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2-BFI Attenuates Experimental Autoimmune Encephalomyelitis in Mice via Regulation of Lymphocytes Subsets

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Research

Keywords: 2BFI, Experimental Autoimmune Encephalomyelitis (EAE), Multiple Sclerosis (MS), T cells, B cells

DOI: https://doi.org/10.21203/rs.3.rs-72159/v1

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// Abstract

Background: Imidazoline compounds are well accepted to exhibit various pharmacological effects including antidepressant, anti-inflammatory, analgesic, anti-morphine tolerance and inhibit the activity of monoamine oxidase. 2-(-2-benzofuranyl)-2-imidazoline (2-BFI), a selective imidazoline 2 receptor (I2R) ligand, has been proven to exhibit therapeutic effects for various neuroimmunological diseases. However, the mechanism behind its neuroprotective properties remains elusive.

Methods: In this study, we used 2-BFI for the treatment of mice with experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG33-55). The clinical signs of neurological deficits were evaluated daily. The demyelination and inflammatory infiltration in the CNS of mice with EAE were examined by Luxol Fast Blue (LFB) staining and hematoxylin-eosin (H&E) staining. Flow cytometry was utilized to examine the ratios of lymphocyte subsets in the periphery and CNS of mice with EAE. We also used Reverse Transcription–Polymerase Chain Reaction (RT-PCR) to observe the changes of expression of inflammatory cytokines by 2-BFI intervention.

Results: We found that 2-BFI significantly reduced the incidence of EAE and attenuated the severity of neurological disability. Pathological staining showed that the infiltration of inflammatory cells and demyelination in the central nervous system (CNS) of the mice were markedly alleviated *via* 2-BFI intervention. To explore the mechanism of action of 2-BFI, we used flow cytometry to determine immunophenotypes in the spleen and CNS of the mice. We discovered that 2-BFI significantly decreased the ratio of CD28+ lymphocytes and B cells in the spleen of EAE mice. In the CNS, the expression of CD4+ T cells was downregulated by 2-BFI, while B cells and CD39+ lymphocytes were dramatically increased. RT-PCR also demonstrated that the level of IFN-γ mRNA secreted by CD4+T cells was lower than that in the CNS of EAE mice, while the levels of TGF- β and IL-10mRNA secreted by Treg and B cells were increased with 2-BFI intervention.

Conclusion: 2-BFI could ameliorate EAE-induced neurobehavioral deficits and reduce the infiltration of inflammatory cells *via* regulating the activation and migration of lymphocyte subsets. This study provides a new explanation for the protective mechanism of 2-BFI in neuroimmune diseases.

Background

Imidazoline receptors (IR) are widely distributed in mammalian tissues including the central nervous system (CNS) and are generally classified into three subtypes: I1R, I2R and I3R [1]. I2R are predominantly located in the brain and liver where they regulate monoamine turnover identified by idazoxan [2]. A recent study revealed that 2-(benzofuranyl)-2-imidazoline (2-BFI) is a specific ligand to I2R [3]. In the CNS, I2R binding is thought to mainly occur in the outer membrane of the mitochondria of neurons and astrocytes [4, 5]. There are enormous evidences that I2R are associated with various neurological disorders, and its ligands, including 2-BFI, manifested protective effects against these diseases. Prolonged treatment with the imidazole compound LSL 60101 could activate I2R and protect motoneurons from death caused by

neurectomy of the facial motor nerve [6]. Selective I2R agonist reduced the size of infarct in rats with focal ischemia, and improved neurobehavioral deficits [7]. Chronical administration of 2-BFI can reduce the development of tolerance to morphine [8], and showed therapeutic potentials in psychiatric disorders [9]. Moreover, advances in the field of neuroinflammation have indicated that 2-BFI can suppress the accumulation of oxidants, inhibit the inflammatory response and enhance neural viability [10]. In a study on neuropathic pain model in rats, 2-BFI showed anti- hypersensitivity and anti-inflammatory effects by downregulating the levels of proinflammatory cytokines [11]. Thus, it is crucial to explore the mechanism underlying the neuroprotection neuroprotective properties of 2-BFI to confirm its potential value in clinical application.

