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Regulation of B cell functions by *S*-nitrosoglutathione in the EAE model

Judong Kim [a,1], S.M. Touhidul Islam [a,1], Fei Qiao [b], Avtar K. Singh [b,c], Mushfiquddin Khan [a], Jeseong Won [b,**], Inderjit Singh [a,d,*]

[a] Department of Pediatrics, Medical University of South Carolina, Charleston, SC, USA
[b] Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC, USA
[c] Pathology and Laboratory Medicine Service, Ralph H. Johnson Veterans Administration Medical Center, Charleston, SC, USA
[d] Research Service, Ralph H. Johnson Veterans Administration Medical Center, Charleston, SC, USA

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ABSTRACT

B cells play both protective and pathogenic roles in T cell-mediated autoimmune diseases by releasing regulatory vs. pathogenic cytokines. B cell-depleting therapy has been attempted in various autoimmune diseases but its efficacy varies and can even worsen symptoms due to depletion of B cells releasing regulatory cytokines along with B cells releasing pathogenic cytokines. Here, we report that *S*-nitrosoglutathione (GSNO) and GSNO-reductase (GSNOR) inhibitor N6022 drive upregulation of regulatory cytokine (IL-10) and downregulation of pathogenic effector cytokine (IL-6) in B cells and protected against the neuroinflammatory disease of experimental autoimmune encephalomyelitis (EAE). In human and mouse B cells, the GSNO/N6022-mediated regulation of IL-10 vs. IL-6 was not limited to regulatory B cells but also to a broad range of B cell subsets and antibody-secreting cells. Adoptive transfer of B cells from N6022 treated EAE mice or EAE mice deficient in the GSNOR gene also regulated T cell balance (Treg > Th17) and reduced clinical disease in the recipient EAE mice. The data presented here provide evidence of the role of GSNO in shifting B cell immune balance (IL-10 > IL-6) and the preclinical relevance of N6022, a first-in-class drug targeting GSNOR with proven human safety, as therapeutics for autoimmune disorders including multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the CNS where autoreactive T and B cells migrate into the CNS through the blood-brain barrier (BBB) and cause inflammatory demyelination [1]. MS has long been considered primarily T-cell-mediated disease based on the findings of major histocompatibility complex class-II (MHC-II; HLA-DR2, critical for presentation of antigen to T cells) as the strongest genetic risk factor for MS [2], the implication of Th1 and Th17 cells and their cytokines (IFN-γ and IL-17) in the inflammatory demyelination in MS and experimental autoimmune encephalomyelitis (EAE; an animal model of MS) [3], development of EAE in B cell-deficient mice, but not in T cell-deficient mice [4,5], and initiation of EAE solely by adoptive transfer of myelin-reactive T cells into the naïve mice [6].

Recently, however, clinical trials of B cell depletion using humanized anti-CD20 monoclonal antibodies (mAbs) [7,8] provided evidence for B cell involvement in MS immunopathogenesis. B cells are known to play a pathogenic role in autoimmune responses beyond autoantibody production through mechanisms such as antigen presentation to T cells [9]. B cells also express effector/proinflammatory cytokines, such as IL-6, IFN-γ, and GM-CSF [10]. Among these, IL-6 is of major importance because B cells from MS patients secrete abnormally high levels of IL-6, and B cell-specific knockout of IL-6 decreases Th17 responses and diminishes EAE severity [11,12]. Moreover, B cell depletion therapy ameliorates MS symptoms by ablating IL-6-producing B cells [11]. At present, ocrelizumab, rituximab, and ofatumumab are approved for both relapsing and/or progressive forms of MS. However, these anti-CD20 mAb therapies provide only limited efficacy in MS patients [13] and no efficacy or adverse effect for patients with other autoimmune diseases, such as systemic lupus erythematosus [14], ulcerative colitis [15], and psoriasis [16], underscoring the need for drugs that efficiently target effector function of B cells for MS therapy.

* Corresponding author. 509 Children's Research Institute, 173 Ashley Avenue, Charleston, SC, 29425, USA.
** Corresponding author. 510 Children's Research Institute, 173 Ashley Avenue, Charleston, SC, 29425, USA.
E-mail addresses: wonj@musc.edu (J. Won), singhi@musc.edu (I. Singh).
[1] These authors have equal contributions to this work.

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Recent studies suggest that the observed inefficacy of anti-CD20 mAbs in autoimmune diseases may be attributed to the depletion of B cells playing regulatory/anti-inflammatory roles in addition to the depletion of B cells playing effector/pro-inflammatory roles [17]. In mouse models of EAE and systemic lupus erythematosus, B cell depletion during the early phase of the disease exacerbated the disease by depleting the regulatory B cells while the B cell depletion during the late phase of the disease attenuated the disease by depleting the effector B cells [18,19]. B cells are known to exert their regulatory functions by producing regulatory/anti-inflammatory cytokines, such as IL-10, IL-35, and TGF-β [10]. Among them, IL-10 has been under intense investigation as a potential target for MS [10,20]. In mice and humans, multiple populations of B cells are known to express IL-10. $CD24^{hi}$ $CD27^{hi}$ B cells [21] and $CD24^{hi}$ $CD38^{hi}$ immature B cells [22] in humans and $CD1d^{hi}$ $CD5^{hi}$ B cells [23] in mice are the best-characterized B cell subsets producing high levels of IL-10. B cell production of IL-10 is known to inhibit Th1 and Th17 cell immune responses and macrophage/dendritic cell-mediated antigen-presentation [18,24,25], as well as induction of T*reg* responses [25,26]. Accordingly, B cells expressing IL-10 are reported to be involved in the remission and recovery of EAE [5,27]. Taken together, these findings suggest that pan-B cell depletion by anti-CD20 mAb targeting the effector functions of B cells could be potentially harmful due to its simultaneous depletion of B cells playing regulatory functions. This, therefore, underscores the need for new therapies that selectively inhibit effector functions of B cells while sparing or promoting regulatory B cell functions.

Recently, our laboratory has reported a novel immunomodulatory mechanism regulated by cellular *S*-nitrosoglutathione (GSNO) homeostasis [28–32]. GSNO is a physiological endogenous nitric oxide (NO) carrier molecule and its cellular homeostasis is maintained by its synthesis from NO and glutathione (GSH) and its catabolism by GSNO reductase (GSNOR) [33]. Our laboratory has documented that both exogenous GSNO treatment or GSNOR inhibitor (N6022) treatment ameliorates EAE disease via shifting Th17 vs. Treg balance toward Treg [30–32]. IL-6 and IL-10 are known to play crucial roles in the regulation of Th17/Treg balance [25,34–36]. Therefore, we next investigated the role of GSNO-mediated mechanisms on effector (IL-6) vs. regulatory (IL-10) B cell functions using the EAE and related cell culture models. The studies described here document that exogenous supplementation of GSNO or endogenous increase in GSNO by inhibiting its catabolism using an inhibitor of GSNOR (N6022) down-regulate IL-6 associated effector B cell function while upregulating the IL-10 associated B cell regulatory function in EAE disease, a balanced immune regulation based potential therapy for MS.

2. Experimental procedures

2.1. Animals

C57BL/6 J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; cat. no. 000664). The GSNOR knockout ($GSNOR^{-/-}$) [37] mice were gifted by Dr. Shyam Biswal of The Johns Hopkins University. Mice were supplied with food and water *ad libitum* and kept in ventilated cages in specific pathogen-free animal care facility maintained by the Medical University of South Carolina throughout the entire study. They were housed at controlled temperature (22 °C), humidity (45–55%), and 12 h light/dark cycle. All animal studies were reviewed and approved by the Medical University of South Carolina's Institutional Animal Care and Use Committee (IACUC) (AR # 2019–00761).

2.2. Induction and evaluation of EAE

EAE was induced by the subcutaneous injection of female C57BL/6 J mice (8–12 weeks old) with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide emulsified in the complete Freund's adjuvant (CFA) as described in the kit instruction (Hook Laboratories, Lawrence, MA). One hundred ng of pertussis toxin (PTX) was injected intraperitoneally on days 0 and 1. Mice were weighed and assessed for clinical signs every day starting from day 0 through the day of experiment termination. EAE score was evaluated as follows: 0 = no clinical signs of disease; 0.5 = partial tail paralysis; 1 = limp tail or waddling gait with tail tonicity; 2 = waddling gait with limp tail (ataxia); 2.5 = ataxia with partial limb paralysis; 3 = full paralysis of one limb; 3.5 = full paralysis of one limb with partial paralysis of second limb; 4 = full paralysis of two limbs; 4.5 = moribund stage; 5 = death. Mice were treated with daily doses of a vehicle (100 μL of 10% dimethylsulfoxide in saline), N6022 (1 mg/kg body weight/ip; Axon Medcam LLC., Reston, VA, USA), or GSNO (1 mg/kg body weight/ip; World Precision Instruments, Sarasota, FL, USA) starting before the disease onset (day 9 of post-immunization).

2.3. ELISA

The concentration of cytokines in the serum or culture supernatants was measured by ELISA (enzyme-linked immunosorbent assay) using the kit for TNF-α (Mybiosource # MBS9135747, San Diego, CA, USA), IFN-γ (Mybiosource # MBS9135739), GM-CSF (Mybiosource # MBS9135768), IL-6 (Mybiosource # MBS9135728), and IL-10 (Mybiosource # MBS9135736).

2.4. Histological and immunohistological analysis

Mice were anesthetized and transcardially perfused first with phosphate-buffered saline (PBS) and then 4% paraformaldehyde in PBS as described previously [38]. The spinal cords were decalcified prior to histological examination. Cryosections (8 μm thick) obtained from the spinal cord lumbar area were used for Hematoxylin and Eosin (H&E) staining or immunostaining for B220 (Invitrogen, Carlsbad, CA USA; cat. no. 14045285). All digital images were taken using a BX-60 microscope equipped with a DP70 camera unit (Olympus, Tokyo, Japan).

