE mice. Spleens from PBS- and SAS-treated mice were analyzed 15 d postinduction of EAE. (A) The percentage of CD4+ T cells, Th1 (IFN-γ+/IL-17+), Th17 (IFN-γ+/IL-17+), and T regulatory cells (Foxp3+) in spleens from PBS-treated (n = 10) and SAS-treated (n = 9) mice from two independent experiments. (B, left panel) The percentage of Ki-67+ cells in the CD4 population from naive spleens (n = 4) as well as from PBS (n = 5) and SAS-treated mice (n = 5) induced with EAE. A one-way ANOVA test demonstrated statistical significance between the proportion of Ki-67+ cells from naive spleens compared with either PBS- or SAS-treated EAE spleens. No significance was observed between PBS- and SAS-treated EAE spleens. (B, right panel) Representative dot plots; numbers indicate proportion of proliferation. (C) Proportion of cells entering into the cell cycle in response to anti-CD3 or MOGp measured by 1-h EdU uptake assay in spleens from EAE mice treated with PBS (n = 6) or SAS (n = 6). Dot plots show percentages. Bar graphs represent two-tailed t test. ***p < 0.001.
Th1 signaling, neutralizing Abs to IFN

determine whether NO produced by Th1 and potentiated in the
produce NO even in the presence of microglia (Fig. 9C). To de-
the presence of microglia (Fig. 9C). In contrast, Th17 cells did not
NO (Fig. 9C). Th1 cells produced NO that was further increased in

A mechanism by which microglia can induce death to OLs is NO
is inhibited by Abs against TNF-α + IFN-γ

A mechanism by which microglia can induce death to OLs is NO
production. Th1 and Th17 cells were next tested for their ability to
induce NO release from microglia. We cocultured Th1 or Th17
cells with mature OLs in the absence or presence of microglia for
48 h, after which supernatants were tested for NO. Mature OLs did
not express NO (Fig. 9C, 9D), consistent with their lack of iNOS
(37). Microglia cocultured with mature OLs also did not express
NO (Fig. 9C). Th1 cells produced NO that was further increased in
the presence of microglia (Fig. 9C). In contrast, Th17 cells did not
produce NO even in the presence of microglia (Fig. 9C). To
determine whether NO produced by Th1 and potentiated in
the presence of microglia was dependent on the cytokines produced by
Th1 signaling, neutralizing Abs to IFN-γ and TNF-α (I+T) were
added to cocultures. Anti–IFN-γ and anti–TNF-α reduced NO
production from Th1 cells as well as Th1-potentiated NO release
from microglia (Fig. 9D). We conclude that MOG-specific Th1
cells, but not Th17 cells, produce NO and potentiate NO release
from microglia that is dependent on IFN-γ and TNF-α signaling.

Th1 but not Th17 cytokines induce NO release from microglia
and subsequent mature OL death

Th1 cells produce IFN-γ, and Th17 cells produce IL-17A and IL-
17F. Both Th1 and Th17 cells produce TNF-α (38). To determine
whether these cytokines produced by Th1 cells activate microglia to
promote mature OL death, microglia were cocultured with mature
OLs in the presence of 100 ng/ml IFN-γ or TNF-α alone or in
combination for 48 h. IFN-γ reduced mature OL number that was
not further enhanced by addition of IL-17A or IL-17F (Fig. 10A,
10B). Addition of TNF-α alone did not induce mature OL death
(Supplemental Fig. 3A, 3B). However, if both IFN-γ and TNF-α
were added to the cultures, almost all mature OLs were killed (Fig.
10B). IFN-γ and TNF-α led to significant production of NO,
whereas all other cytokines individually or in combination did not
(Fig. 10C). Treatment of microglia/OL cocultures with the Th17
cytokines IL-17A or IL-17F alone or in combination with each
other did not cause OL death (Supplemental Fig. 3A, 3B) nor in-
crease NO production (Fig. 10C). No combination of cytokine
treatment in the absence of microglia induced mature OL death
(data not shown).

