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S-nitrosoglutathione a Physiologic Nitric Oxide Carrier Attenuates Experimental Autoimmune Encephalomyelitis

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Abstract

S-nitrosoglutathione (GSNO) is a physiological nitric oxide molecule which regulates biological activities of target proteins via s-nitrosylation leading to attenuation of chronic inflammation. In this study we evaluated the therapeutic efficacy of GSNO in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. Oral administration of GSNO (0.5 or 1.0 mg/kg) reduced disease progression in chronic models (SJL and C57BL/6) of EAE induced with PLP_(139–151) or MOG_(35–55) peptides, respectively. GSNO attenuated EAE disease by reducing the production of IL17 (from Th₁ or Th17 cells) and the infiltration of CD4 T cells into the central nervous system without affecting the levels of Th1 (IFN γ) and Th2 (IL4) immune responses. Inhibition of IL17 was observed in T cells under normal as well as Th17 skewed conditions. In vitro studies showed that the phosphorylation of STAT3 and expression of ROR γ , key regulators of IL17 signaling, were reduced while phosphorylation of STAT4 or STAT6 and expression of T-bet or GATA3 remained unaffected, suggesting that GSNO preferentially targets Th17 cells. Collectively, GSNO attenuated EAE via modulation of Th17 cells and its effects are independent of Th1 or Th2 cells functions, indicating that it may have therapeutic potential for Th17-mediated autoimmune diseases.

Keywords

EAE; T cells; autoimmunity; GSNO; IL-17; inflammation; MS; STAT; T-bet; ROR-gamma

Introduction

Experimental autoimmune encephalomyelitis (EAE) is mediated by the infiltration of CD4⁺ (Th1 and/or Th17) T cells and their sequential expansion leading to an inflammatory autoimmune disease of central nervous system (CNS; Segal 2003; Imitola et al. 2005; Becher et al. 2006). Animals with EAE serve as a model for multiple sclerosis (MS) representing both

inflammation and demyelination with chronic relapsing–remitting and progressive-relapsing forms of the disease (Skundric 2005). The inflammatory environment both in EAE and MS is characterized by the expression of high levels of pro-inflammatory mediators (IL17, TNF α , IFN γ , and iNOS; Sredni-Kenigsbuch 2002; Segal 2003; Imitola et al. 2005; Langrish et al. 2005) secreted by infiltrating immune and resident glial cells (astrocytes and microglia; Izikson et al. 2002; Imitola et al. 2005) which damage oligodendrocytes, myelin sheaths, and neurons/axons resulting in demyelination and axonal loss (Hendriks et al. 2005; Imitola et al. 2005). Inducible nitric oxide synthase (iNOS) is up-regulated in EAE and MS (Bogdan 1998, 2001a; Hendriks et al. 2005), and high levels of nitric oxide (NO), produced by iNOS, react with superoxide to form peroxynitrite, a highly toxic reactive molecule, which modifies proteins, lipids, nucleic acids resulting in the loss of oligodendrocytes (Bogdan 1998, 2001a; Pannu and Singh 2006). In fact, overproduction of NO is thought to play a significant role in the pathology of various autoimmune diseases such as EAE (Weinberg et al. 1994; Hoey et al. 1997; Fenyk-Melody et al. 1998), arthritis (EAA; McCartney-Francis et al. 1993), and uveitis (EAU; Tarrant et al. 1999). However, studies in iNOS-deficient mice showed a more severe EAE disease than wild-type mice suggesting the complexity of NO-mediated mechanisms in immune responses (Fenyk-Melody et al. 1998; Dalton et al. 2000; Shi et al. 2001; Kahl et al. 2004; Dalton and Wittmer 2005).

GSNO is an endogenous NO-modulating signaling molecule involved in the regulation of physiological as well as pathological events (Bogdan 2001b; Foster et al. 2003). Furthermore, NO and GSNO have two distinct actions. GSNO may S-nitrosylate thiol groups directly through transnitrosylation while metalloenzyme or oxygen is needed to generate S-nitrosylated proteins in the presence of NO (Stamler 1994; Hogg 1999). Incorporation of the NO moiety by covalent bonding to a thiol group of the cysteine residue of a protein and peptide is termed S-nitrosylation (Lipton et al. 1993). It is generally accepted that direct reaction of the NO (radical) with thiol (-SH) does not yield nitrosothiol (Wink et al. 1994). Previous reaction of NO with oxygen is considered to be necessary for S-nitrosylation via formation of higher nitrogen oxides among which N₂O₃ is thought to be the quintessential S-nitrosylating species (Wink et al. 1993; Hogg 2002). The mechanism of nitrosothiol formation and protein S-nitrosylation is of biological relevance especially due to the high concentrations of intracellular low molecular mass thiols such as glutathione and cysteine in blood and brain (Kluge et al. 1997).

Nitrosylation is considered significant because of the high reactivity, reversibility, occurrence in physiological conditions, regulation by redox, and its influence on many protein functions (Foster et al. 2003). It plays an important and novel regulatory role in signal transduction (Martinez-Ruiz and Lamas 2004; Hess et al. 2005). S-nitrosylation has been shown to modify the function of many proteins in vitro including NF- κ B (Fortenberry et al. 2001; Prasad et al. 2006) and caspases (Mohr et al. 1997; Khan et al. 2005).