2.5. Isolation of mononuclear cells from the CNS and spleen

Mononuclear cells in the CNS (brain and spinal cord) were isolated as described previously with modifications [31]. Mice were anesthetized and transcardially perfused with phosphate-buffered saline (PBS) to remove blood cells. Then, the brain and spinal cord were isolated. In EAE mice, B cells are highly enriched in the area between the meninges and neural tissue, thus the meningeal membrane was included for mononuclear cell extraction. Finely minced brain and spinal cord tissues were incubated with 2 ml digestion buffer containing 1 mg/ml collagenase D (Roche, Mannheim, Germany; cat. no. 11088866001) and 50 μg/ml DNAse I (Roche cat. no. 10104159001) in RPMI 1640 (ThermoFisher, Foster City, CA, USA; cat. no. 61870036) for 30 min at 37 °C. The digested tissues were gently mashed onto a 100-μm nylon mesh strainer and then the filtered cells were placed into a 15-ml conical tube and centrifuged at 500 *g* for 5 min at 4 °C. The pellet was resuspended in 4 ml of ice-cold 90% percoll, overlaid with 3 ml 60% percoll, 4 ml of 40% percoll, and 3 ml of Hanks' balanced salt solution (HBSS; Sigma-Aldrich, St-Louis, MO, USA; cat. no. H9269), and centrifuged at 500 *g* for 20 min with no brake. Following centrifugation, the layer of debris from the top of the tube (4 ml) was discarded, and then 6 ml of the interphase layer containing the mononuclear cells was placed in a new tube, added with 10 ml of HBSS, and centrifuged at 500 *g* for 10 min at 4 °C. The pellet at the bottom was washed with HBSS.

Splenocytes were isolated as described previously with modifications [31]. In brief, the spleen was placed in a sterile 70 μm cell strainer mesh in a Petri dish containing ice-cold HBSS and mashed with the syringe plunger. The cell suspension was then transferred into a 15 ml conical tube and washed twice with ice-cold HBSS at 500×*g* for 5 min at 4 °C. The pellet was re-suspended with 1 ml red blood cell (RBC) lysis buffer (Thermofisher cat. no. A1049201) and then washed with ice-cold HBSS and the splenocytes were spun down at 500 g for 5 min at 4 °C.

2.6. Mouse and human B cell cultures

Naïve B cells were purified from the spleen of C57BL6/J mice by positive selection with mouse anti-CD19 coated microbeads (Miltenyi Biotec, Auburn, CA, cat. no. 130-121-301) according to the manufacturer's instruction. The purified B cells (>95% $CD19^+$ cells) were resuspended in RPMI 1640 media containing 10% FBS, 1% antibiotics, L-glutamine (4 mM), and 2-mercaptoethanol (50 μM). B cells were then stimulated with lipopolysaccharide (LPS; 10 μg/ml; Sigma-Aldrich; *Escherichia coli* 0111:B4; cat. no. L2630) or *anti*-IgM (10 μg/ml of F (ab')2-goat anti-mouse IgM; Thermo Fisher cat. no. 16-5092-85) and co-stimulated with anti-CD40 mAb (10μg/ml; FGK45; Enzo Life Science Farmingdale, NY; cat. no. ALX-805-046-C500) and recombinant BAFF (B-cell activating factor 100 ng/ml; R&D system, Minneapolis, MN, USA; cat. no. 8876-BF) for 48 h. The B cells were treated with GSNO (50μM; World Precision Instrument, Sarasota, FL, USA; cat. no. GSNO-100) or vehicle (PBS) 1 h before the stimulation/co-stimulation. Human blood B cells were purchase from Stemcell Technology Inc (Cambridge, MA, USA; cat#: 70,023) and cultured in RPMI 1640 media containing 10% FBS. The cells were stimulated with *anti*-IgM/IgG Ab (10 μg/ml; eBioscience# 16–509985) and costimulated with recombinant BAFF (100 ng/ml; R&D# 2149 B F) and human CD40 ligand (hCD40L; R&D system # 6245-CL-050) for 48 h in the presence or absence of GSNO pretreatment (50μM/1hr).

2.7. Fluorescence flow cytometry

Before immunofluorescence staining, all cells were cultured in complete RPMI 1640 medium (10% fetal bovine serum, 4 mM L-Glutamine, 200 μg/ml penicillin, 200 U/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol) containing phorbol 12-myristate 13-acetate (PMA; 50 ng/ml, Sigma-Aldrich cat. no. P8139), ionomycin (500 ng/ml; Sigma-Aldrich cat. no. I9657), and 1x brefeldin A/monensin (BD bioscience, San Jose, CA, USA; cat. no. 555029/554,724) for 5 h to ensure intracellular accumulation of cytokines. The cells were then incubated with mouse Fc blocker (BD Biosciences, cat. no. 553142) or human Fc blocker (Biolegend # 422,302) for 15 min and with surface antigen-specific antibodies for another 30 min at 4 °C in 100 μl of staining buffer (BD Biosciences, cat. no. 554656). The cells were then fixed with fixation buffer (BD Biosciences, cat. no. 554655) at 4 °C for 20 min, washed twice with Perm/Wash buffer (BD Biosciences, cat. no. 554723), and incubated with intracellular cytokines-specific antibodies for 30 min in Perm/Wash buffer at 4 °C (Please see supplementary material S1 for the list of antibodies against the cell surface antigens used for this study). The cells were then washed twice with stain buffer and subjected to flow cytometry using BD LSRFortessa™ Flow Cytometer (BD Bioscience). All flow cytometric data were analyzed using FlowJo software (Treestar, Ashland, OR, USA). The samples were gated to remove debris based on forward scatter (FSC) versus side scatter (SSC). Next, the resulted B cells were pre-gated with $B220^+$ and then gated with each subset specific cell surface antigens and/or IL-10 and IL-6.

2.8. Adoptive transfer of B cells

Female C57BL/6 J mice (8–12 weeks old) or $GSNOR^{-/-}$ mice were immunized with MOG_{35-55} peptide emulsified in the CFA as described above. A day after the immunization, control and MOG-immunized C57BL/6 J mice were received daily N6022 (1 mg/kg body weight/ip) treatment. On day 10 post-immunization, splenic B cells were isolated by B cell isolation kit mouse (Anti-CD19 MACS; Miltenyi Biotech # 130-121-301) from control and EAE mice treated/untreated with N6022 as well as $GSNOR^{-/-}$ EAE mice. The isolated B cells (2×10^6 cells) were then transferred to recipient EAE mice (MOG_{35-55}-immunized/PTX-treated) on day 10 post-immunization.

2.9. Statistical analysis

Statistical analysis was performed with Graphpad Prism 8. Values are expressed as mean ± standard error mean (SEM). Multiple comparisons were performed using one-way ANOVA followed by a Bonferroni test. Correlation assay of $IL\text{-}10^+$ and $IL\text{-}6^+$ B cells was performed with Graphpad Prism 8 b y nonlinear regression (curve fit) XY analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. GSNO and GSNOR inhibitor (N6022) reduce B cell infiltration into the spinal cord and ameliorate EAE disease

We earlier reported that exogenously supplemented GSNO and endogenously generated GSNO by inhibition of GSNOR activity using its reversible inhibitor N6022 ameliorated the disease in different models of EAE [28–32]. Similarly, we observed that treatment of MOG_{35-55} immunization-induced EAE mice with a daily dose of exogenous GSNO (1 mg/kg/day/i.p.) or N6022 (1 mg/kg/day/i.p.) starting one day before the onset of EAE disease (day 9 post-immunization) reduced the severity of EAE clinical disease observed by daily disease severity (Fig. 1A–i) and quantitative disease severity (area under the curves) (Fig. 1A–ii), and decreased the loss of body weight (Fig. 1B–i and ii). EAE mice showed increased blood levels of pro-inflammatory cytokines, such as TNF-α (Fig. 1C–i), IFN-γ (Fig. 1C–ii), GM-CSF (Fig. 1C–iii), and IL-6 (Fig. 1C–iv) as well as anti-inflammatory cytokine IL-10 (Fig. 1C–v), and either GSNO or N6022 treatment decreased the blood levels of the most pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, and IL-6) while increasing the IL-10 levels. However, the EAE-increased GM-CSF levels were not significantly affected by either GSNO or N6022 treatment (Fig. 1C–iii). GSNO or N6022 treatment also reduced the CNS infiltration of mononuclear cells (Fig. 1D) and B cells (Fig. 1E) into the spinal cord, especially near the area of the anterior median fissure (AMF) of lumbar spinal cords as shown by H&E staining and B220 immunostaining (Fig. 1D–i and 1E-i) and their quantifications (Fig. 1D–ii and 1 E-ii).

3.2. GSNO/N6022 treatments reduce CNS B cell-infiltration while inducing the proportion of $IL\text{-}10^+$ B cells and reducing the proportion of $IL\text{-}6^+$ B cells in the CNS

We next investigated the effects of GSNO and N6022 treatments on the infiltration of B cells into the CNS in EAE mice using fluorescence flow cytometry. As expected, EAE mice had increased infiltration of $B220^+$ B cells in the spinal cords (Fig. 2A–i) as well as the brains (Fig. 2B–i) which were significantly reduced by treatment with either GSNO or N6022. Next, we investigated the effects of GSNO or N6022 treatment on the proportions of $IL\text{-}10^+$ vs. $IL\text{-}6^+$ B cells in the CNS. Fig. 2A–ii and 2B-ii show that either GSNO or N6022 treatment increased the proportion of $IL\text{-}10^+$ cells among B cells ($B220^+$) in the spinal cords and the brains of EAE mice. Conversely, GSNO or N6022 treatment decreased the proportion of $IL\text{-}6^+$ cells (Fig. 2A–iii and 2B-iii). Accordingly, either GSNO or N6022 treatment restored the decreased ratio of $IL\text{-}10^+$ vs. $IL\text{-}6^+$ B cells close to the control levels in the spinal cords as well as the brain (Fig. 2A–iv and 2B-iv).

