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Regulation of IL-10 and IL-17 mediated experimental autoimmune encephalomyelitis by S-nitrosoglutathione

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Abstract

In this study, we investigated IL-10 and IL-17 specific immunomodulatory potential of S-nitrosoglutathione (GSNO), a physiological nitric oxide carrier molecule, in experimental autoimmune encephalomyelitis (EAE). In active EAE model, GSNO treatment attenuated EAE severity and splenic CD4⁺ T cells isolated from these mice exhibited decreased IL-17 expression without affecting the IFN- γ expression compared to the cells from untreated EAE mice. Similarly, adoptive transfer of these cells to naive mice resulted in reduction in IL-17 expression in the spinal cords of recipient mice with milder EAE severity. CD4⁺ T cells isolated from GSNO treated EAE mice, as compared to untreated EAE mice, still expressed lower levels of IL-17 under T_H17 skewing conditions, but expressed similar levels of IFN- γ under T_H1 skewing condition. Interestingly, under both T_H17 and T_H1 skewing condition, CD4⁺ T cells isolated from GSNO treated EAE mice, as compared to untreated EAE mice, expressed higher levels of IL-10 and adoptive transfer of these T_H17 and T_H1 skewed cells seemingly exhibited milder EAE disease. In addition, adoptive transfer of CD4⁺ T cells from GSNO treated EAE mice to active EAE mice also ameliorated EAE disease with induction of spinal cord expression of IL-10 and reduction in of IL-17, thus suggesting the participation of IL-10 mechanism in GSNO mediated immunomodulation. GSNO treatment of mice passively immunized with CD4⁺ T cells either from GSNO treated EAE mice or untreated mice further ameliorated EAE disease, supporting efficacy

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Conflicts of interest

We confirm there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced the outcome.

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of GSNO for prophylaxis and therapy in EAE. Overall, these data document a modulatory role of GSNO in IL-17/IL-10 axis of EAE and other autoimmune diseases.

Graphical Abstract

Keywords

Experimental autoimmune encephalomyelitis (EAE); IL-10; IL-17; S-nitrosoglutathione (GSNO); T_H1 ; T_H17 ; adoptive transfer

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T helper (T_H) cell mediated autoimmune disease of central nervous system (CNS) that serves as an animal model of multiple sclerosis (MS) [20]. The disease is characterized by differentiation and expansion of myelin specific immune cells, their infiltration into the CNS, and chronic encephalitogenic inflammation leading to damage to myelin, oligodendrocytes, and axons [4]. Earlier, $IFN-\gamma$ producing T_H1 cells were believed to be solely responsible for the initiation and progression of MS and EAE [19, 27]. However, there is now a general consensus that IL-17 producing T cells (T_H17) are also involved in the onset and progression of EAE [7, 16, 18]. Moreover, recent studies have also described an important role for IL-10 producing regulatory T cells (Tregs) in regulation T_H1 and T_H17 mediated immune responses in EAE disease [9, 11].

There is a growing body of evidence that nitric oxide (NO) plays an important role in regulation of cellular processes involved in immune and inflammatory responses [2]. Cellular NO is synthesized by three distinct forms of NO synthases (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [10]. NO is reported to play an inhibitory role in differentiation of T_H17 cells and their release of IL-17 [12, 22]. Accordingly, genetic ablation of iNOS (but not nNOS and eNOS) increased the severity of EAE disease via inducing T_H17 cells [12]. NO was also reported to induce a specific subset of Treg cells, called NO-Treg, that reduce EAE disease via inducing the release of IL-10 [23].

At the molecular level, NO exerts its physiological activities by interacting with and modulating the activities of enzymes containing iron-sulfur centers (e.g. guanylyl cyclase) [5] and/or by secondary modification of protein thiols directly or by low-molecular-weight-nitrosothiol mediated trans-S-nitrosylation [29]. S-nitrosoglutathione (GSNO), the most

abundant cellular low-molecular-weight-nitrosothiol, is formed by a redox based reaction between NO and glutathione (GSH) [29]. GSNO has been identified as a potential carrier and reservoir of NO [29] and is known to inhibit platelet activation [8] and inflammatory processes [24, 25], and is also reported to induce cardiovascular protection [17].