Administration of GSNO has been reported to be neuroprotective not only in cerebral ischemia but also in EAE. The treatment of GSNO inhibited proinflammatory cytokines and endothelial–leukocyte interactions through down-regulation of cellular adhesion molecules modulated by S-nitrosylation and inhibition of NF- κ B pathway proteins (Khan et al. 2005; Khan et al. 2006; Prasad et al. 2006).

CD4⁺ T cells differentiate into Th1, Th2, or Th17 cells upon T cell receptor engagement with major histocompatibility complex on antigen presenting cells (APC; Murphy and Reiner 2002; Wynn 2005; Dong 2008). The fate of T cell differentiation depends on the cytokine milieu of APCs interacting with T cells (Murphy and Reiner 2002; Dong 2008). Regardless of conflicting reports as to the role of Th1 vs Th17 cells in terms of EAE induction (Ferber et al. 1996; Langrish et al. 2005; McGeachy et al. 2007; Kroenke et al. 2008), both Th1 as well as

Th17 cells express proinflammatory mediators associated with disease initiation, whereas Th2 cells secreting anti-inflammatory cytokines are reported to be protective (Chitnis and Khoury 2003; Langrish et al. 2005; Kroenke et al. 2008). This study was designed to evaluate the efficacy of GSNO treatment in mediating anti-inflammatory activities in chronic rodent models (SJL or C57BL/6) of EAE disease and on CD4⁺ T cell immune responses during EAE induced by myelin PLP_(139–151) or MOG_(35–55) peptides, respectively. The results document that GSNO treatment preferentially limits the production of IL17 from CD4⁺ Th_{inflammatory} (Th_i or Th17) cells independent of Th1 or Th2 immune responses and thus attenuated EAE disease progression. These results define for the first time, a novel role of a physiological NO carrier in CD4⁺ T-cell-mediated immune responses during an autoimmune disease of CNS.

Materials and methods

Mice

Female SJL and C57BL/6 mice, 4- to 5-week-old, were purchased from the National Cancer Institute (Bethesda, MD, USA), Jackson (Bar Harbor, ME, USA), or Harlan Laboratories (Indianapolis, IN, USA). Mice were housed in the animal care facility of Medical University of South Carolina. Paralyzed mice were provided alternate food/water source (transgel).

Peptides and reagents

Myelin proteolipid protein (myelin PLP_(139–151), HSLG TKWLGHDPDKF) and myelin oligodendrocyte protein (MOG_(35–55), MEVGWYRSPFSRVVHLYRNGK) were purchased from Peptide International Inc. Louisville, KY, USA. GSNO was purchased from World Precision Instruments, Sarasota, FL, USA. Recombinant murine IL4, IL12, IL23, IL6, IL12/23p40 homodimer, TGFβ, and IL12p35 were purchased from BD Pharmingen, San Diego, CA, USA; R&D Systems, Minneapolis, MN, USA; and Pros-pec, Israel. Purified (NA/LE) anti-CD3ε (145-2C11) and CD28 (37.51) antibodies were purchased from BD Pharmingen. OPT EIA systems (for IFNγ and IL4 ELISA) were purchased from BD Pharmingen and Duo-Set for IL17 ELISA from R&D Systems. The anti-pSTAT3/STAT3, pSTAT4/STAT4, pSTAT6/STAT6, pSTAT1, T-bet, GATA3, and RORγ antibodies were purchased from Cell Signaling, MA, USA; Zymed Laboratories, CA, USA; Santa Cruz Biotechnology, CA, USA; and Abcam, MA, USA.

Clinical evaluation of peptide-induced EAE

For PLP_(139–151) or MOG_(35–55)-induced EAE, 8- to 10-week-old female SJL or C57BL/6 mice were immunized with an emulsion (100 μl, subcutaneous; sc) containing 200 μg of killed *Mycobacterium tuberculosis* (Mtb) H37Ra (Difco, Detroit, MI, USA) and 100 μg myelin PLP_(139–151) or MOG_(35–55) distributed over two spots on the flank with a booster given on day 7. Each mouse additionally received pertussis toxin (PTx, 200 ng, intravenous; iv; Sigma) in 300 μl of phosphate-buffered saline (PBS) on day 0 and 72 h post-immunization. Individual animals were observed daily for clinical disease scores in a blinded fashion on a 0–5 scale as follows: 0 = no abnormality; 1 = piloerection, sluggish; 2 = limp tail; 2.5 = hind limb weakness (legs slip through cage top); 3 = hind limb paralysis; 4 = hind and forelimb paralysis; and 5 = moribund (Nath et al. 2006).

Treatment of EAE with S-nitrosoglutathione

To test prophylactic and therapeutic efficacy mice were treated with GSNO (0.5 or 1 mg/kg, oral administration, 100 μl/PBS mouse) daily starting from day 0 of immunization (prophylactic) or day ~11–13 when all mice had visible EAE symptoms (therapeutic). Mice in control group received 100 μl PBS.