We next investigated the effect of N6022 on B cell subset-specific expression of IL-10 and IL-6. The EAE mice exhibited increased CNS (spinal cord + brain) infiltrations of B cell subsets, such as B1a, B1b, $CD1d^{hi}$ $CD5^{hi}$, and memory B cells (Fig. 2C–i). Among them, the CNS infiltration of $CD1d^{hi}CD5^{hi}$ and memory B cells are remarkable and account for approximately 35% and 41% of CNS B cells, respectively (Fig. 2C–i). It is of interest to note that $CD1d^{hi}$ $CD5^{hi}$ B cells are one of the major B cell subsets known to express higher levels of IL-10 (so-called B10 or regulatory B cells/*Breg*) [10]. On the other hand, memory B cells are known to secrete higher levels of IL-6 [39–41] and are associated

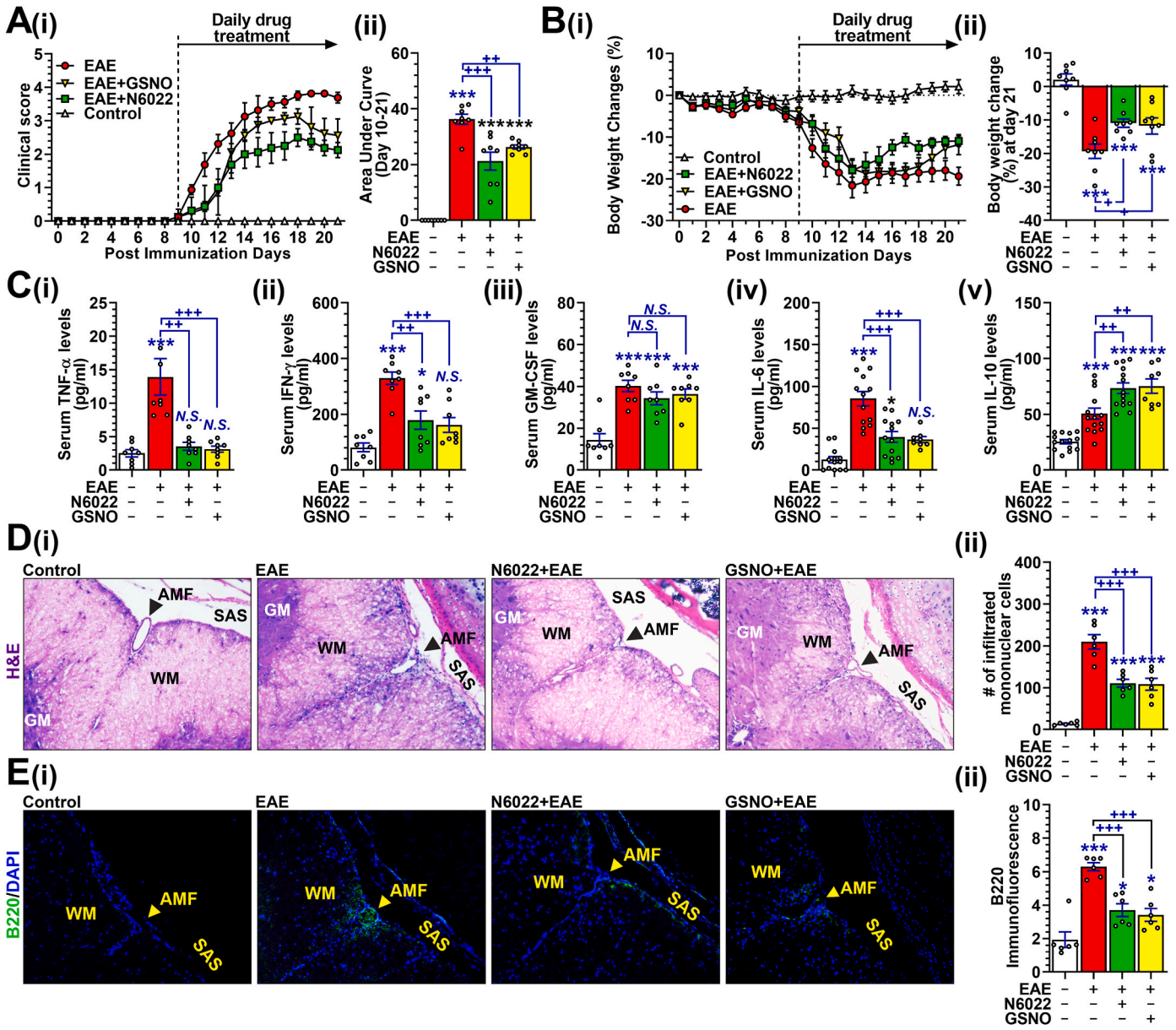

Fig. 1. GSNO and GSNOR inhibitor (N6022) inhibit EAE immunopathologies and decrease B cell infiltration into CNS. C57BL/6 mice were immunized with MOG_{35-55} for the induction of EAE and received a daily dose of saline, GSNO (1 mg/kg/day/ip), or N6022 (1 mg/kg/day/ip) starting before the onset of the disease (day 9 post-immunization). Daily clinical scores (**A-i**), area under each curve for quantitative measurement of clinical disease (**A-ii**), daily body weight changes (**B-i**), and statistic analysis of body weight changes on day 21 post-immunization (**B-ii**) of control mice, EAE mice (treated with saline), and EAE mice treated with GSNO or N6022 were analyzed. On day 21 post-immunization, blood levels of TNF-α (***C*-i**), IFN-γ (***C*-ii**), GM-CSF (***C*-ii**), IL-6 (***C*-iv**), and IL-10 (***C*-v**) were analyzed by ELISA. On day 21 post-immunization, spinal lumbar cords were analyzed for mononuclear cell infiltration (H&E staining; **D-i**) and the number of infiltrated cells (**D-ii**) were analyzed. In addition, the spinal cord sections were immunostained for B220 (B cell marker; **D-i**) and the B220 immunofluorescence was analyzed by Image J software. Abbreviations: AMF = anterior median fissure, GM = grey matter, SAS = subarachnoid space, WM = white matter. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^{+}$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

with neurodegeneration in the relapsing MS [42,43]. In contrast to B cells, the N6022 treatment increased the numbers of antibody-secreting cells (ASCs), such as plasmablasts and plasma cells, in the CNS of EAE mice (Fig. 2C–i). ASCs are reported to be one of the main sources of IL-10 in the spleen and draining lymph nodes of EAE mice [44,45].

Next, we analyzed the proportions of IL-10^{+} vs. IL-6^{+} B cells in each subset of B cells and ASCs. We observed that N6022 treatment increased the proportion of IL-10^{+} cells (Fig. 2C–ii), while decreasing the proportion of IL-6^{+} cells (Fig. 2C–iii), in all subsets of B cells and ASCs tested. These data indicate that the N6022/GSNO-mediated mechanism for the induction of IL-10 expression and the inhibition of IL-6 expression in the CNS B cells is not restricted to specific subsets of B cells or ASCs but across all B lineage cells including ASCs in the MOG_{35-55}-induced EAE model.

3.3. GSNO/N6022 treatments induce B cell expression of IL-10 but decrease IL-6 expression in the spleen of EAE mice

Next, we investigated the effect of GSNO/N6022 treatment on the splenic B cell expression of IL-10 vs. IL-6 under EAE conditions. Fig. 3A–i shows that EAE induction had no significant effect on the number of total B cells in the spleen and the proportion of IL-10^{+} cells in the splenic

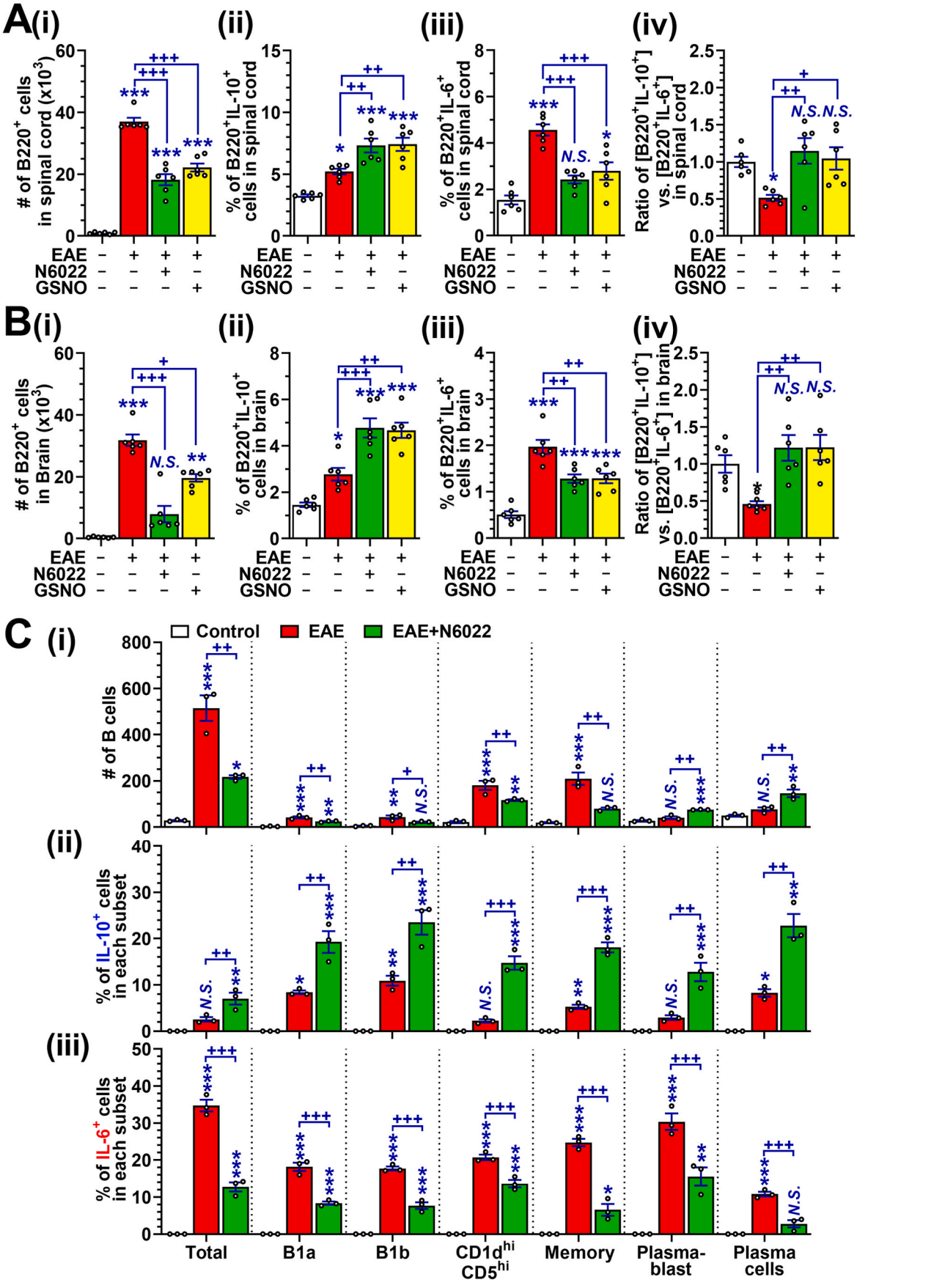

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Fig. 2. GSNO and GSNOR inhibitor (N6022) increase IL-10$^+$ B cells but decrease IL-6$^+$ B cells in the CNS of EAE mice. C57BL/6 mice were immunized with MOG_{35-55} for the induction of EAE and received a daily dose of saline, GSNO (1 mg/kg/day/ip), or N6022 (1 mg/kg/day/ip) starting before the onset of the disease (day 9 post-immunization). On day 21 post-immunization, mononuclear were isolated from the spinal cords (**A**) and the brain (**B**) and the number of B220$^+$ B cells (total B cells: **A-i** and **B-i**) and the proportions of IL-10$^+$ (**A-ii** and **B-ii**) and IL-6$^+$ cells (**A-ii** and **B-ii**) in the B220$^+$ B cells, and the ratio of IL-10$^+$ vs. IL-6$^+$ B cells (**A-iv** and **B-iv**) were analyzed by fluorescence flow cytometric analysis. Next, the B cells were isolated from the spinal cords and the brains, and numbers of total B cells (B220$^+$) and each subset of B-lineage cells, such as B1a, B1b, Breg (CD1d^{hi} CD5^{hi}), memory B cells, plasmablasts, and plasma cells, were analyzed (***C*-i**). Next, the proportions of IL-10$^+$ (***C*-ii**) and IL-6$^+$ (***C*-ii**) in total B cells (B220$^+$) and each subset of B-lineage cells were analyzed by fluorescence flow cytometry. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^+$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