We previously evaluated the immuno-modulatory efficacy of GSNO in different EAE models and reported prophylactic and therapeutic efficacy of GSNO against the clinical disease of EAE [22, 26]. GSNO inhibited the IL-6-induced STAT3 activation (Tyr⁷⁰⁵ phosphorylation) by S-nitrosylation of the STAT3 protein on Cys²⁵⁹ [14] and also downregulated the IL-6 and TGF- β induced expression of ROR γ t, a T_H17 cell specific transcription factor [22]. Additionally, GSNO treatment also inhibited the T_H17 cell polarization induced by IL-6 and TGF- β and their effector function induced by IL-23 in in vitro cell culture conditions, but without significant changes in T_H1 (IFN- γ) and T_H2 (IL-4) immune responses, suggesting GSNO as a potential T_H17 specific immune-modulatory agent under EAE conditions [22]. Previous studies from our laboratory and others have described that GSNO directly modulates the activities of proinflammatory transcription factors, such as NF- κ B, AP-1, and STAT3 [6, 26, 31] and thus modulates gene expression for various proinflammatory effectors, such as iNOS, ICAM-1, and VCAM-1 [6, 13, 15, 26, 31]. It is also of interest to note that some of these transcription factors (e.g. NF- κ B and AP-1) play a role in IL-17 mediated proinflammatory processes [30]. These studies, therefore, indicate that GSNO-mediated mechanisms effectively modulate both differentiation and effector function of T_H17 cells and thus suggest its potential for prophylactic and therapeutic intervention of EAE and other autoimmune diseases.

In this study, we report that GSNO modulates autoimmune responses in EAE by specifically targeting the T_H17 cells and by inducing IL-10 expression. This conclusion is supported by 1) splenic CD4⁺ T cells isolated from GSNO treated EAE animals expressed lower levels of IL-17, while did not exhibited any difference in IFN- γ expression compared to the cells from untreated EAE animals; accordingly, adoptive transfer of these cells to naïve mice exhibits milder disease with lesser expression of IL-17 in the spinal cord; 2) CD4⁺ T cells from GSNO treated EAE animals express higher levels of IL-10 under both T_H1 and T_H17 skewing conditions, than the cells from untreated EAE animals, and adoptive transfer of both T_H1 and T_H17 skewed cells exhibited milder EAE disease; 3) Adoptive transfer of CD4⁺ T cells from GSNO treated EAE mice to active EAE mice ameliorated active EAE disease with induction of spinal cord expression of IL-10 and reduction in of IL-17; 4) GSNO treatment of mice passively immunized with CD4⁺ T cells either from GSNO treated EAE mice or untreated mice further ameliorated EAE disease.

2. Materials and methods

2.1 Mice

Female SJL and C57BL/6 mice, purchased from the Jackson Laboratory (Bar Harbor, ME), were housed in the animal care facility of Medical University of South Carolina and received standard laboratory food and water *ad libitum*. Paralyzed mice were provided with Transgel (Charles River Laboratories, Wilmington, MA) as an alternate food/water source. All animal protocols were in accordance with the animal experiment guidelines of the Medical

University of South Carolina and National Institute of Health and accepted by Institutional Animal Care and Use Committee in Medical University of South Carolina (Approved number: AR#1644).