Ex vivo recall responses

Mice (SJL or C57BL/6) were killed at peak of disease and spleens were removed from EAE-diseased and GSNO-treated animals. The spleens were harvested from mice and a single-cell suspension was prepared. Red blood cells were lysed ($1\times$ pharma lyse buffer BD Pharmingen) and cells were washed twice with RPMI-1640 and resuspended in RPMI-complete media [containing RPMI 1640 (Mediatech), 10% FBS (Hyclone), and 100 $\mu\text{g/ml}$ streptomycin and penicillin (Atlanta Biologicals), 1 mM glutamine, 1 mM nonessential amino acids, and 5×10^{-5} M 2-ME (Sigma)]. Cells concentration was adjusted to 2.5×10^6 cells/ml and plated in 96-well round-bottom microculture plates (Falcon Labware, CA, USA) in 0.1 ml of RPMI-complete media with PLP_(139–151) (5 $\mu\text{g/ml}$) or MOG_(35–55) (25 $\mu\text{g/ml}$) peptide followed by incubation for 48 h. Recall responses were measured with [^3H]thymidine (1 $\mu\text{Ci/well}$) added at 48 h and further incubated for 18–24 h and the mean incorporation of thymidine was measured by 1450 Microbeta Wallac Trilux Liquid Scintillation Counter (Perkin-Elmer Life Sciences).

Ex vivo and in vitro effects of S-nitrosoglutathione on Th1, Th2, and Th17 cytokine generation

For ex vivo cytokine analysis, spleen cells from GSNO-treated (0.5 or 1 mg/kg) and untreated (SJL or C57BL/6) mice at the peak of EAE disease were cultured for 48 h (for Th1, IFN γ and Th17, IL17) and 96 h (for Th2, IL4 cytokines) in presence of PLP_(139–151) (5 $\mu\text{g/ml}$), MOG_(35–55) (25 $\mu\text{g/ml}$), or killed *Mtb* (10 $\mu\text{g/ml}$). Total CD3 positive T cells were isolated from SJL or C57BL/6 mice, purified with T cell enrichment columns and assessed for purity by FACS. The T cells (2×10^5 cells/well) suspended in RPMI-complete media were pretreated with GSNO (10 to 400 μM) and then stimulated with anti-CD3 ϵ and CD28 antibodies. Culture supernatants were collected at 36 to 96 h and measured for Th1 (IFN γ), Th2 (IL4), or Th17 (IL17) cytokines production by ELISA.

Histological analysis

To assess the degree of CNS inflammation SJL mice (treated with GSNO or left untreated) were euthanized on day 17 (at the peak of EAE disease) by CO $_2$ asphyxiation. The spinal cords were fixed in 10% buffered formalin (VWR PA) and transverse sections (5 μm) were taken (four sections/mouse), and stained with H&E to assess leukocyte infiltration and inflammation (Nath et al. 2006; Prasad et al. 2006). To characterize the infiltrated cells spinal cord sections were stained with anti-CD4 $^+$ antibodies and detected by immunohistochemical analysis. Briefly, the sections were incubated overnight with mouse polyclonal IgG CD4 $^+$ antibody (Santa Cruz, CA, USA). Secondary biotinylated anti-mouse IgG was incubated for 30 min followed by an avidin–biotin HRP complex (Vectastain ABC-Elite Kit, Vector Labs) with DAB as substrate. Sections were analyzed with an Olympus microscope and images were captured with a digital video camera.

For the quantitation of infiltrates in EAE- and GSNO-treated mice spinal cords digital images of H&E sections were opened in Image J. Area covered by infiltrating cells (nuclei stained with hematoxylin) was quantitated by converting images to grayscale and adjusting threshold. The image was then analyzed and the area fraction was calculated for eight images from each group. Statistical analysis was performed using Student's *t* test.

Western blot analysis

Cells were incubated in the presence or absence of GSNO (1 to 500 μM) and stimulated with CD3/28 antibodies for 24 to 48 h and then harvested, washed with Hank's buffer, and lysed in lysis buffer (20 mM HEPES-NaOH, pH 7.6, containing protease inhibitor cocktail: 20 mM NaCl, 1% triton X-100, 50 mM NaF, 10 mM sodium orthovanadate, and 1 mM DTT). Proteins were resolved with 4–20% gradient SDS-PAGE (BioRad) and transferred to nitrocellulose

membranes which were then blocked for 1 h with 5% nonfat dry milk in TTBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween20, pH 7.6) and incubated overnight at 4°C in primary antibody (pSTAT3/STAT3, pSTAT4/STAT4, pSTAT6/STAT6, pSTAT1, GATA3, T-bet, ROR γ , or β -actin) containing 3% BSA (for phospho-antibodies) or nonfat dry milk (for non-phospho antibodies). Secondary antibody HRP-conjugated (anti-rabbit or anti-mouse dilution 1:5,000) was added for 1 h and then developed with an ECL detection system (Amersham).

Statistical analysis

Clinical disease scores as average maximal scores over the treatment period (mean $\pm$ SD) and analyzed using Kruskal–Wallis test. Statistics for proliferation and cytokine responses were analyzed with one-way multiple-range analysis of variance using Graph Pad Prism 3.0 software. Significances (p value) between groups were determined using the Newman–Keul test. A value of $p < 0.05^*$ and above was considered significant.