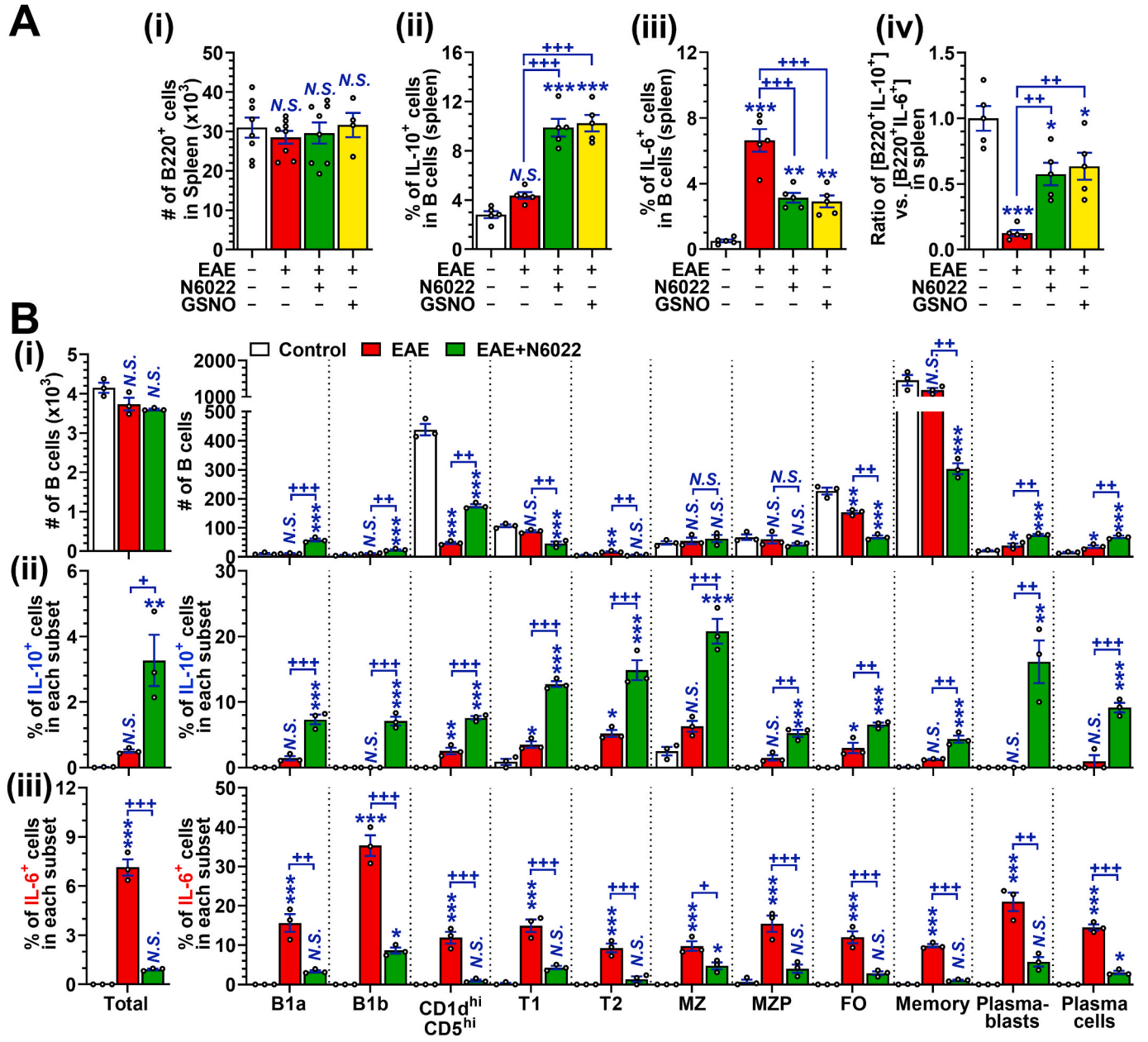

Fig. 3. GSNO and GSNOR inhibitor (N6022) increase IL-10$^+$ B cells but decrease IL-6$^+$ B cells in the spleen of EAE mice. C57BL/6 mice were immunized with MOG_{35-55} for the induction of EAE and received a daily dose of saline, GSNO (1 mg/kg/day/ip), or N6022 (1 mg/kg/day/ip) starting before the onset of the disease (day 9 post-immunization). On day 21 post-immunization, splenocytes were isolated and the number of B220$^+$ B cells (total B cells: **A-i**) and the proportions of IL-10$^+$ (**A-ii**) and IL-6$^+$ cells (**A-ii**) in the B220$^+$ B cells, and the ratio of IL-10$^+$ vs. IL-6$^+$ B cells (**A-iv**) were analyzed by fluorescence flow cytometric analysis. Next, the splenocytes from control, EAE, and N6022-treated EAE mice were analyzed for the numbers of total B cells (B220$^+$) and each subset of B-lineage cells, such as B1a, B1b, Breg (CD1d^{hi} CD5^{hi}), transitional B cells (T1 and T2), marginal zone (MZ) B cells, marginal zone precursor (MZP) B cells, follicular (FO) B cells, memory B cells, plasmablasts, and plasma cells, were analyzed (**B-i**). Next, the proportions of IL-10$^+$ (**B-ii**) and IL-6$^+$ (**B-ii**) in total B cells and each subset of B-lineage cells were analyzed by fluorescence flow cytometry. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^+$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

B cells (Fig. 3A-ii). However, EAE induction increased the proportion of IL-6^{+} cells in the splenic B cells (Fig. 3A-iii). Either N6022 or GSNO treatment of EAE mice increased the proportion of IL-10^{+} cells in the splenic B cells (Fig. 3A–ii) but reduced the proportion of IL-6^{+} cells (Fig. 3A–iii). Accordingly, N6022 and GSNO treatments reversed the decreased ratio of IL-10^{+} vs IL-6^{+} B cells in the spleen of EAE mice (Fig. 3A–iv).

To characterize B cell subsets expressing IL-10 and IL-6, B cells were purified from the spleen of control, EAE, and N6022 treated EAE mice, and the numbers of total and each subset of B cells were analyzed. As expected, we observed no significant changes in the number of total B cells (B220^{+}) in the spleens of EAE mice and N6022 treated EAE mice as compared to control mice (Fig. 3B–i). In control mice, memory B cells, CD1d^{hi} CD5^{hi} B cells, and follicular (FO) B cells were the most abundant B cell subtypes. The number of memory B cells was not significantly altered by the induction of EAE, but the numbers of CD1d^{hi} CD5^{hi} B cells and FO B cells were significantly decreased by the induction of EAE (Fig. 3B–i). N6022 treatment of EAE mice increased the numbers of B1a, B1b, and CD1d^{hi} CD5^{hi} B cell subsets, but decreased the type 1 and type 2 (T1 and T2) transitional B cell subsets and FO and memory B cell subsets (Fig. 3B–i). However, N6022 had no obvious effect on marginal zone (MZ) B cell and MZ precursor (MZP) B cell subtypes in EAE mice (Fig. 3B–i). Interestingly, induction of EAE increased the numbers of plasmablasts and plasma cells in the spleen and N6022 treatment of EAE mice further increased the cell numbers of these ASCs.

Next, we analyzed the proportions of IL-10^{+} vs. IL-6^{+} B cells in each subset of splenic B cells and ASCs. Similar to the observations in the CNS (Fig. 2C), N6022 treatment increased the proportion of IL-10^{+} B cells (Fig. 3B–ii), while reducing the IL-6^{+} B cells (Fig. 3B–iii) in all subsets of B cells and ASCs. These data again indicate that N6022 upregulates IL-10 expression while downregulating IL-6 expression in the splenic B cells and ASCs regardless of their subtypes.