2.2 Induction of EAE disease

EAE disease was induced in 8- to 12-week-old female SJL or C57BL/6 mice by immunization with proteolipid protein peptide (PLP_{139–151}; Peptide International, Louisville, KY) or MOG_{35–55} peptide (MOG_{35–55}; 200ug; Peptide International) emulsified (1:1) in 100ul complete Freund's adjuvant (CFA) on day 0 and day 7. Additionally, 300 ng of Pertussis toxin (Sigma-Aldrich, St Louis, MO) was given on day 0 and day 2 by i.p. injection. Pertussis toxin used as per the standardized protocol reported by us and other investigators for the induction of EAE [21]. On the day of immunization, one group of mice received 100 µl phosphate buffered saline (PBS) and the second group of mice received daily GSNO (1.0 mg/kg, 100 µl/PBS) via oral route. GSNO was purchased from World Precision Instruments (Sarasota, FL) and its concentration was adjusted spectrophotometrically at 334 nm. Individual animals were observed daily for clinical disease severity by an investigator, blinded to experimental treatments, 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 [22].

2.3 Ex vivo culture of T cells and characterization of CD4⁺ T cell lineages

At the peak of EAE disease, the mice were sacrificed and CD4⁺ T cells were purified from spleens by CD4⁺ T cell isolation kit (Miltenyi, Auburn, CA). The purified T cells (2.5×10^6 cells/ml) were cultured in 96-well round-bottom microculture plates (Falcon Labware, Oxnard, CA) in RPMI-complete media containing RPMI 1640 (Life Technologies, Gaithersburg, MD), 10% FBS, and 100 µg/ml streptomycin and penicillin (Atlanta Biologicals Norcross, GA), 1 mM glutamine, 1 mM nonessential amino acids, and 50 µM 2-mercaptoethanol (Sigma-Aldrich).

For skewing of different CD4⁺ T cell subsets and their expansion, the isolated CD4⁺ T cells were stimulated with PLP_{139–151} (5 µg/ml) with IL-2 (10ng/ml) for T_H0, IL-2 (10ng/ml), rhIL12p35 (10ng/ml), and anti-IL-4 (1µg/ml) for T_H1, or rmlL12/23p40 homodimer (10ng/ml), anti-IFN-γ (1µg/ml), anti-IL-4 (1 µg/ml) for T_H17. All cytokines and antibodies were purchased from BD Biosciences (San Diego, CA). Following stimulation, the cells were harvested for adoptive transfer of EAE disease and the culture supernatants were collected for analysis of IFN-γ, IL-17, and IL-10 expression by ELISA (BioLegend Cat# 430802, 432505, and 431411; San Diego, CA).

2.4 Adoptive transfer model of EAE

Cultured T cells ($20\text{--}30 \times 10^6$ T cells in 300 µl RPMI media per mouse) were injected to naïve female SJL or C57BL/6 mice (8–12 week old) via intraperitoneal route. The recipient mice were also given two doses of pertussis toxin (200ng/300 µl of PBS/i.p.) on day 0 and 2 of post immunization. Clinical EAE disease was measured as described above.

2.5 Statistical analysis

Clinical disease scores are presented as average maximal scores over the treatment period (mean + SD) and analyzed using a nonparametric Kruskal-Wallis test. Statistical significance was set at 0.05. Statistics for proliferation and cytokine responses were analyzed with a one-way multiple-range analysis of variance (ANOVA). All analyses were conducted 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.

3. Results and discussion

Previously, we reported prophylactic and therapeutic efficacy of GSNO in chronic (C57BL/6 mice immunized with MOG₃₅₋₅₅) and relapsing-remitting models (SJL mice immunized with PLP₁₃₉₋₁₅₁) of active EAE [22]. In both models, GSNO was reported to attenuate the EAE disease by inhibiting STAT3/ROR γ t and thus T_H17 specific immune responses, but without altering T_H1 (STAT4/T-bet) and T_H2 (STAT6/GATA3) specific immune responses. Moreover, in ex vivo and in vitro T cell culture studies, GSNO treatment specifically inhibited IL-6 and TGF- β induced polarization and expansion of T_H17 cells and their effector function (IL-17 production) induced by IL-23 [22], suggesting a role for GSNO mediated mechanisms in modulation of differentiation, expansion, and effector functions of T_H17 cells. Recently, we further reported the effect of GSNO as well as N6022 (an inhibitor of GSNO degrading enzyme GSNO-reductase) in induction of IL-10 via inducing CD4⁺ CD25⁺ FOXP3⁻ specific subset of regulatory T (T_{reg}) cells [28]. At present, however, the role of T_{reg}/IL-10 in IL-17/T_H17 mediated immuno-pathobiology in EAE is not well understood.