Results

Prophylactic and therapeutic administration of GSNO delayed the induction of EAE

Here we examined the prophylactic and therapeutic efficacy of GSNO in pathogenesis in chronic models of EAE (SJL or C57BL/6) in mice. EAE was induced in SJL or C57BL/6 mice with PLP_(139–151) or MOG_(35–55) peptide, respectively. All animals in the EAE group (that were not treated with GSNO) developed clinical paralysis starting from day ~12 and reached a maximum mean clinical score of 2.69 ± 0.25 on day 17 in SJL and 3.2 ± 0.25 on day 19 in C57BL/6 mice (Fig. 1A, B). Mice treated with GSNO (0.5 or 1.0 mg/kg) developed paralysis starting on day ~13, and disease peaked on day ~17 in SJL and day 21 in C57BL/6 mice. Clinical disease severity in SJL mice, a relapsing–remitting model was reduced with GSNO treatment in a dose-dependent manner and the most effective dose of GSNO was 1.0 mg/kg which lowered the disease index significantly (1.75 ± 0.21 , $p < 0.001$). Similarly in C57BL/6 mice with chronic disease both doses of GSNO (on day 21, 0.5 mg/kg, 1.6 ± 0.35 , $p < 0.001$ or 1 mg/kg, 2.6 ± 0.24 , $p < 0.05$ and on day 24 onwards 1.8 ± 0.2 , $p < 0.001$) were effective. For therapeutic efficacy GSNO (1 mg/kg, daily, oral) was given to SJL mice with disease score ~2.0, which decreased the clinical severity of EAE disease (Fig. 1C) documenting its potential in controlling EAE disease when given after the onset of disease. Animals used in the study are summarized in Table 1.

GSNO-mediated effects on recall responses to neural antigen-specific T cells and their cytokine profile

Spleen cells were isolated from PLP_(139–151) or MOG_(35–55)-peptide-induced EAE-diseased and GSNO (0.5 or 1.0 mg/kg) treated mice, stimulated ex vivo with PLP_(139–151) or MOG_(35–55) peptides. As shown in Fig. 1 (SJL, D and B6, G), stimulation with myelin peptides increased immune T cell responses as proliferation, which were unaffected by GSNO treatment. Furthermore, these cultures were tested for Th1, Th2 or Th17 cytokine profile. As shown in Fig. 1 (SJL, F and B6, J), in vivo treatment with GSNO did not alter the production of IFN γ (Th1) compared to untreated EAE mice, whereas IL4 (Th2) cytokine was not detected in these cultures (data not shown). Furthermore, the production of the newly identified inflammatory cytokine IL17 was significantly ($p < 0.0001$) reduced by GSNO treatment as shown in Fig. 1 (SJL, E and B6, H). The production of IL17 from spleen cells of treated and untreated mice upon ex vivo stimulation with killed *Mtb* was also significantly ($p < 0.0001$) inhibited in treated cells (Fig. 1I, B6 mice). These results clearly showed that GSNO treatment inhibited the production of IL17 from myelin specific and *Mtb* stimulated T cells without affecting the Th1 or Th2 immune responses.

Infiltration of immune cells into spinal cords of EAE was reduced by GSNO treatment

The infiltration of activated immune cells into the CNS of untreated EAE and treated with different doses of GSNO (0.5 or 1 mg/kg) were examined in sections of spinal cords. As shown in Fig. 2A, untreated EAE mice had profound infiltration of mononuclear cells representing a high degree of inflammation in CNS, which was significantly reduced by GSNO treatment (Fig. 2B, C).

Furthermore, we observed that the infiltration of CD4⁺ immune T cells was increased in the CNS during EAE disease in spinal cord sections (Fig. 2D). However, GSNO (0.5 or 1.0 mg/kg) treatment reduced the infiltration of CD4⁺ T cells (Fig. 2E, F). The area with infiltrates were quantitated in H&E sections and found that infiltration of cells was significantly reduced by GSNO (Fig. 2G). These observations document that treatment of EAE animals with GSNO attenuated the infiltration of immune CD4⁺ T cells into CNS.

Th1 cell differentiation: status of phosphorylation of STAT4, STAT1, and expression of T-bet

Role of IFN γ producing Th1 cells is controversial in term of EAE induction but during inflammation induced by endotoxin it is one of the key defensive molecules against intracellular pathogens and recently it has been shown to induce EAE with the help of macrophages (Murphy and Reiner 2002; Langrish et al. 2005; Wynn 2005; Kroenke et al. 2008). Therefore, we investigated the effect of GSNO on Th1 cell differentiation. Total CD3 positive T cells were pretreated with GSNO (1 to 400 μ M) followed by stimulation with CD3/28. As shown in Fig. 3, GSNO did not hamper the production of Th1 (IFN γ) cytokine from (A) SJL or (B) C57BL/6 mice. Phosphorylation of signal transducers and activators of transcription protein (STAT)-4 is involved in Th1 cell differentiation, and can be measured by its phosphorylation and production of IFN γ (Murphy and Reiner 2002). As depicted in Fig. 3C, D phosphorylation of STAT4 under normal and Th1-skewed (rmIL12) conditions was not affected by GSNO suggesting that it did not alter Th1 cell differentiation. T-bet, a key transcription factor for Th1 (Murphy and Reiner 2002) which is downstream of STAT4, is shown in Fig. 3E and F. Its expression was not modulated with GSNO treatment under Th0 conditions; however, it was inhibited in Th1 skewed conditions. STAT1 phosphorylation is an integral part of IFN γ signaling and involve in an autocrine loop to further induce the production of IFN γ , we have observed (Fig. 3G) that up to 100 μ M there was no inhibition; however, at 200 μ M it was inhibited. As we observed no inhibition in the production of IFN γ from naïve CD3 positive T cells (Fig. 3A and B) and myelin-specific CD4 T cells (Fig. 1F, J), we reasoned that this may be due to the common p40 subunit of IL12 and 23 cytokines. To rule out this possibility we stimulated naïve CD3 positive T cells with anti-CD3/28 in the presence of IL12p35 for 36 h in the presence or absence of GSNO. As shown in Fig. 3H expression of T-bet was not affected. This strengthens our hypothesis that GSNO does not affect the Th1 pathway and its associated transcription factors and thus IFN γ production.