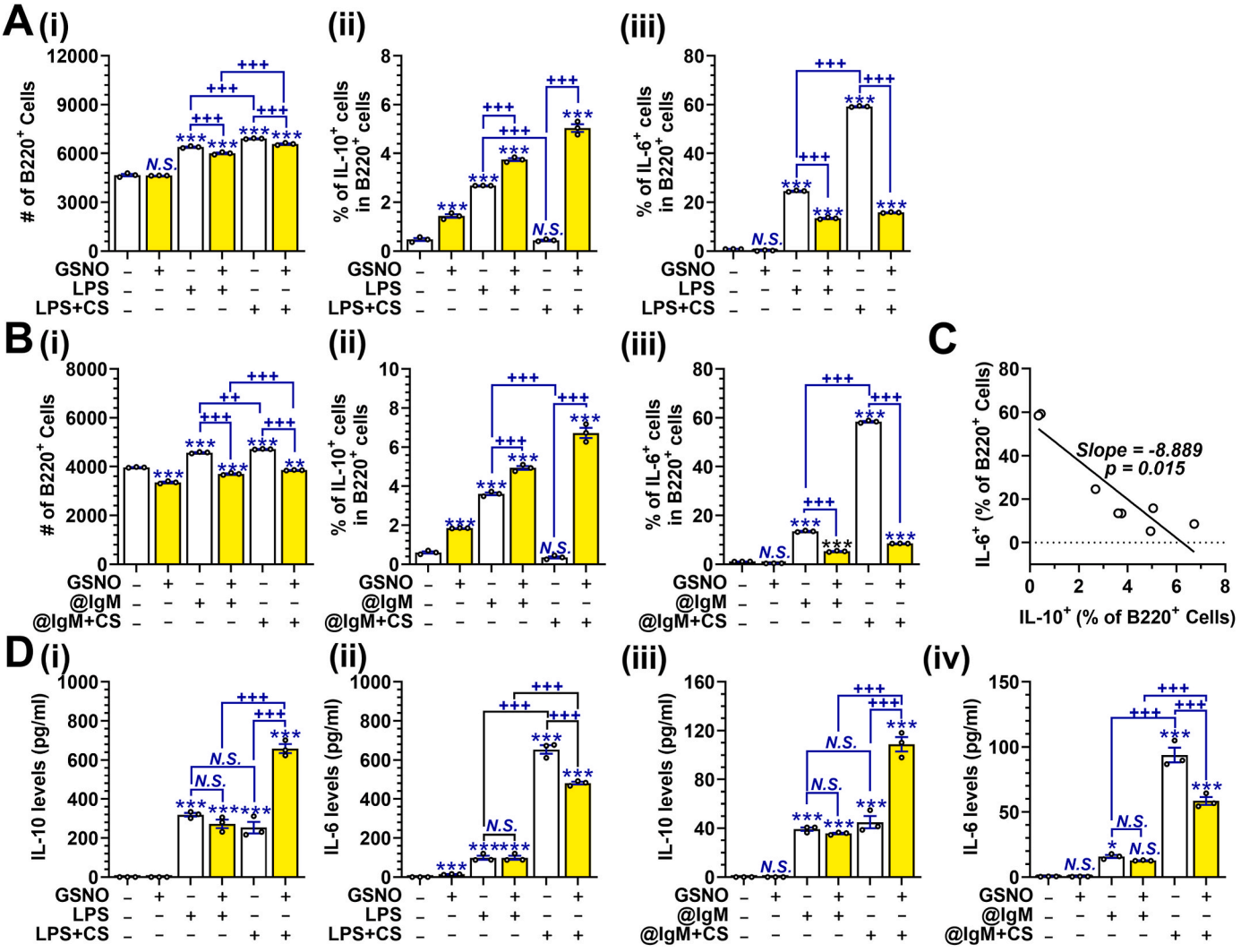

Fig. 4. GSNO increases IL-10^{+} expression but decreases IL-6^{+} expression in B cells under in vitro stimulatory conditions. Naïve splenic B cells were purified from C57BL/6 mice and stimulated with lipopolysaccharide (LPS) for TLR4 activation (**A**) or *anti*-IgM mAb (@IgM) for BCR activation (**B**) and co-stimulated (CS) with anti-CD40 mAb for activation of CD40 and recombinant BAFF for activation of BAFFR in the presence or absence of GSNO (50μM/1hr) pretreatment. Following the incubation for 43 h, the cells were treated with PMA, ionomycin, and brefeldin-A for 5 h for induction of intracellular cytokine accumulation and then the numbers of B220^{+} total B cells (**A-i** and **B-i**), and the proportions of IL-10^{+} (**A-ii** and **B-ii**) and IL-6^{+} (**A-ii** and **B-ii**) B cells (B220^{+}) were analyzed by fluorescence flow cytometric analysis. By using the above data, a correlation between the numbers of IL-10^{+} vs. IL-6^{+} B cells was analyzed by nonlinear regression (curve fit) XY analysis (**C**). For analysis of B cell release of IL-10 and IL-6, the cells were incubated without brefeldin A treatment under identical experimental conditions, and the levels of IL-10 (**D-i** and **-ii**) and of IL-6 (**D-ii** and **-iv**) were analyzed by ELISA. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^{+}$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

3.4. GSNO treatment induces B cell expression of IL-10 but decreases IL-6 expression in in-vitro splenic B cell culture conditions

Under autoimmune disease conditions, B cells can be activated in a T cell-dependent manner [46]. To avoid the T cell involvement in GSNO-mediated regulation of B cell expression of IL-10 vs. IL-6, in vitro cultured pure splenic B cells were pretreated with GSNO (50 μM) for 1 h and stimulated with lipopolysaccharide (LPS: 10 μg/ml) for activation of toll-like receptor 4 (TLR4) or *anti*-IgM monoclonal antibody (*anti*-IgM mAb: 10 μg/ml) for activation of B-cell receptor (BCR) as reported previously [47–49]. The B cells were also co-stimulated (CS) with anti-CD40 monoclonal antibody (anti-CD40 mAb: 10 μg/ml) for activation of CD40 and recombinant B-cell-activating factor belonging to the TNF family (rBAFF: 100 ng/ml) for activation of BAFF-receptor. CD40 and BAFF-R co-stimulation of stimulated B cells is known to increase IL-6 expression and decrease IL-10 expression [49,50]. Fig. 4A–i and 4 B-i show that the number of total B ($B220^+$) cells was increased by both LPS and *anti*-IgM mAb stimulations and further increased by their co-stimulation with anti-CD40 mAb and rBAFF. GSNO treatment significantly reduced these increases, thereby indicating that GSNO-mediated mechanism(s) inhibits B cell expansion under stimulated and co-stimulated conditions.

Next, we investigated the effect of GSNO treatment on the proportions of $IL\text{-}10^+$ and $IL\text{-}6^+$ B cells under stimulatory and co-stimulatory conditions. Stimulation of naïve B cells with LPS and *anti*-IgM mAb increased the numbers of $IL\text{-}10^+$ B cells (Fig. 4A–ii and 4B-ii) and $IL\text{-}6^+$ B cells (Fig. 4A–iii and 4B-iii). Co-stimulation of these cells with anti-CD40 mAb and rBAFF reduced the number of $IL\text{-}10^+$ B cells under both *anti*-IgM mAb and LPS stimulatory conditions (Fig. 4A–ii and 4B-ii) but further increased the number of $IL\text{-}6^+$ B cells as reported previously [47–49]. GSNO treatment of LPS- or *anti*-IgM mAb stimulated B cells significantly increased the number of $IL\text{-}10^+$ B cells both in the absence and the presence of co-stimulatory factors but the highest proportion of $IL\text{-}10^+$ B cells was observed in the presence of co-stimulatory factors (Fig. 4A–ii and 4B-ii). Conversely, GSNO treatment of the stimulated B cells significantly decreased the number of $IL\text{-}6^+$ B cells both in the absence and presence of co-stimulatory factors (Fig. 4A–iii and 4B-iii). It is of interest to note that the statistically significant linear regression between the numbers of $IL\text{-}10^+$ B cells vs. $IL\text{-}6^+$ B cells under stimulatory/co-stimulatory and GSNO treated/untreated conditions (Fig. 4C) indicates a negative correlation between the expansion of $IL\text{-}10^+$ B cells vs. $IL\text{-}6^+$ B cells.

Next, we investigated the effect of GSNO on B cell secretions of IL-10 and IL-6. Fig. 4D–i and 4D-iii show that stimulation of naïve B cells with LPS or *anti*-IgM mAb increased the secretion of IL-10. GSNO treatment had no significant effect on IL-10 secretion under LPS or *anti*-IgM mAb stimulatory conditions without co-stimulation. However, GSNO treatment significantly increased IL-10 production under co-stimulatory conditions (Fig. 4D–i and -iii). Stimulation of naïve B cells with LPS or *anti*-IgM also increased the secretion of IL-6 and which was further increased by co-stimulation with anti-CD40 and BAFF (Fig. 4D–ii and -iv). GSNO treatment had no obvious effect on *anti*-IgM mAb- or LPS-stimulated IL-6 secretion but it significantly decreased IL-6 secretion under co-stimulatory conditions (Fig. 4D–ii and -iv). In summary, these data demonstrate that GSNO treatment has no significant effect on B cell secretion of IL-10 and IL-6 under stimulatory conditions. However, GSNO treatment increases IL-10 secretion while decreasing IL-6 secretion under co-stimulatory conditions, supporting the role of the GSNO-mediated mechanism(s) in co-stimulatory signaling pathways of B cells for the secretions of IL-10 vs. IL-6.

3.5. GSNO treatment induces the B cell expression of IL-10 but decreases IL-6 expression in mouse splenic breg cells as well as human B cells

Co-expression of CD1d and CD5 has been classically used to characterize a population of splenic B cells predominantly expressing IL-10 in mice, so-called Breg or B10 cells [23]. Therefore, we next investigated the effect of GSNO treatment on the regulation of the $CD1d^{hi}$ $CD5^{hi}$ B cell populations. Fig. 5A–i and -ii show that naïve splenic B cells included a very low proportion of $CD1d^{hi}$ $CD5^{hi}$ B cells. Stimulation of these cells with LPS or *anti*-IgM mAb increased the proportion of $CD1d^{hi}$ $CD5^{hi}$ B cells, which was further increased by co-stimulation with anti-CD40 mAb and rBAFF (Fig. 5A–i and -ii). GSNO treatment of stimulated B cells had no significant effect on the proportion of $CD1d^{hi}$ $CD5^{hi}$ B cells but it decreased the proportion of this cell population under co-stimulatory conditions (Fig. 5A–i and -ii).

Next, we investigated the effect of GSNO treatment on the proportions of $IL\text{-}10^+$ vs. $IL\text{-}6^+$ cells among the $CD1d^{hi}CD5^{hi}$ B cells. Fig. 5A–iii and -iv show that stimulation of naïve B cells with LPS or *anti*-IgM mAb resulted in an increased proportion of $IL\text{-}10^+$ $CD1d^{hi}$ $CD5^{hi}$ B cells, which was decreased by co-stimulation with anti-CD40 mAb and rBAFF. Interestingly, stimulation of naïve B cells also resulted in an increased proportion of $IL\text{-}6^+$ $CD1d^{hi}$ $CD5^{hi}$ B cells and which were further increased by co-stimulation with anti-CD40 mAb and rBAFF (Fig. 5A–v and -vi). As expected, we observed that GSNO treatment increased the proportion of $IL\text{-}10^+$ $CD1d^{hi}$ $CD5^{hi}$ B cells under stimulatory conditions and even more under co-stimulatory conditions (Fig. 5A–iii and -iv). On the other hand, GSNO treatment inhibited the proportion of $IL\text{-}6^+$ $CD1d^{hi}CD5^{hi}$ B cells under stimulatory and co-stimulatory conditions (Fig. 5A–v and -vi). It is of interest to note that GSNO treatment of stimulated and/or co-stimulated B cells resulted in increased proportions of $IL\text{-}10^+$ $CD1d^{lo}$ $CD5^{hi}$ B cells while decreasing $IL\text{-}6^+$ $CD1d^{lo}$ $CD5^{hi}$ B cells (Supplementary data S2). Taken together with in vivo data (Figs. 2 and 3), these data document that GSNO mediated B cell regulation for IL-10 vs. IL-6 expression is not limited to Breg ($CD1d^{hi}$ $CD5^{hi}$) cells but other B cell subtypes as well.

Next, we investigated the effects of GSNO on the subset-specific expression of IL-10 vs. IL-6 in human B cells stimulated with anti-human IgM/IgG Ab and costimulated with rBAFF and human CD40 ligand (hCD40L) (Fig. 5B). GSNO treatment had no obvious effect on the number of total B cells but decreased the numbers of some subsets of B cells (e.g. immature B cells and memory B cells) while increasing the numbers of ASCs (plasmablasts and plasma cells) (Fig. 5B–i). Similar to EAE mice, GSNO treatment increased the proportion of $IL\text{-}10^+$ cells but decreased $IL\text{-}6^+$ cells not only in B10/Breg subset-specific cells ($CD24^{hi}CD27^+$ B cells [21,51] and $CD24^{hi}$ $CD38^{hi}$ immature B cells [22]) but also in all subsets of B cells and ASCs (Fig. 5B–ii and -iii). Taken together, these data document that GSNO/N6022-mediated induction of IL-10 and suppression of IL-6 is not limited to Breg/B10 cells but applies to all subsets of B cells and ASCs in EAE mice and human peripheral B cells.