In the present study, we provide further evidence supporting the role of GSNO in inhibition of T_H17 cell differentiation and effector function by using murine passive-immunization model of EAE. Using this model, we investigated the regulatory role of GSNO in differentiation and effector functions of T cells associated with EAE disease. SJL mice were immunized with PLP₁₃₉₋₁₅₁ peptide and treated with 1.0 mg/kg GSNO (denoted as “GSNO +EAE group” hereafter) or the same volume of PBS (denoted as “EAE group” hereafter) daily starting on the day of immunization. At the peak of EAE disease (~ day 10 post immunization), CD4⁺ T cells were isolated from the spleens of the mice in both groups (EAE and GSNO+EAE). The isolated T cells were cultured in ex vivo in the presence or absence of PLP₁₃₉₋₁₅₁ peptide, then, lineage specific activation of T cells (T_H17 vs. T_H1) was analyzed for media levels of T_H1 (IFN- γ) and T_H17 (IL-17) cytokines. Figure 1A shows that T cells isolated from the EAE group or GSNO+EAE group produced comparable levels of IFN- γ in response to PLP₁₃₉₋₁₅₁ stimulation. However, T cells isolated from GSNO+EAE group produced significantly lower levels of IL-17 as compared to those from T cells isolated from the EAE group.

To evaluate the role of GSNO-mediated mechanisms in immune responses of EAE, ex vivo cultured T cells were re-stimulated with PLP₁₃₉₋₁₅₁ peptide and then adoptively transferred to naïve SJL mice as passive immunization. Development of EAE disease was assessed by daily evaluation of mean clinical score. Figure 1B shows that the mice passively immunized

with T cells from GSNO+EAE group exhibited significantly delayed and milder disease than the mice passively immunized with T cells from EAE group. Next, T_H17 vs T_H1 lineage specific expressions of IL-17 vs. IFN- γ were investigated in spinal cords of the passively immunized EAE mice. Figure 1C shows that the mice passively immunized with T cells from EAE group and GSNO+EAE group expressed comparable levels of IFN- γ in the spinal cords. However, the mice passively immunized with T cells from GSNO+EAE group expressed significantly lower levels of IL-17 than the mice passively immunized with T cells from EAE group (Fig. 1C). These data, along with the data from actively-immunized EAE model [22], indicate that GSNO mediated attenuation of EAE disease is mediated via modulation of T_H17 differentiation without altering the T_H1 lineage cell function (IFN- γ).