Th2 cell differentiation: status of phosphorylation of STAT6 and expression of GATA3

Since GSNO had no effect on Th1 cells, we examined the effects GSNO has on the Th2 cell differentiation pathway. The total CD3 positive T cells were pretreated with GSNO followed by stimulation with CD3/28 and production of Th2 (IL4) cytokine was measured. As shown in Fig. 4(A) SJL or (B) C57BL/6, mice Th2 responses were not hampered by GSNO treatment. Previous studies suggested that Th1 and Th2 cytokines antagonize each other (Murphy and Reiner 2002; Imitola et al. 2005) while GSNO treatment did not alter the balance between these two immune responses. The phosphorylation of STAT6 and expression of GATA3, a downstream signaling molecule to STAT6 and a key transcription factor involved in Th2 cell differentiation (Murphy and Reiner 2002), was also examined after GSNO treatment. As depicted in Fig. 4C, D, phosphorylation of STAT6 and expression of GATA3 (Fig. 4E, F) were unchanged by GSNO treatment both under normal and Th2-skewed (rmIL4) conditions. These

observations are consistent with the observed lack of effects on the production of IL4. GSNO-mediated mechanisms attenuated EAE disease but without altering the T cell behavior under Th0 (non-polarizing) and Th1- or Th2-skewed paradigm.

Th17 cell differentiation: status of phosphorylation of STAT3 and expression of ROR γ

Recent reports documenting the induction of EAE by Th17 cells underscores the role of IL17 in the pathogenesis of EAE (Langrish et al. 2005; Bettelli et al. 2006; Komiyama et al. 2006; Kroenke et al. 2008). The pathobiology of IL17 in EAE disease was further supported by the fact that an antibody to IL17 attenuated the autoimmune disease and IL17 gene-deficient mice were resistant to EAE disease (Langrish et al. 2005; Park et al. 2005; Komiyama et al. 2006). Phosphorylation of STAT3 and its downstream signaling through orphan nuclear receptor (ROR γ) are an absolute requirement for the differentiation of CD4⁺ Th17 cells since STAT3^{-/-} and ROR γ ^{-/-} mice were unable to mount Th17 cell immune responses and resistant to EAE (Ivanov et al. 2006; Harris et al. 2007; Yang et al. 2007). In light of these studies we investigated the effect of GSNO on newly identified CD4⁺ Th_i or Th17 cell differentiation and production of IL17 from these cells. As shown (Fig. 5A–E), GSNO effectively limited the production of IL17 (36 and 72 h) under neutral as well as Th17 skewed (rmIL23 or rmIL6+TGF β) conditions from purified CD3 positive T cells as well as in total spleen cells (Fig. 5F). Recent studies have described that while IL6/TGF β is required for differentiation of Th17 cells from naïve T cells (Harrington et al. 2005; Mangan et al. 2006) IL23 is needed for the maintenance of these cells in vitro as well as in vivo (Mangan et al. 2006). GSNO treatment blocked the production of IL17 at early (Fig. 5A and B) and late time points (Fig. 5C, D and E) both in the presence of IL6/TGF β or IL23 suggesting that it inhibits both the differentiation and maintenance of these cells. These results are further supported by the observed reduced phosphorylation of STAT3 and expression of ROR γ under neutral and Th17 skewed condition in CD3 positive T cells (Fig. 5G and H). Similar effects were observed in the spleen cells of EAE disease mice upon ex vivo stimulation with myelin antigen under neutral or Th17 skewed (IL6/TGF β) conditions in the presence or absence of GSNO (Fig. 5I). These data clearly document that IL17 production was preferentially inhibited by GSNO in T cells from EAE-diseased mice even when the cells were driven to Th_i or Th17 cell differentiation.

Discussion

EAE or MS pathology is primarily mediated by CD4⁺ T cells which contribute to disease progression or attenuation upon activation and differentiation into either Th1, Th17, or Th2 cells producing inflammatory cytokines (IFN γ , TNF α , and IL17) or anti-inflammatory cytokines (IL4; Murphy and Reiner 2002; Segal 2003; Imitola et al. 2005; Langrish et al. 2005; Park et al. 2005). Several studies suggest that modulation of activated T cells to produce anti-inflammatory cytokines can be useful in attenuation of the EAE disease process (van der Veen 2001; Imitola et al. 2005; Pannu and Singh 2006). Here, we examined the efficacy of an endogenous physiological carrier of nitric oxide (GSNO) on EAE disease progression in chronic models of EAE and measured its effect on T cell modulation. These studies document that GSNO treatment preferentially limits the production of IL17 from CD4⁺ Th17 or Th_i cells in vitro and in vivo but without inhibiting Th1 or Th2 cell-mediated immune responses, which eventually led to the attenuation of EAE disease.