3.6. GSNOR inhibition ameliorates EAE disease via regulating B cell expression of IL-10 vs. IL-6

We next investigated the role of GSNOR and its inhibitor (N6022) in B cell-mediated regulatory functions in EAE mice using B cell adoptive transfer models. For this, B cells were purified from control, EAE, or N6022-treated EAE mice one day before the onset of the disease (day 10 post-immunization) and transferred to EAE mice on day 10 post-immunization. Fig. 6A–i (daily clinical disease) and -ii (area under the curves) show that adoptive transfer of B cells from untreated EAE donor mice (magenta) had no obvious effect on the clinical disease in the recipient EAE mice as compared to the untreated EAE mice (red). However, adoptive transfer of B cells from N6022-treated EAE donor mice (green) significantly ameliorated the clinical disease in the recipient EAE mice (Fig. 6A–i and -ii). Accordingly, the recipient mice had increased blood IL-10 levels (Fig. 6A–iii) and the number of Treg ($CD4^+CD25^+FOXP3^+$) cells in the CNS (Fig. 6A–v), while they had decreased blood IL-6 levels (Fig. 6A–iv) and the number of Th17 ($CD4^+IL\text{-}17^+$) cells (Fig. 6A–vi) in the CNS. These data document the role of N6022-treated B cells in the regulation of T cell-mediated (Treg

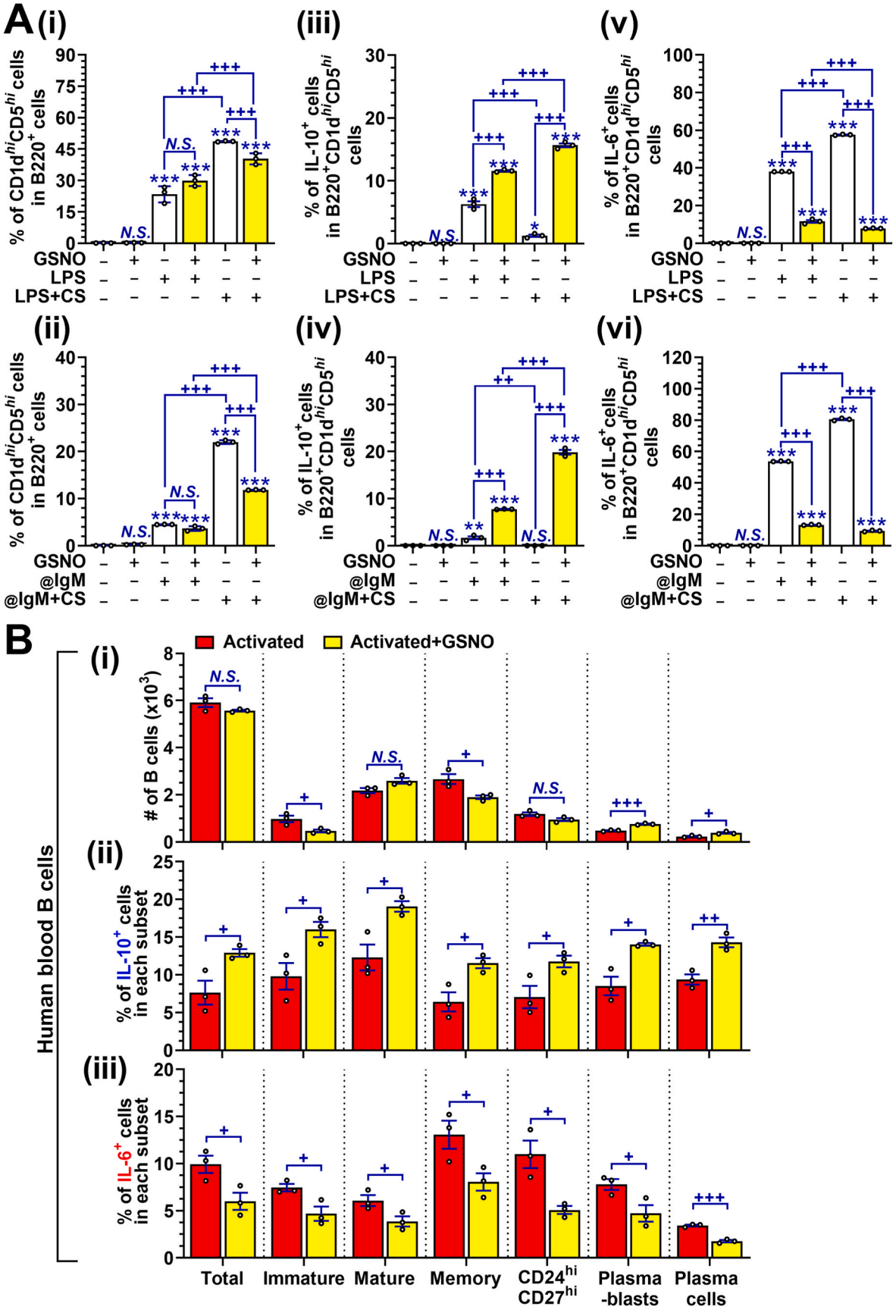

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Fig. 5. GSNO increases IL-10$^+$ expression but decreases IL-6$^+$ expression in CD1d^{hi} CD5^{hi} mouse B cells and in human blood B cells under in vitro stimulatory conditions. Naïve splenic B cells were purified from C57BL/6 mice and stimulated with lipopolysaccharide (LPS) for TLR4 activation (**A-i, -ii,** and **-v**) or *anti*-IgM mAb (@IgM) for BCR activation (**A-ii, -iv,** and **-vi**) and co-stimulated (CS) with anti-CD40 mAb for activation of CD40 and recombinant BAFF for activation of BAFFR in the presence or absence of GSNO (50μM/1hr) pretreatment. Following the incubation for 43 h, the cells were treated with PMA, ionomycin, and brefeldin-A for 5 h for induction of intracellular cytokine accumulation and then the numbers of Breg cells (B220$^+$ CD1d^{hi} CD5^{hi}) (**A-i** and **-ii**), and the proportions of IL-10$^+$ (**A-ii** and **-iv**) and IL-6$^+$ (**A-ii** and **-vi**) in Breg cells (B220$^+$ CD1d^{hi} CD5^{hi}) were analyzed by fluorescence flow cytometric analysis. Human blood B cells were stimulated with *anti*-IgM/IgG antibody (@IgM) and costimulated with recombinant BAFF and human CD40 ligand (hCD40L) in the presence or absence of GSNO (50μM/1hr) pretreatment. 43hr after the cells were further incubated with PMA/ionomycin/Brefeldin-A for 5 h and the numbers of total B cells, immature (transitional) B cells, mature B cells, memory B cells, Breg cells (CD24^{hi} CD27 ^{hi}), plasmablasts, or plasma cells (**B-i**) and the proportions of IL-10$^+$ (**B-ii**) and IL-6$^+$ (**B-ii**) cells in the total and each subset of B-lineage cells were analyzed. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^+$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

> Th17) EAE disease. Interestingly, adoptive transfer of B cells from N6022-treated control (non-EAE) mice had no effect on the clinical disease, blood IL-10/IL-6 levels, as well as the numbers of CNS Treg and Th17 cells in the recipient EAE mice, indicating that the observed B cell-mediated regulation of Treg and Th17 cells in EAE mice is an antigen (MOG_{35-55})- or disease-specific.

N6022 is a first-in-class drug targeting GSNOR [52]. To further confirm the role of N6022 in the GSNOR-mediated mechanism, EAE was induced in GSNOR knockout (GSNOR$^{-/-}$) and wild type (WT; C57BL/6) mice. We observed that GSNOR$^{-/-}$ mice as compared to WT mice had increased IL-10$^+$ B cells and decreased IL-6$^+$ B cells in the spleen on day 10 post-immunization (Fig. 6B–i and -ii). For adoptive transfer, B cells isolated from GSNOR$^{-/-}$ EAE mice or WT mice (on day 10 post-immunization) were transferred into the EAE mice on day 10 post-immunization. Adoptive transfer of B cells from GSNOR$^{-/-}$ EAE mice ameliorated clinical disease in the recipient EAE mice (green) as compared to the mice receiving B cells from WT EAE donor mice (red) (Fig. 6B–iii and iv). Accordingly, adoptive transfer of B cells from GSNOR$^{-/-}$ EAE donor mice increased IL-10 levels, while decreasing IL-6 levels, in the blood of the recipient EAE mice. These studies provide evidence for the role of N6022 and GSNOR-mediate mechanisms in the regulation of effector vs. regulatory B cell functions in EAE disease, which is highly relevant to autoimmune disease mechanisms.

4. Discussion

Activated and differentiated B cells are known to produce various regulatory and effector cytokines that can regulate autoimmune and inflammatory neuropathy in various disease conditions including MS but the related mechanisms are not well understood [10]. Here, we investigated the potential role of GSNO in the regulation of B cell expression of regulatory vs. effector cytokines in relation to the autoimmune disease of EAE. Our study demonstrates, for the first time, that exogenous GSNO treatment or increase in endogenous GSNO by treatment with GSNOR inhibitor N6022 shifts the balance of B cell expression of regulatory (IL-10) vs. effector (IL-6) cytokines towards IL-10 under in vitro B cell stimulatory/co-stimulatory conditions as well as in vivo EAE conditions. The GSNO/N6022-mediated B cell regulation is not limited to specific subtypes of B cells, but rather affects a broad range of B cell subsets as well as ASCs. GSNO/N6022 treatment protected against the neuroinflammatory disease of EAE. Moreover, adoptive transfer of B cells from EAE mice treated with N6022 or EAE mice deficient in GSNOR gene led to a change in T cell balance (Treg > Th17) and decreased clinical disease in the recipient EAE mice, thus documenting the potential efficacy of GSNO and N6022 in B cell-mediated regulation of T cell disease of EAE.