To further evaluate the role of GSNO in lineage specific inhibition of T_H1 vs. T_H17 differentiation during the immunization, T cells isolated from EAE or GSNO+EAE group of mice were skewed under T_H1 (IL-2, *rhIL*-12p35, and anti-IL-4) or T_H17 (IL-2, *rhIL*-12/23p40, anti-IFN- γ 1, and anti-IL-4) cytokine conditions for lineage specific expansion in the presence or absence of PLP_{139–151} peptide. Figure 2A-i shows that T cells isolated from EAE and GSNO+EAE groups produced comparable amounts of IFN- γ , but not IL-17 under T_H1 skewing conditions. On the other hand, T cells isolated from EAE group produced higher levels of IL-17 than T cells isolated from GSNO+EAE group under T_H17 skewing conditions, while T cells from both groups did not produce any IFN- γ under the same T_H17 skewing conditions (Fig. 2B-i). T cells isolated from both EAE and GSNO +EAE groups produced similarly increased levels of GM-CSF, a non-lineage specific cytokine, under both T_H1 and T_H17 skewing conditions (Figs. 2A-i and B-i). Again, these data indicate a lineage specific inhibitory action of GSNO on T_H17 differentiation during the development of EAE disease. Interestingly, T cells isolated from GSNO+EAE group produced significantly higher amounts of IL-10 than the T cells isolated from EAE group under both T_H1 and T_H17 skewing conditions (Figs. 2A-i and B-i). Accordingly, adoptive transfer of both T_H1 and T_H17 skewed T cells from GSNO treated EAE mice expressing high levels of IL-10, as compared to T cells from untreated EAE mice, produced significantly milder EAE disease (Figs. 2A-ii and 2B-ii). IL-10 is an anti-inflammatory cytokine and its potential on the attenuation of EAE disease was shown in transgenic mice expressing IL-10 in T cells [1]. Therefore, this study, for the first time, reports the role of GSNO-mediated mechanisms in induction of IL-10 expression under both T_H1 and T_H17 skewing conditions and its potential participation in attenuation of EAE disease. IL-10 is known to inhibit immune responses mediated by both T_H1 and T_H17 cells [9, 11]. Therefore, GSNO-induced IL-10 production under T_H1 and T_H17 skewing conditions should inhibit effector functions of both T_H1 and T_H17 cells. However, GSNO inhibited only T_H17 pathway without affecting T_H1 pathway (Figs. 2A-i and B-i) and the underlying mechanism for GSNO mediated selective inhibition of T_H17 is not well understood at present.

NO induced IL-10 production via induction of specific lineage of regulatory T (Treg) cells was described previously [23]. These cells expressed cell surface markers for Treg (e.g. CD4 and CD25) but not FOXP3 and thus are distinguished from neutral and inducible Tregs (nTreg and iTreg; CD4⁺/CD25⁺/FOXP3⁺). In addition, these cells are different from Tr1 (CD4⁺/CD25⁺/FOXP3⁻) in that the induction of these is IL-10-independent [23]. These NO-

inducible CD4⁺/CD25⁺/FOXP3⁻ cells, coined as 'NO-Treg', had a potent immunomodulatory effect by producing anti-inflammatory IL-10 in the active EAE mouse model [23]. According to this report, GSNO-mediated mechanisms may contribute to inhibition of T_H17 immune response and EAE disease via inducing NO-Treg [23]. However, the observed induction of high levels of IL-10 by T cells from GSNO treated EAE mice under both T_H1 and T_H17 skewing conditions (Figs. 2A-i and B-i) documents the role of T_H1/T_H17 cell produced IL-10 in immunomodulation of EAE.

Next, we investigated immunomodulatory role of GSNO treated T cells in active EAE disease. For it, CD4⁺ T cells were isolated from MOG₃₅₋₅₅ immunized C57BL/6 mice, which were treated with GSNO (1mg/kg/day) starting on the day of disease onset (day14 post-immunization) and then adoptively transferred to active EAE mice on the day of disease onset (day14 post-immunization). Fig. 3A show that adoptive transfer of GSNO-treated CD4⁺ T cells (denoted as "GSNO-T_H" hereafter) to active EAE mice significantly attenuated the clinical signs of EAE disease. In addition, active EAE mice treated with GSNO-T_H cells, as compared to untreated active EAE mice, expressed higher levels of IL-10, lower levels of IL-17, and comparable levels of IFN- γ in spinal cords (Fig. 3B). Previous study reported that NO inhibits T_H17 response via inducing IL-10 production by activation of specific subset of CD4⁺ CD25⁺ FOXP3⁻ regulatory T cells, namely NO-Treg [23]. Recently, we also reported that treatment of EAE mice with GSNO or N6022, an inhibitor of GSNO catabolizing enzyme (GSNO reductase), also attenuated EAE disease via inducing IL-10 production by CD4⁺ CD25⁺ FOXP3⁻ T cells [28]. Therefore, we expect that IL-10 pathway plays critical role in T_H17 specific immunomodulation by GSNO treatment.