The GSNO-mediated mechanisms in inhibition of IL17 generation were supported by the following observations (1) GSNO treatment did not inhibit the production of Th1 (IFN γ) or Th2 (IL4) cytokines from T cells or the phosphorylation of STAT4 or STAT6 and expression of T-bet or GATA3 transcription factors required for Th1 or Th2 immune responses, respectively. (2) GSNO treatment reduced the phosphorylation of STAT3 and expression of

ROR γ transcription factors required for Th17 immune responses. (3) GSNO administration attenuated the infiltration of vascular immune cells into the CNS and thus the EAE disease. The observed GSNO-mediated mechanisms of EAE disease attenuation indicate a critical role for Th17 responses in EAE. These results are consistent with inhibition of EAE with antibodies to IL17 in addition to the resistance of IL17 gene-deficient mice to EAE disease (Harrington et al. 2005; Park et al. 2005; Wynn 2005; Komiyama et al. 2006). Our observations led us to hypothesize that preferential inhibition of IL17 by GSNO was the key to attenuating EAE disease, while CD4⁺ T cells producing Th1 (IFN γ) or Th2 (IL4) immune responses were not affected.

The role of Th1 cells is controversial as adoptive transfer of these cells was unable to induce EAE in mice (Langrish et al. 2005) suggesting that IFN γ producing Th1 cells could be beneficial, an idea supported by the fact that IFN γ gene-deficient mice were more susceptible to EAE than wild-type littermates (Ferber et al. 1996). In opposition to these studies it was reported that adoptive transfer of Th1 cells induce EAE with higher infiltration of CD11b positive macrophages, whereas Th17 cells induced EAE was associated with more accumulation of Ly6G positive neutrophils in the CNS (Kroenke et al. 2008). Our results suggest that inhibition of T-bet in the presence of Th1 skewed conditions (IL12p70; including both p35 and p40 subunits) upon CD3/28 stimulation may be because the p40 subunit is common in IL12 and IL23. We did not observe any inhibition of T-bet with IL12p35 alone suggesting that Th1 cells generated in the presence of total IL12p70 may not be able to reflect the true pure Th1 population and still has Th17 cells due to the presence of p40 subunit.

NO has been reported to modulate immune functions with contrasting effects: either up- or down-regulating proliferation, inducing glucose uptake, cytokine induction, and intracellular signaling in T lymphocytes (Lander et al. 1993; Bogdan 1998; Williams et al. 1998; Allione et al. 1999; Bogdan 2001a; van der Veen 2001). The benefits of NO can be extrapolated in studies with iNOS-deficient animals where these mice succumbed to severe autoimmune diseases (EAE, myasthenia gravis) compared to wild-type littermates, suggesting that NO release can control inflammation and autoimmune diseases mediated by T cells (Bogdan 1998; Fenyk-Melody et al. 1998; Shi et al. 2001; Dalton and Wittmer 2005). We have investigated the use of a NO donor (GSNO) that releases NO for nitrosylation of proteins rather than nitration observed with peroxynitrite (ONOO). Recently, nitrosylating mechanisms have been reported to regulate critical cellular events such as activation of NF- κ B (Moilanen and Vapaatalo 1995; Pannu and Singh 2006; Prasad et al. 2006). We have also shown that GSNO regulates NF- κ B activation via S-nitrosylation of p65 which limits its binding ability to consensus sequence, thereby inhibiting nuclear translocation and the expression of adhesion molecules and subsequent transmigration of inflammatory immune cells which, in turn provides protection from inflammation and demyelination in the CNS during EAE disease (Khan et al. 2005; Khan et al. 2006; Prasad et al. 2006). Compared to other NO donors our studies document that GSNO does not hamper T cell activation or cytokine (Th1/Th2) generation either in vitro or in vivo. These results were in contrast to previous reports where NO donors [S-nitroso-N-acetyl-penicillamine (SNAP) or sodium nitroprusside (SNP)] inhibited both Th1 (IFN γ , IL2) and Th2 (IL4, IL5, and IL10) responses in human T cells (Bauer et al. 1997) and induced IFN γ without affecting Th2 cytokines in mice T cells (Taylor-Robinson et al. 1994; Niedbala et al. 1999).