We previously reported that GSNO treatment exhibits anti-inflammatory and immunomodulatory activities in EAE models [29,30,32]. GSNO, an endogenous *S*-nitrosothiol (SNO) compound biosynthesized by the reaction between NOS-produced NO and glutathione (GSH), is now recognized to regulate various cellular processes by *S*-nitrosylation of respective proteins [53]. Depletion of NO and/or GSH causes depletion of cellular GSNO and thus impairs GSNO-mediated cellular processes [54–57]. EAE is known to involve increased expression of inducible nitric oxide synthase (iNOS) and increase the production of NO [58,59]. However, macrophages and microglia in EAE mice, as well as MS patients, are known to express the increased level of γ-glutamyl transferase (GGT) that causes oxidative stress by degrading glutathione [60]. Under oxidative stress conditions, NO reacts with superoxide anion to form peroxynitrite, the most powerful oxidative/nitrosative agent implicated in the pathogenesis of MS and EAE [61]. These studies, therefore, suggest that the increased expression of GGT during the course of EAE/MS and the subsequent depletion of glutathione could drive not only induction of peroxynitrite-induced nitrosative stress, but also depletion of GSNO and thus dysregulation of GSNO-mediated immune regulation, suggesting central roles of both GGT and GSNO/GSNOR in MS/EAE immunopathogenesis.

In this study, we provided evidence that GSNO and N6022 regulate T cell-mediated immunopathogenesis via regulating B cell functions. At present, however, it is unclear whether the effects of GSNO and N6022 are peripheral, or in CNS, or both. However, Fig. 6 shows that splenic B cells adoptively transferred from N6022 treated EAE mice or GSNOR deficient EAE mice ameliorated EAE disease in the recipient EAE mice, thus suggesting that the effects of GSNO and N6022 observed with EAE mice may be peripheral. GSNOR plays a key role in the cellular GSNO homeostasis. However, the inhibition of GSNOR activity by N6022 or by its null mutation may not increase the cellular GSNO levels if the cells do not synthesize GSNO. Genetic studies with GSNOR null mice have established that the level of cellular protein *S*-nitrosylation is controlled not only through enzymatic degradation by GSNOR but also at the level of synthesis by nitric oxide synthase [62–64]. In support, genetic deletion of GSNOR did not increase cellular protein *S*-nitrosylation in the absence of the iNOS gene [64]. EAE mice have increased expression of iNOS and NO production in the spleen [58,59]. Therefore, deficiency or inhibition of GSNOR under EAE conditions may increase GSNO level in the spleens. In support, we previously reported that systemic treatment of EAE mice with N6022, as well as GSNO, increased the level of *S*-nitrosylated proteins (PrSNOs) in the spleen [52]. Cellular PrSNOs are known to be in *trans*-*S*-nitrosation equilibrium with GSNO and thus can be a measurement of cellular GSNO level [53]. Taken together, these data suggest that GSNO/N6022 treatment-induced optimization of GSNO level in the periphery contributes to the immunomodulation of EAE. In addition, the observation of the increased expression of IL-10 and decreased expression of IL-6 in the cultured B cells by GSNO treatment (Figs. 4 and 5) further supports the role of GSNO and GSNOR in B cell-mediated immunomodulation of EAE. Since GSNOR is a ubiquitous enzyme, the non-targeted effect of N6022 is a concern. However, studies with N6022 in animal models of asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, stroke, MS, and traumatic spinal cord injury did not observe any toxicity [31,65–67]. Moreover, N6022 is well tolerated with minimal side effects, even at high concentrations, in both animals [68,69] and humans (clinicaltrials.gov – NCT01147406, NCT01746784), thus suggesting the clinical utility of GSNOR inhibitor N6022 for MS.

In the MOG-EAE model, amelioration of clinical symptoms of EAE by N6022 or GSNO treatment (Fig. 1A and B) was consistent with decreased levels of proinflammatory/effector cytokines (e.g. TNF-α, IFN-γ, and IL-6) and increased levels of anti-inflammatory/regulatory cytokine IL-10

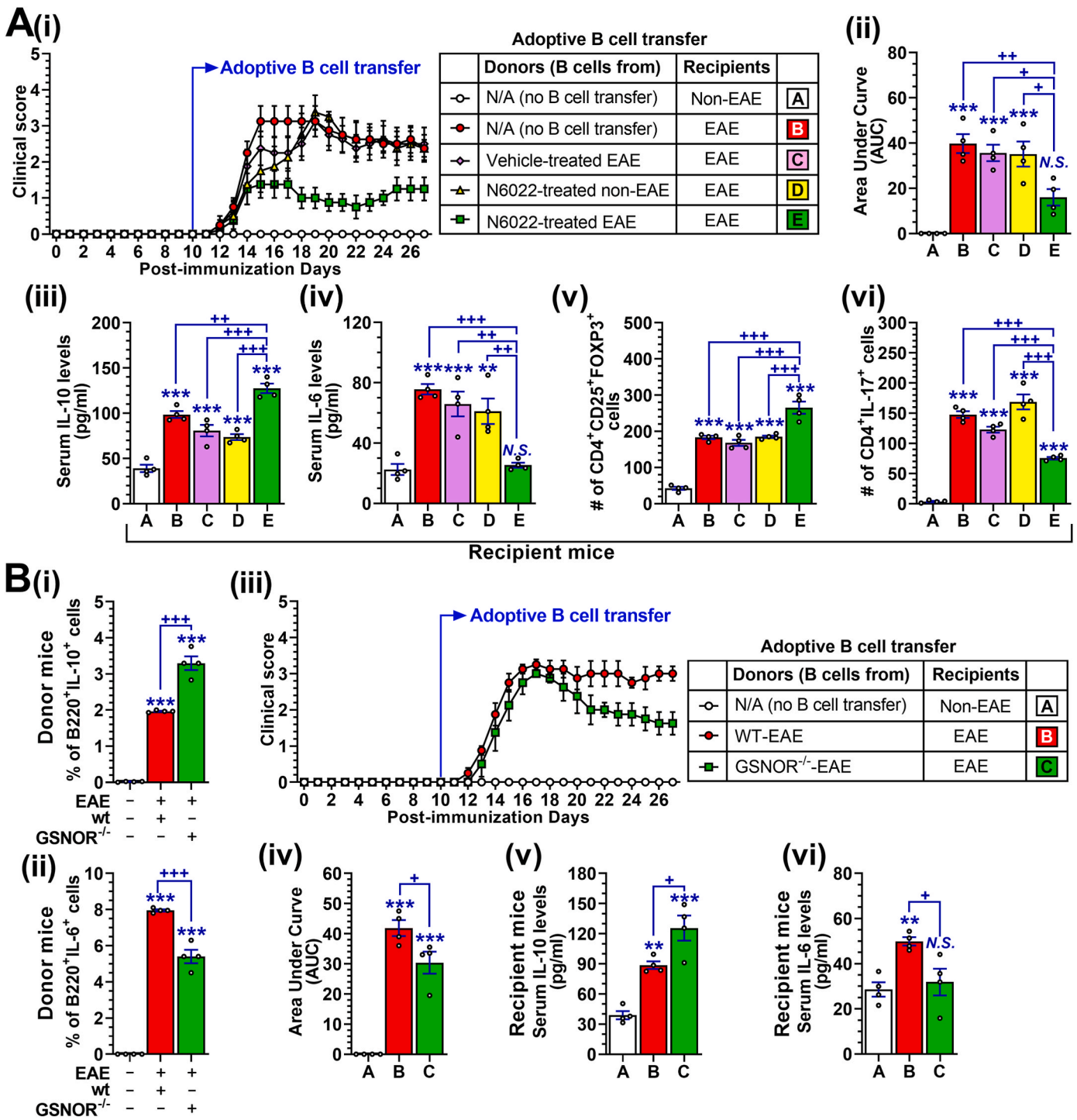

Fig. 6. Adoptive transfer of B cells from N6022-treated EAE mice or GSNOR deficient EAE mice to active EAE mice decreases EAE disease. C57BL/6 mice were immunized with MOG_{35-55} for the induction of EAE. Control (non-EAE) and EAE mice received daily doses of saline, N6022 (1 mg/kg/day/ip) starting day 1 post-immunization. On day 10 post-immunization, B cells were purified from control, EAE, N6022-treated control mice, and N6022-treated EAE mice. The purified B cells (2×10^6 cells) were then transferred to EAE mice (MOG_{35-55}-immunized) on day 10 post-immunization. Following the adoptive B cell transfer, the recipient mice were analyzed for daily clinical scores (**A-i**) and their quantitative summaries (area under the curves; AUC) (**A-ii**), blood levels of IL-10 (**A-ii**) and IL-6 (**A-iv**), and the numbers of Treg ($CD4^+CD25^+FOXP3^+$) (**A-v**) and Th17 ($CD4^+$ IL-$17a^+$) cells (**A-vi**) in the CNS. Wild type (WT) and GSNOR deficient mice ($GSNOR^{-/-}$) were immunized with MOG_{35-55} for the induction of EAE. On day 10 post-immunization, B cells were purified from the spleens of these mice and the proportions of IL-10^+ (**B-i**) and IL-6^+ (**B-ii**) cells in total B cells (B220+) were analyzed. The purified B cells (2×10^6 cells) were then transferred to EAE mice (MOG_{35-55}-immunized) on day 10 post-immunization and daily clinical scores (**B-ii**) and their AUC (**B-iv**) and blood levels of IL-10 (**B-v**) and IL-6 (**B-vi**) of the recipient mice were analyzed. The bar graph represents the mean ± standard error mean (SEM) and the scatter dot plot represents an individual data point. *P < 0.05; **P < 0.01, ***P < 0.001 vs. control. $^{+}$P < 0.05; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. as indicated. NS; not significant.

in the blood (Fig. 1C). It is of interest to note that both N6022 and GSNO have no obvious effect on the blood levels of GM-CSF (Fig. 1C–iii), which is mainly expressed by activated leukocytes [70], suggesting that the efficacy of N6022/GSNO may not involve general suppression of leukocytes. It is consistent with no effects of GSNO or N6022 on IFN-γ expression by Th1 cells while it inhibits the expression of IL-17 by Th17 cells [31]. Along with the altered cytokine profiles, N6022/GSNO treatment also reduced the CNS recruitment of mononuclear cells (Fig. 1D), including B cells (Fig. 1E), near the meningeal area of the anterior median fissure which usually shows heavy demyelination in EAE mice. Previously, our laboratory reported that GSNO protects the blood-brain barrier (BBB) under neuropathological conditions [71,72] and inhibits immune cell infiltration into the CNS by inhibiting endothelial expression of cell adhesion molecules (e.g. ICAM-1, VCAM-1, and *E*-selectin) in EAE animals [29]. Taken together. These studies provide evidence that neurovascular protection or inhibition of CNS recruitment of immune cells participates, at least in part, as a mechanism of action underlying the anti-inflammatory activities of GSNO and N6022 in EAE pathogenesis.