GSNO is known for its anti-inflammatory activity in various disease conditions (see review [6]). Under EAE conditions, GSNO was reported to inhibit CNS infiltration of peripheral immune cells via inhibiting endothelial expression of proinflammatory adhesion molecules (e.g. ICAM and VCAM) [26]. At molecular levels, GSNO is known to inhibit activities of a series of transcription factors (e.g. NF- κ B, AP-1, CREB, and STAT3) via S-nitrosylation mechanisms [6, 26, 31]. It is of interest to note that some of these transcription factors also play critical roles in IL-23 mediated T_H17 effector function (e.g. STAT3) [3] as well as IL-17 mediated inflammatory reaction (e.g. NF- κ B and AP-1) [30]. Therefore, GSNO may exert its efficacy on EAE disease not only via regulating the T cell differentiation, but also via regulating effector functions of polarized T cells and thus neuroinflammation. To further investigate the efficacy of GSNO on the T_H17 cell effector function in EAE disease, SJL naïve mice were passively immunized by adoptive transfer of T cells isolated from EAE or GSNO+EAE group and further received daily GSNO treatment during the course of the disease (Fig. 4A). Figure 4B shows that passive immunized mice with the T cells isolated from GSNO treated EAE mice, but without receiving GSNO during the disease, exhibited milder disease (solid triangles) and the disease severity was further reduced when these mice were treated with GSNO during the disease (cross markers). On the other hand, passive immunized mice with T cells from untreated EAE mice exhibited the severest EAE disease (solid diamonds) and GSNO treatment of these mice during the disease also reduced the EAE disease (blank squares). These observations indicate that GSNO-mediated modulation of T cell differentiation as well as T cell effector function participate in attenuation of EAE disease.

5. Conclusions

T_H1 /IFN- γ and T_H17 /IL-17 cells are primary effector T cells and cytokines involved in immune pathogenesis of EAE and MS. We previously reported role of GSNO-mediated mechanism in inhibition of T_H17 and induction of T_{reg} [22][28]. In this study, we further reports the role of GSNO in induction of IL-10 and its specific function on T_H17 cell mediated autoimmune response in EAE. Under ex vivo conditions, splenic $CD4^+$ T cells isolated from GSNO treated EAE mice exhibited decreased T_H17 response (IL-17) with unaltered T_H1 response (IFN- γ) even under T_H1 and T_H17 skewing conditions. Interestingly, $CD4^+$ T cells from GSNO treated EAE mice under both T_H1 and T_H17 skewing conditions express higher levels of IL-10, as compared to the cells from untreated EAE mice, and adoptive transfer of these cells to naïve mice resulted in milder disease, suggesting the role of IL-10 in modulation of EAE disease. Moreover, adoptive transfer of $CD4^+$ T cells from GSNO treated EAE mice to active EAE disease also resulted in amelioration of disease severity of active EAE mice with induction of IL-10 and reduction of IL-17, but without affecting IFN- γ . These data suggest that GSNO/IL-10 mechanism is specific to T_H17 (IL-17), but not T_H1 /IFN- γ , mediated immune pathogenesis of EAE.

In summary, we report, for the first time, that the GSNO mediated mechanisms induce expression of IL-10 by T_H1 and T_H17 polarized cells and that adoptive transfer of these cells, producing IL-10, to naïve mice or active EAE mice produces milder disease. The observed findings using EAE adoptive transfer model indicate that GSNO-mediated mechanisms in T_H1 or T_H17 cells downregulate the immune activity of these cell types by acquiring expression of IL-10 in addition to their respective signature cytokines (IFN- γ / T_H1 or IL-17/ T_H17).