Previously, we had reported that GSNO protects against focal cerebral ischemia by reducing inflammation compared to other NO donors (Khan et al. 2005). Structurally different NO donors releasing NO either as a free radical (NO $\cdot$) or as an NO ion (NO⁺/NO⁻) have been shown to reduce oxidative stress/inflammation and increase cerebral blood flow (Li and Forstermann 2000; Cirino et al. 2003; Khan et al. 2006). GSNO was a potent neuroprotective compared to other NO donors such as SNAP, SNP, methylamine hexamethylene methylamine

NONOate (MAHMA), propylamine propylamine NONOate (PAPA), and 3-morpholinodisodnonimine (SIN-1). GSNO decreased the formation of nitrotyrosine, lipid peroxidation in blood and increase the ratio of reduced versus oxidized glutathione (GSH/GSSG) in brain (Chiueh 1999; Chiueh and Rauhala 1999; Khan et al. 2005; Khan et al. 2006; Prasad et al. 2006). These studies clearly showed that the immediate release of NO was not as effective as slow release of NO⁻, GSNO is a stable NO modulator and formed during the oxygen dependent oxidation of NO in the presence of GSH (Singh et al. 1996; Hogg 2000; Megson 2000; Jourdain et al. 2003; Schrammel et al. 2003). It is present in high concentrations in rat blood as well as brain and regulates nitrosylation/denitrosylation under physiological conditions (Kluge et al. 1997). It protects the brain by reducing inflammation in a rat model of experimental stroke (Khan et al. 2005). Additionally, GSNO is a more potent antioxidant than GSH against ONOO⁻ and HO[•] (Rauhala et al. 1998). NMDA receptor activity is also down regulated by GSNO (Li and Forstermann 2000). Recently, we have shown that the neuroprotective action of NO donors in experimental stroke depends on their ability to reduce oxidative stress both in brain and blood (Khan et al. 2006).

GSNO-mediated nitrosylating NO effects requires an enzyme (reductase) or a thioredoxin system for its metabolism to NO, GSH, or GSSG (Singh et al. 1996; Megson 2000). Endogenous NO is involved in IL12 signaling as its regulation was impaired in iNOS-deficient mice (Diefenbach et al. 1999). Our results are in agreement with these observations as GSNO is an endogenous physiological regulator of NO and may enhance IL12 signaling, a key cytokine for Th1 pathway, and because it was unable to inhibit the production of IFN γ . Furthermore, we report that phosphorylation of STAT1 and STAT4 was not altered in the presence of GSNO (up to 100 μ M) under Th1 skewed conditions suggesting that GSNO did not interfere with Th1 pathway mediated by IL12 signaling.

GSNO efficiently assists in CD4⁺ T cell differentiation either to Th1 or Th2 by maintaining the expression of T-bet or GATA3 responsible for effective Th1 or Th2 immune responses. The attenuation of EAE disease by GSNO has been observed in both the relapsing–remitting and chronic form of disease while controlling the inflammatory sequelae in EAE by targeting the IL17 generating pathways. IL6/TGF β is required for the differentiation of Th17 cells (Harrington et al. 2005; Mangan et al. 2006) whereas IL23 is needed for the maintenance of Th17 phenotype (Mangan et al. 2006); however, GSNO can inhibit both differentiation and maintenance of these cells by blocking the production of IL17 at early (36 h) as well as late (72 h) time points in the presence of IL6/TGF β or IL12/23p40 homodimer.

In summary, this study describes for the first time the immune regulation of CD4⁺ T cells in EAE by a compound which is physiologically available in the mammalian cells. The attenuation of EAE disease by GSNO-mediated preferential down-regulation of Th₁ or Th17 cells but independent of Th1 and Th2 immune responses documents the potential of GSNO therapeutic by itself or in combination with existing drugs for the treatment of autoimmune diseases mediated by Th17 cells like MS and rheumatoid arthritis.

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Fig. 1.

GSNO inhibits clinical disease of EAE. Paralysis in mice were assessed based on clinical symptoms after active immunization with PLP₍₁₃₉₋₁₅₁₎ or MOG₍₃₅₋₅₅₎ myelin peptides in SJL (A and C) or C57BL/6 (B) mice and treated with GSNO (0.5 or 1.0 mg/kg) daily from day 0 of immunization for prophylactic (A and B) and day 13 for therapeutic (C) efficacy. Mean disease score for EAE- and GSNO-treated mice plotted in A (EAE; *n*=15, GSNO; 0.5 or 1.0 mg/kg, *n*= 16 each dose), B (EAE; *n*=10, GSNO; 0.5 or 1.0 mg/kg, *n*=11 each dose), and C (EAE; *n*=9, GSNO; 1.0 mg/kg, *n*=10) at each time point. D–J Spleen cells from untreated EAE and GSNO-treated mice were stimulated ex vivo with PLP₍₁₃₉₋₁₅₁₎ (5 μg/ml), MOG₍₃₅₋₅₅₎ (25 μg/ml), or killed *M. tuberculosis* H37RA (10 μg/ml) for 72 h. For recall responses spleen cells

were stimulated with PLP_(139–151) or MOG_(35–55) peptides from SJL (**D**) or C57BL/6 (**G**) mice, respectively, thymidine [³H] (1 µCi/well) was added at 48 h and further incubated for 24 h and uptake of radioactivity was measured at 72 h. Production of IL17 (SJL, **E** or C57BL/6, **H** and **I**) and IFN γ (SJL, **F** or C57BL/6, **J**) from antigen-specific T cells were measured by ELISA after 72 h of incubation. Data are represented from all the mice used (Table 1) in each experiment where **A** $P < 0.01$ EAE vs GSNO 1.0 mg/kg, **B** $p < 0.01$ EAE vs GSNO 1.0 mg/kg, $p < 0.001$ EAE vs GSNO 0.5 mg/kg. **C** $p < 0.01$ EAE vs GSNO 1.0 mg/kg. **E** and **I** IL17 production $p < 0.0001$ compared EAE vs GSNO treated