MS and EAE have long been considered a $CD4^+$ T-cell disease [3]. However, recent findings of the regulatory and effector functions of B cells in the modulation of $CD4^+$ T cell immunity [73] suggest the importance of B cells in MS immunopathogenesis. Regulatory and effector functions of B cells are primarily mediated by B cell expression of regulatory (e.g. IL-10, IL-35, and TGF-β) and effector (e.g. IL-6, IFN-γ, and GM-CSF) cytokines [10]. Since IL-10 and IL-6 play crucial roles in the regulation of Treg/Th17 balance [25,26,34–36], we focused our study on B cell expression of IL-10 and IL-6. We previously reported that N6022/GSNO treatments shift the Treg vs. Th17 immune balance toward Treg in EAE mice [30–32]. In this study, we observed that N6022/GSNO treatment also increased IL-10 expression, while decreasing IL-6 expression, in B cells in the CNS (Fig. 2) and in the spleen (Fig. 3) of EAE mice as well as in vitro mouse (Fig. 4) and human (Fig. 5B) B cell cultures. Moreover, the observed amelioration of EAE disease and changes in T cell immune balance (Treg > Th17) in the recipient mice by adoptive transfer of B cells from N6022 treated EAE mice or $GSNOR^{-/-}$ EAE mice (Fig. 6) highlight the role of N6022/GSNO-mediated mechanisms in B cell-mediated modulation of T cell disease of EAE.

In humans and mice, specific subtypes of B cells are known to express high levels of IL-10. These B cells, also known as regulatory B cells (Breg) or IL-10 expressing B cells (B10), include $CD24^{hi}CD27^+$ B cells [21,51] and $CD24^{hi}CD38^{hi}$ immature B cells [22] in humans and $CD5^{hi}CD1d^{hi}$ B cells in mice [23]. In this study, we observed that N6022 or GSNO treatment increased the IL-10 expression in these subtypes of B cells in the CNS (Fig. 2C) and in the spleen (Fig. 3B) of EAE mice as well as in vitro stimulated/costimulated mouse (Fig. 5A) and human (Fig. 5B) B cell cultures. However, the increased induction of IL-10 was not limited to these cell types but extended to almost all subsets of B cells in the CNS and spleen in EAE mice as well as in vitro B cell culture. Moreover, N6022/GSNO treatment also decreased IL-6 expression in all subsets of B cells tested. These data indicate that the N6022/GSNO-mediated mechanism for B cell expression of IL-10 vs. IL-6 is not B cell subset specific but rather applies to all B cells in a non-specific manner. Fig. 4A shows that GSNO treatment increased IL-10 expression at much lower levels in unstimulated B cells than in stimulated and costimulated B cells. In addition, adoptive transfer of B cells from GSNO-treated control mice (non-EAE) did not produce any beneficial effects on EAE disease of recipient mice while that of B cells from GSNO-treated EAE mice protected against the EAE disease in the recipient mice (Fig. 6A). These data document that B cell activation is a prerequisite for N6022/GSNO-mediated regulation of regulatory vs effector function or IL-10 vs. IL-6 expression of B cells.

Memory B cells are highly abundant in the human spleen [74]. In MS, an increased number of circulating memory B cells are associated with neurodegeneration in RRMS [42] and memory B cells are also detected in MS post-mortem lesions [43]. Memory B cells are known to secrete elevated levels of IL-6 [39–41], therefore, the potential target for effective immunotherapy in RRMS [75]. In this study, we observed that GSNO treatment reduces the number of memory B cells in the CNS (Fig. 2C–i) and in the spleen (Fig. 3B–i) of EAE mice as well as in the stimulated/costimulated human blood B cell cultures (Fig. 5B–i). As expected, N6022/GSNO treatment also shifted the balance of IL-10 vs. IL-6 expression toward IL-10. Interestingly, N6022/GSNO increased the numbers of ASCs (plasmablasts and plasma cells) and their expression of IL-10 while decreasing IL-6 expression. ASCs are reported to be the main source of IL-10 in the spleen and lymph node of EAE mice [44,45]. At present, however, it is unclear whether N6022/GSNO induces maturation of memory B cells into these ASCs.

At present, the cellular mechanisms for GSNO-mediated differential regulation of IL-10 vs. IL-6 in B cells is not well understood. Transcriptional regulation of IL-10 and IL-6 genes is complex and involves the convergence of multiple transcription factors, such as NF-κB, *c*-Maf, CREB, STAT3, C/EBPβ, AP1 for IL-10 [76–78], and NF-κB, AP-1, STAT3, CREB, NF-IL6, C/EBP for IL-6 [79–82]. It is of interest to note that some of these transcription factors involved in the transcriptional activation for both IL-10 and IL-6 genes (e.g. NF-κB, STAT3, C/EBPβ, and AP-1) are repressed by a NO/GSNO-mediated *S*-nitrosylation mechanism [28, 83–86]. On the other hand, *S*-nitrosylation of CREB is known to increase its binding to the responsible DNA motifs [87]. Therefore, these transcription factors do not appear to be involved in GSNO/*S*-nitrosylation mediated differential regulation of IL-10 and IL-6 genes in B cells.

The deletion of nuclear factor of activated T-cells c1 (NFATc1) gene was reported to lead to decreased Th17 cells and increased IL-10 producing B cells and thus milder EAE disease [88,89]. NFATc1 is activated by calcineurin-mediated dephosphorylation leading to subsequent nuclear translocation [90] and *S*-nitrosylation of calcineurin inhibits its activity for NFATc1 activation [91]. Therefore, calcineurin-NFATc1 signaling is one of the potential pathways involving GSNO-mediated differential regulation of IL-10 vs. IL-6. Secondly, hypoxia-inducible factor 1α (HIF-1α) is highly expressed in B cells and is involved in the expansion of $CD1d^{hi}CD5^{hi}$ B cells and their expression of IL-10 [47]. Mice lacking HIF-1α in B cells were reported to have increased Th17 cells and decreased $IL\text{-}10^+$ B cells and thus exacerbation of EAE [47]. HIF-1α is maintained at low levels in normoxic cells by prolyl hydroxylase (PHD)- and von Hippel-Lindau tumor-suppressor protein (VHL)-dependent degradation of HIF-1α protein [92]. *S*-nitrosylation is known to stabilize HIF-1α protein [93] and inhibit PHD [94,95] and VHL [96] activities. Therefore, HIF-1α mediated cell signaling is also potentially involved in GSNO-mediated differential regulation of IL-10 vs. IL-6 in B cells. Along with NFATc1 and HIF-1α, interferon regulatory factor 4 (IRF-4) could be another candidate in GSNO-mediated differential regulation of IL-10 vs. IL-6. IRF-4 is known to play a key role in the differentiation of B cells into plasmablasts [45] and is involved in optimal IL-10 expression in B cells [45,97] and inhibition of IL-6 expression [98]. Accordingly, mice lacking IRF-4 are reported to develop severe EAE [45]. At present, the precise roles of these transcription factors in GSNO-mediated differential regulation of IL-10 vs. Il-6 are not fully understood. However, we observed that GSNO treatment of stimulated/costimulated B cells increased the numbers of $HIF1\alpha^+$ and $IRF4^+$ B cells, specifically in $IL\text{-}10^+$ B cells, but not in $IL\text{-}6^+$ B cells (supplementary data S3), thus suggesting the potential role of HIF1α/IRF4 in GSNO mediated regulation of IL-10 vs. IL-6 in B cells.

Humanized or engineered anti-CD20 mAbs inducing B cell depletion have been approved for relapsing and progressive types of MS. The B cell depletion is known to inhibit Th1 and Th17 cell responses [11,99] and pro-inflammatory myeloid-cell responses (which in turn could drive Th1 and Th17 responses) [100] in MS patients and EAE animals, which has emerged as an essential mechanism of B cell depletion therapy [101]. B cell depletion therapy, however, potentially involves adverse effects, such as opportunistic infections, autoimmune pathologic effects, and the formation of neutralizing antibodies [102,103]. Moreover, B cell

depletion therapy also impairs the regulatory B cell functions that lead to the inhibition and recovery of autoimmune diseases [5,27]. In mouse models of EAE [18] and systemic lupus erythematosus [19], global B cell depletion in the early phase of the disease exacerbated the disease by depleting B cells playing regulatory functions while the depletion of B cells in the late phase of the disease attenuated the disease by depleting B cells playing effector functions. This, therefore, underscores the need for new therapies that selectively inhibit effector functions of B cells while sparing or promoting regulatory B cell functions. In this study, we observed that GSNO/N6022 inhibited IL-6 mediated effector B cell function while inducing IL-10-mediated regulatory B cell function, thus suggesting that GSNO/N6022 is potentially a better approach than B cell-depleting therapies (e.g. ocrelizumab, rituximab, and ofatumumab) as well as general immunosuppressive therapies (e.g. teriflunomide, mitoxantrone, cyclophosphamide, and fingolimod) for the treatment of MS. B cell-depleting therapy (e.g. rituximab) is approved for other autoimmune diseases, such as refractory rheumatoid arthritis (RA) [104]. Since IL-6 and IL-10 expressing B cells play a critical role in the disease pathology of RA [105], GSNO/N6022-based treatment may be of therapeutic benefit in RA as well.

Currently, more than 20 immunomodulators and immunosuppressants are approved by FDA or in active clinical trials as disease-modifying therapies (DMTs) for MS. However, most of these DMTs have limited efficacies as CNS disease progression continues into progressive MS despite treatment [106]. The presence of ectopic lymphoid follicle (eLF) in the CNS is associated with aggressive clinical disease and greater CNS tissue injury in the progressive types of MS [107]. Mechanism(s) underlying CNS eLF formation is not fully understood at present but the role of IL-6 in Th17- and follicular T helper (Tfh)-mediated eLF formation has been described [108–111]. B cell-produced IL-6 and IL-10 have been implicated in induction and inhibition of Th17 and Tfh cell differentiation in the germinal center, respectively [11,112–114], thus suggesting for potential therapeutic efficacy of GSNO/N6022 in B cell cytokine (IL-10 vs. IL-6)-mediated regulation in eLF formation and thus CNS disease of progressive MS.

In conclusion, the data presented here provide the first insight into the role of GSNO in shifting the balance of B cell expression of regulatory (IL-10) vs. effector (IL-6) cytokines toward IL-10 and preclinical relevance of N6022, a first-in-class drug targeting GSNOR as therapeutics for MS and other autoimmune disorders.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102053.

Abbreviations

@IgM	anti-immunoglobulin M antibody
ASC	antibody-secreting cell
BAFF	B-cell activating factor belonging to the tumor necrosis factor (TNF) family
BBB	blood-brain barrier
CFA	complete Freund's adjuvant
CS	co-stimulatory factors
EAE	experimental autoimmune encephalomyelitis
GSNO	*S*-nitrosoglutathione
GSNOR	*S*-nitrosoglutathione reductase
LPS	lipopolysaccharide
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PTX	pertussis toxin

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