In the experiments above, MOGp-expanded Th1 and Th17 cells
were of mouse origin, whereas mature OLs and microglia were
from rat (Fig. 10). Because the mechanism by which Th1 and
Th17 cells induce death of mature OLs is strikingly different, an
allogeneic response does not appear to be a confounding factor in
our interpretation. To further limit this possibility, the effect of
murine cytokines on rat mature OL and microglia cocultures was
tested. We again observed that treatment with IL-17A, IL-17F, and
TNF-α (100 ng/ml each for 48 h) alone or in combination did not
cause death of or induce production of NO (Supplemental Fig.
3C). IFN-γ treatment was toxic to OLs, and combined treatment
with murine IFN-γ and TNF-α further significantly reduced ma-
ture OL number (Supplemental Fig. 3B). As with rat cytokines
only IFN-γ and TNF-α induced the production of NO from
microglia. These results provide evidence that the cytokines pro-
duced from MOGp-expanded Th1 cells potentiate death of OLs in
the presence of microglia.

iNOS inhibitors block death of mature OLs induced by
cytokine-activated microglia

We used inhibitors of iNOS to directly test whether production of
NO by microglia after exposure to Th1-associated cytokines
caus ed death to mature OLs. Microglia/Mature OL cocultures
were pretreated with iNOS inhibitor 1400W before exposure to
IFN-γ and TNF-α (100 ng/ml each) for 48 h. IFN-γ- and TNF-α-
stimulated microglia caused significant death to mature OLs that
was blocked by 1400W (Fig. 11A, 11B). Similar results were
observed with aminoguanidine, another inhibitor of iNOS (data
not shown). Western immunoblotting of lysate from microglia
unstimulated or stimulated with IFN-γ and TNF-α resulted in
a 7.5-fold increase in iNOS expression (Fig. 11C). These data
indicate that cytokines associated with Th1 signaling induce NO
release from microglia and cause death of mature OLs.

FIGURE 6. Mice lacking functional systemXc are resistant to EAE.
(A) Mean clinical scores of EAE in C3H/HeSnJ+/+ (n = 6) and littermate
C3H/HeSnJ/sut/sut mice (n = 9). Two-tailed nonparametric Mann–Whitney
U test, mean ± SEM, ***p < 0.0001. (B) Incidence of EAE.

FIGURE 7. SAS attenuates relapsing-remitting EAE. (A) EAE in SJL mice immunized with PLP peptide treated with PBS (n = 8) or SAS (n = 8) from
day 24 postinduction (dashed line). Data are mean ± SEM of clinical scores. Statistical difference was determined using a nonparametric two-tailed Mann–
Whitney U test. Top line represents values used for statistical analysis. (B) Luxol fast blue immunohistochemistry of spinal cords from EAE mice treated
with PBS and SAS. Representative areas of demyelination are in boxed or bracketed regions. Quantitation of demyelination (mean ± SEM, n = 3 mice from
each group, six sections per mouse). Two-tailed t test was used for bar graph. *p < 0.05, ***p < 0.001.
of the system $\chi_-^C$ transporter was elevated in IFN-$\gamma$- and TNF-$\alpha$-treated microglia (Fig. 12C). IFN-$\gamma$- and TNF-$\alpha$-stimulated microglia produced an increase in extracellular glutamate that was blocked by SAS, a system $\chi_-^C$ inhibitor (19, 45) (Fig. 12D). Treatment with SAS in the microglia/OL inflammatory paradigm is protective to mature OLs (Fig. 12E, 12F). In addition to blocking glutamate release from the system $\chi_-^C$ transporter, SAS attenuates NF-$\kappa$B, a key regulator of inflammation. $S$-4-$CPG$, another inhibitor of the system $\chi_-^C$ transporter that has no effect on NF-$\kappa$B (16), also prevents excitotoxicity to mature OLs. These results show that glutamate release from microglia through the system $\chi_-^C$ transporter contributes to excitotoxicity in mature OLs.

**Discussion**

These studies reveal an important link between inflammation and glutamate dysregulation in autoimmune inflammatory demyelination as well as identify the system $\chi_-^C$ transporter on reactive glia as an important source of excitotoxic glutamate release. Moreover, these studies provide evidence that the system $\chi_-^C$ transporter has an important role in regulating immune cell infiltration into the CNS and in the progression of autoimmune demyelination.