Fig. 2. GSNO limit infiltration of CD4⁺ T cells in spinal cord. Active EAE was induced in SJL mice and GSNO was given as described in legend for Fig. 1. Spinal cords (s.c.) (lumbar regions) were harvested from animals in EAE (A), treated with GSNO, 0.5 mg/kg (B), or 1.0 mg/kg (C) on day 17 after active EAE induction. Fixed (10% buffered formalin) s.c. sections were stained with H&E and visualized at $\times 10$ to determine infiltration. A, B, or C Infiltrated cells were shown in artery regions of spinal cord. For CD4⁺ T cells s.c. sections were stained with anti-CD4⁺ mouse polyclonal IgG antibody and reaction was developed with secondary biotinylated anti-mouse IgG followed by an avidin-biotin HRP complex with DAB as substrate. Sections of s.c. show CD4⁺ T cells infiltration in EAE (D) and treated with GSNO

0.5 (**E**) or 1.0 mg/kg (**F**). Quantitations of areas with infiltrates are shown in (**G**). Representative sections from five different animals are shown

Fig. 3.

Th1 immune responses with GSNO. **A–H** Total CD3 positive T cells isolated from spleens of SJL or C57BL/6 mice were stimulated (at 2×10^6 cells/ml) with anti-CD3/28 antibodies in presence or absence of GSNO (1 to 400 μ M). Supernatants from cultured cells of **A** SJL or **B** C57BL/6 mice were collected at 48 h for IFN γ measurements. **C, D, E, F, and G** For phosphorylation of STAT4, STAT1, and expression of T-bet in T cells (from SJL mice) were stimulated as above under normal and Th1 skewed (rmIL12; 10 ng/ml) conditions for 24 h (pSTAT4 or pSTAT1) or 48 h (T-bet). **H** T cells were stimulated with rmIL12p35 (10 ng/ml) and GSNO (100–400 μ M) for 36 h and analyzed for T-bet. Cells were harvested in all culture conditions and subjected to western blot analysis. Blots were stripped and re-probed with total STAT4 or β -actin for equal loading. Data are representative of two independent experiments

Fig. 4.

Th2 immune responses with GSNO. **A–F** Total CD3 positive T cells were isolated and were stimulated as described previously. Supernatants **A** SJL and **B** C57BL/6 mice were collected at 96 h for IL4 measurements. T cells were stimulated with CD3/28 for phosphorylation of STAT6 (24 h) and expression of GATA3 (48 h) under normal and Th2 skewed (rmIL4; 10 ng/ml) conditions. **C, D, E,** and **F** showing western blot of pSTAT6 and GATAT3 under normal or Th2 conditions. Blots of STAT6 or GATA3 were stripped and re-probed with total STAT6 or β -actin as above. Data are representative of two independent experiments

Fig. 5.

Th17 immune responses with GSNO. **A–I** Total CD3 positive T and spleen cells were isolated from C57BL/6 mice and were stimulated as described in “Materials and methods”.

Supernatants were collected at 36 h (**A, B**) and 72 h (**C, D, E, and F**) for IL17 measurements under normal and Th17 conditions. Total CD3 positive T cells from B6 mice were stimulated with CD3/28 (1 µg/ml) for phosphorylation of STAT3 (24 h) and expression of RORγ (48 h) under normal and Th17 conditions (rmIL23; 10 ng/ml or rmIL6+TGFβ; 10 ng/ml) the blots are shown for pSTAT3 (**G**) and RORγ (**H**) in CD3 positive T cells. **I** The spleen cells from EAE mice were stimulated with MOG_(35–55) (25 µg/ml) under normal or Th17 conditions (rmIL6+TGFβ) and after 24 h the cells were analyzed for phosphorylation of STAT3. Blots were stripped and re-probed with total STAT3 and β-actin in case of RORγ. Data are representative of two independent experiments

Table 1

The active EAE was induced in SJL or C57BL/6 mice with PLP₍₁₃₉₋₁₅₁₎ or MOG₍₃₅₋₅₅₎ peptides, respectively

	Number of mice	Incidence	% incidence	Peak day	Peak score
SJL mice EAE	15	15/15	100	17	2.69±0.25
SJL mice immunized+GSNO treated (0.5 mg/kg)	16	16/16	100	17	2.5±0.22
SJL mice immunized+GSNO treated (1.0 mg/kg)	16	16/16	100	18	1.75±0.21***
C57BL/6 mice EAE	10	10/10	100	19	3.2±0.25
C57BL/6 mice immunized+GSNO treated (0.5 mg/kg)	11	11/11	100	21	1.6±0.35***
C57BL/6 mice immunized+GSNO treated (1.0 mg/kg)	11	11/11	100	21	2.6±0.24**
Therapeutic EAE SJL mice	9	9/9	100	15	2.93±0.27
Therapeutic EAE+GSNO treated (1.0 mg/kg)	10	10/10	100	16	2.4±0.18

GSNO was given at 0.5 or 1.0 mg/kg daily by oral gavages. Total incidence and percent of incidence are reported from all experiments. Mean maximum score are given at the peak of disease