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Highlights

- GSNO selectively inhibits T_H17, but not IFN- γ , mediated immune pathogenesis of EAE.
- GSNO induces IL-10 expression of both T_H1 and T_H17 skewed autoreactive T cells.
- Adoptive transfer of both GSNO treated T_H1 and T_H17 skewed cells resulted in milder EAE.
- Transfer of CD4⁺ T cells from GSNO-EAE mice to active EAE also alleviates EAE disease.
- GSNO has prophylactic and therapeutic efficacy against T cell mediated EAE disease.

Fig. 1. Effect of adoptive transfer of T cells isolated from EAE or GSNO treated EAE mice in development of passive EAE disease.

PLP₁₃₉₋₁₅₁ specific T cells isolated from spleens and lymph-nodes of EAE mice or GSNO treated EAE mice were cultured ex vivo and re-stimulated with PLP₁₃₉₋₁₅₁ (10μg/ml) under T_H0 condition (IL-2). A. For characterization of T_H1 vs. T_H17 differentiation, the media from cultured CD4⁺ cells from GSNO treated and untreated EAE mice were analyzed for IFN-γ or IL-17. B. The cultured T cells stimulated with PLP₁₃₉₋₁₅₁ were adoptively transferred to the naïve host SJL mice and the development of passive EAE disease was monitored daily by blinded investigators. C. At the peak of EAE disease, T cells were

isolated from the spinal cord and release of IFN- γ and IL-17 were analyzed by ELISA in the presence or absence of ex *vivo* PLP_{139–151} stimulation.

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Fig. 2. Development of passive EAE disease by adoptive transfer of T_H1 or T_H17 skewed T cells isolated from GSNO treated or untreated EAE mice.

T cells isolated from spleens and lymph-nodes of GSNO treated or untreated EAE mice were cultured under T_H1 (IL12p35, anti-IL-4, and anti-IL-17) (A) or T_H17 (IL12/23p40) (B) skewing conditions in the presence or absence of PLP₁₃₉₋₁₅₁ peptide. Then, release of IFN- γ , IL-17, IL-10, and GM-CSF were analyzed by ELISA (A-i and B-i). T_H1 and T_H17 skewed T cells were then adoptively transferred to naïve host mice to induce passive EAE disease and clinical disease scores were analyzed daily as described experimental procedure (A-ii and B-ii).

Fig. 3. Immuno-modulatory role of GSNO treated CD4⁺ T cells in active EAE disease. A. MOG₃₅₋₅₅ immunized EAE mice were treated with daily GSNO (1mg/kg/day/i.p.) starting at the day of disease onset (day 14) and CD4⁺ T_H cells were isolated from spleen at the peak of disease. The isolated CD4⁺ T_H cells were adoptively transferred to MOG₃₅₋₅₅ immunized active EAE mice at the day of disease onset and then clinical score of each mice was evaluated as described in Materials and Methods. **B.** At the peak of disease, expressions of IFN- γ , IL-17, and IL-10 were analyzed in the spinal cords of EAE mice.

Fig. 4. Effect of GSNO treatment in T cell differentiation and effector function in adoptive transfer EAE disease.

The PLP-immunized T cells from spleens and lymph-nodes of EAE mice or GSNO treated EAE mice were transferred to naïve SJL mice. On the day of passive immunization, the recipient mice were further treated vehicle (saline) or GSNO during the course of the disease (A). Following immunization, the severity of EAE disease was analyzed as described in materials and methods (B). Each group denotes saline treated recipient mice immunized with T cells from saline treated EAE mice (line with solid diamonds), GSNO treated recipient mice immunized with T cells from saline treated EAE mice (line with open squares), (c) saline treated recipient mice immunized with T cells from GSNO treated EAE

mice (line with solid triangles), or GSNO treated recipient mice immunized with T cells from GSNO treated EAE mice (line with cross marks).

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