Our data demonstrate that myelin-specific CD4$^+$ Th1 and Th17 cells initiate different signaling mechanisms for myelin destruction. Th17 cells caused direct death of mature OLs, whereas Th1 cells required a mediator. Th17-induced mature OL death is independent of cytokines associated with Th17 signaling, because direct stimulation with cytokines did not result in mature OL death. Important clues for how Th17 cells may trigger mature OL death are provided by a recent in vivo imaging study demonstrating that Th17 cells may make direct contact with neurons, causing increased neuronal calcium signaling (46). Th17 cells killed neurons in an in vitro coculture system, and cell death was partially blocked by NMDA receptor antagonists, suggesting a possible role for glutamate in death by Th17 cells (46).

Although NO produced by neuronal NO synthase plays an important role in neurotransmission, NO derived from iNOS is produced mainly by activated astrocytes and microglia and is not present in OLs (37, 47–49). Cytokines and/or LPS have been shown to induce NO production in microglia and astrocytes (37, 50–53). However, we demonstrate that Th1 cells can produce NO as well as potentiate NO release from microglia, unlike Th17 cells. This production of NO is contributed by TNF-$\alpha$/IFN-$\gamma$ signaling because neutralizing Abs to these cytokines blocked NO production. In our study, IFN-$\gamma$-stimulated microglia did cause mature OL death, which was potentiated in the presence of TNF-$\alpha$. Cell death occurred presumably due to the production of NO because neither cytokine alone caused NO release from microglia. Direct cytokine exposure to mature OLs did not produce cell death. A previous study reported that direct IFN-$\gamma$ exposure to OL progenitor cells induced cell death (54), most likely through activation of IFN-$\gamma$ receptors located on OLs (55). The difference in these findings may be due to the fact that the OLs used in our study were fully differentiated, rather than progenitor OLs. Cytokine-stimulated production of NO from microglia also resulted in an increase in iNOS. Inhibiting iNOS protected mature OLs from TNF-$\alpha$/IFN-$\gamma$-stimulated microglia, demonstrating a role for NO in triggering mature OL death. The relevance of iNOS contributing to the neuroinflammatory disease state is demonstrated in an in vivo study in which iNOS inhibitors ameliorated clinical scores in mice subjected to EAE (56).

NO production also has relevance to excitotoxic mechanisms. NO production from LPS-activated glia inhibits cellular respiration...
causing energy failure and glutamate release in culture, resulting in neuronal NMDA glutamate receptor-mediated excitotoxicity (40, 57–59). In this work, we demonstrate that Th1-associated cytokines TNF-α and IFN-γ induce production of NO from microglia that causes AMPA glutamate receptor-mediated excitotoxicity to mature OLs. Glutamate transporters (excitatory amino acid trans-

FIGURE 9. Microglia are required for MOG-specific Th1, but not Th17-mediated mature OL death. Th1 or Th17 cells were cocultured with mature OLs in the absence or presence of microglia for 48 h. Phase-contrast images and Olig2 immunocytochemistry show the mature OLs remaining after 48-h coculturing with Th1 (A) or Th17 (B) cells, in the absence or presence of microglia (MG). Lower bar graphs represent quantification of Olig2-positive cells in each experimental condition expressed as percentage of control of mature OLs alone (mean ± SEM from three independent experiments). Th1 cells, but not Th17 cells, induce NO release from microglia that is inhibited by Abs against TNF-α and IFN-γ. Scale bars, 100 μm. (C) Th1 or Th17 cells (4 × 10^5) were cultured with or without mature OLs and/or microglia (1 × 10^5) for 48 h. Changes in supernatant NO concentration as a result of coculture of mature OLs with microglia (MG) and/or T cells are shown as fold change compared with control (mature OLs; mean ± SEM, n = 3 independent experiments with four replicates per experiment). (D) Th1 (4 × 10^5) cells were cultured with and without mature OLs and/or microglia in the presence and absence of Abs for TNF-α and IFN-γ. Changes in supernatant NO concentration as a result of coculture of mature OLs with microglia (MG) and/or T cells with or without Abs for IFN-γ (I) and TNF-α (T) are shown as fold change compared with control (mature OLs; mean ± SEM, n = 3 independent experiments with four replicates per experiment). Statistical differences compared with control (mature OLs) were determined using one-way ANOVA, followed by Bonferroni multiple comparison posttest (**p < 0.01, ***p < 0.001).
porters) are efficient in maintaining proper physiological concentrations of glutamate (60); however, perturbations in glutamate transporter expression can also contribute to excitotoxic cell death (61, 62). Cytokines are known to reduce glutamate transporter expression in astrocytes (63, 64), suggesting that during inflammatory conditions glutamate release through the system \( \text{Xc}^- \) transporter cannot be properly regulated by glutamate transporters. Pharmacologically inhibiting glutamate receptors or increasing glutamate transporter expression to confer protection is not likely feasible due to the imperative nature of glutamatergic signaling for normal physiological processes. This rationale makes identifying and blocking the source of excitotoxic glutamate a more practical therapeutic strategy.

As demonstrated in this manuscript, inflammation leads to excitotoxicity because cell death is blocked by the glutamate receptor antagonist NBQX. Release of glutamate from the system \( \text{Xc}^- \) transporter is a well-known source of glutamate toxicity during energy failure (16, 21, 42, 43). In exchange for the import of L-cystine, an important metabolite for glutathione, the system \( \text{Xc}^- \) transporter exports glutamate. Glutamate release from the

**FIGURE 10.** IFN-\( \gamma \) plus TNF-\( \alpha \) induces death of mature OLs and production of NO in the presence of microglia. (A) Purified microglia (1 \( \times \) 10\(^5\)) were plated onto mature OLs and left unstimulated or treated with 100 ng/ml IFN-\( \gamma \), IFN-\( \gamma \) + IL-17A, IFN-\( \gamma \) + IL-17F, or IFN-\( \gamma \) + TNF-\( \alpha \) for 48 h. Phase-contrast images and corresponding immunocytochemistry for Olig2 are shown. Scale bars, 50 \( \mu \)m. (B) Quantification of Olig2-positive cells shown in (A) expressed as percentage of unstimulated control (mean \( \pm \) SEM, \( n = 3 \) independent experiments with four replicates per experiment). (C) Purified microglia (1 \( \times \) 10\(^5\)) were plated onto mature OLs, and cocultures were treated with 100 ng/ml of each of the indicated cytokines for 48 h. Supernatants were then tested for NO production (mean \( \pm \) SEM, \( n = 3 \) with two replicates per experiment). Statistical differences compared with control (unstimulated) were determined using one-way ANOVA, followed by Bonferroni multiple comparison posttest (*\( p < 0.05 \), **\( p < 0.001 \)).

**FIGURE 11.** The iNOS inhibitor 1400W blocks mature OL death induced by cytokine-activated microglia. (A) Phase-contrast images of microglia (1 \( \times \) 10\(^5\)) plated onto mature OLs and stimulated with IFN-\( \gamma \) and TNF-\( \alpha \) in the presence or absence of 25 \( \mu \)M 1400W. Scale bars, 50 \( \mu \)m. (B) Differences in cell counts for IFN-\( \gamma \) and TNF-\( \alpha \) in the presence or absence of 1400W are shown as percentage of control (unstimulated; mean \( \pm \) SEM, \( n = 3 \) independent experiments with four replicates per experiment). Statistical differences determined using one-way ANOVA, followed by Bonferroni multiple comparison posttest (*\( p < 0.05 \), **\( p < 0.01 \)). Similar results were obtained with 200 \( \mu \)M aminoguanidine hydrochloride, another iNOS inhibitor (data not shown). (C) Western blot (left panel) and densitometric analysis (right panel) of iNOS expression in microglia in the absence or presence of IFN-\( \gamma \) and TNF-\( \alpha \) (mean \( \pm \) SEM, \( n = 3 \)). Statistical differences compared with control (unstimulated) were determined using a \( t \) test (**\( p < 0.001 \)).
system $X_c^-$ transporter has been demonstrated in cultured microglia after exposure to LPS and human peripheral monocytes from MS patients (30). Furthermore, in animal models of glioma, blocking glutamate release from the system $X_c^-$ transporter prevented excitotoxicity to neurons (45, 65, 66). Consistent with these data, blocking the system $X_c^-$ transporter ameliorated mature OL death from TNF-$\alpha$/IFN-$\gamma$-stimulated microglia. In addition to blocking glutamate release from the system $X_c^-$ transporter, SAS attenuates NF-$\kappa$B, a key regulator of inflammation. Therefore, we tested S-4-CPG, another inhibitor of system $X_c^-$ that does not have any effect on NF-$\kappa$B (19), and demonstrated its effectiveness in alleviating OL excitotoxicity.
icity in vitro. Although neonatally derived microglia is the most widely used approach, the activation profile of neonatally derived microglia in culture may not completely reflect what is observed in the adult rodent in vivo. These data warrant the exploration of this therapeutic target in animal models of MS.

Using the C57BL/6 animal model of EAE, treatment with system $X_c^-$ transporter inhibitors SAS and S-4-CPG improved clinical scores. These data are consistent with myelin preservation in the spinal cord. It seems unlikely that the protective nature of SAS can be attributed to its role in inhibiting NF-$k_B$ because S-4-CPG, which has no mechanism of action at NF-$k_B$, produced similar results. In culture, system $X_c^-$ has been shown to affect T cell activation by redox modulation via macrophage and dendritic cells (67, 68). Therefore, i.p. injection of SAS was performed on day 7, after T cell activation has already occurred. This suggests that the lack of immune cell infiltration into the CNS in SAS-treated mice is an effect on T cell migration and not activation. Inhibitors of NF-$k_B$ do not have any effect on T cell infiltration into the CNS (69), supporting an important role for system $X_c^-$ in T cell migration. Additionally, no reduction in T cell proliferation was found in spleens, suggesting that altering the function of system $X_c^-$ did not affect T cell activation, but rather perturbed infiltration into the CNS. To further validate the role of the system $X_c^-$ transporter in T cell migration, a non-pharmacological approach was undertaken. In this regard, we found that mice harboring a natural mutation in the gene encoding xCT were resistant to EAE compared with littermate controls. Similar to results from SAS-treated mice, T cell proliferation was not affected in system $X_c$-deficient mice. Taken together, these data support a novel role for the system $X_c^-$ transporter in regulating T cell infiltration into the CNS. Currently, two other studies have explored the effect of SAS in EAE induced in guinea pigs, but only report clinical assessments (70, 71).

The unexpected finding that SAS attenuates T cell infiltration into the CNS suggests that its protective nature may be due to attenuating T cell infiltration. To further explore the role of system $X_c^-$ in CNS protection, we used a relapsing-remitting form of EAE and introduced SAS after the first relapse, therefore after T cell infiltration. SAS-treated animals showed improved clinical scores compared with PBS treated. Consistent with clinical outcomes, there was a reduction in reactive astrocytes, reactive microglia, and myelin damage in both the brain and spinal cord. These data support a novel role for the system $X_c^-$ transporter in regulating pathogenesis after T cell infiltration in EAE.

Common therapies for MS block T cell infiltration into the CNS, which reduces, but does not eliminate, relapses. This warrants understanding the underlying mechanisms of how immune cells initiate CNS destruction to devise treatment strategies that also include CNS protection. We demonstrate that SAS is also protective to the CNS after T cell infiltration. This may have relevance to the clinical trial using SAS as a stand-alone therapy in MS. SAS treatment was beneficial during the first 18 mo, but at the 3-yr time point no difference in Expanded Disability Status Scale score was observed compared with MS patients with no treatment (72). We propose that SAS may be more efficacious as an add-on therapy rather than a stand-alone treatment. Current therapies for MS do not protect the brain during symptomatic attacks, and using SAS as an add-on therapy may prove beneficial to quality of life and even disease progression.

The clinical data do support the efficacy of SAS as a treatment during the early acute phase of the inflammatory event because it was beneficial during the first 18 mo of treatment in MS. We show the efficacy of SAS in attenuating immune cell infiltration into the spinal cord during the initial inflammatory insult using the C57BL/6 chronic model of EAE. These data are consistent with preservation of spinal cord function and less damage to the myelin sheath. Transverse myelitis is an acute inflammatory demyelinating disease in which the immune system becomes primed to attack myelin in a specific segment of the spinal cord. Our data show that SAS blocks immune cell infiltration into the spinal cord in the acute onset of EAE, suggesting that SAS may be beneficial in an acute inflammatory demyelinating disease state such as transverse myelitis.