Multiple sclerosis (MS), a prototypical autoimmune inflammatory disorder of the CNS characterized by demyelination and inflammatory infiltration, is believed to be mediated by autoreactive T cells. Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model of MS, which mimics the clinical features and pathological manifestations found in this disease [12]. The migration of autoreactive myelin-specific T cells across the blood-brain barrier (BBB) leads to a diverse accumulation of T cells, B cells and macrophages in the CNS, which is considered to be a crucial step in the initiation of inflammation and progressive paralysis in EAE [13, 14]. Recent evidence suggests that perturbation in lymphocyte subsets including CD4 + T cells, CD8 + T cells and B cells is a significant hallmark in the progression of EAE. Meanwhile, extensive research confirms that cytokines derived from T cells such as interferon-γ (IFN-γ) play a key role in the development of EAE [15–17], whereas B cells ameliorate the severity of the disease *via* the production of suppressive cytokines like interleukin-10 (IL-10) and TGF-β [18, 19].

In our previous studies, 2-BFI was found to significantly attenuate spinal cord injury and exhibited neuroprotection against EAE [20]. We also found that 2-BFI could regulate the expression of cytokines in EAE with dose and time dependency [21]. It is reasonable to deduce that 2-BFI serves important functions in inflammatory and immune responses of EAE, but the mechanism has not been fully elucidated. In this study, 2-BFI was administrated to mice with EAE induced by myelin oligodendrocyte glycoprotein (MOG33-55). We evaluated the therapeutic effect of 2-BFI against the clinical neurological deficit and pathological damage. We used flow cytometry to detect the influence of 2-BFI on the changes of lymphocyte subsets in the periphery and CNS. We further examined the changes of mRNA levels of proinflammatory cytokines such as IFN-γ, IL-10 and TGF-β to explore the potential immunological protective mechanisms of 2-BFI.

Methods

Materials

Chemicals and reagents including 2-BFI, pertussis toxin (PT), incomplete Freund’s adjuvant (IFA) and mycobacterium tuberculosis H37RA were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). MOG35-55 (Myelin oligodendrocyte glycoprotein35–55 amino acid peptide,

MEVGWYRSPFSRVVHLYRNGK, 95% purity or greater) was synthesized from Lianmei Chemical Co. (Xi'an, China). Percoll and red blood cell lysis buffer was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Antibodies used in flow cytometry such as anti-CD4-FITC, anti-CD3-APC, anti-CD8-PE and anti-B220-PE-cy5.5 were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Mice and EAE induction

Female C57BL/6 mice, 6-8 weeks old, weighing 17-20 g were obtained from the Experimental Animal Center of Beijing Military Region and housed under specific pathogen-free conditions. Animals were maintained with free access to food and water. Each mouse received subcutaneously injections into multiple sites in the flanks with 0.2 mg of MOG35–55 emulsified in IFA supplemented with 8 mg/ml of heat-killed mycobacterium tuberculosis H37Ra (complete Freund's adjuvant, CFA). In addition, 0.2 ml phosphate-buffered saline (PBS) containing 200 ng of PT were injected intraperitoneally (i.p.) at days 0 and 2 post-immunization. The body weight and clinical signs of EAE micewere examined every day.

2-BFI treatment and treatment groups

Twenty-four mice were randomly divided into three groups for the present experiments. EAE mice were subtyped into one 2-BFI-treated groups (EAE-2BFI group) and one saline-treated group (EAE-control group,). Mice in the EAE-2BFI group were given twice daily i.p. injection of 2BFI 20 mg /kg for 14 days from the day of immunization, while the EAE-control group mice were given twice daily i.p. injection of the same dose of saline. The remaining mice (CFA-control group) were immunized with only CFA and the equivalent amount of saline instead of MOG35–55 used in EAE mice.

Clinical and pathological evaluation of EAE

Clinical signs of neurological deficits were observed and evaluated daily, the severity was graded as follows scale [22]: (1) for the tail, 0 = no signs; 1 = half paralyzed tail; 2 = complete limp tail; (2) for each of the hind- or forelimbs assessed separately, 0 = no signs; 1 = a weak or altered gait; 2 = paresis; 3 = a fully paralyzed limb. The sum of the state of the tail and all four limbs was from 0 to 15, thus a fully paralyzed quadriplegic mouse would attain a score of 14. Mortality equals a score of 15.

EAE mice and CFA-control mice were euthanized at 20 days after immunization, approximately the peak clinical signs exhibited. The mice were perfused by intracardiac injection pre-cooling PBS, then the lumbar cords were dissected out and fixated in 4% paraformaldehyde for 12h. Transverse sections of the spinal cord were stained with H&E staining. The severity of inflammatory cell infiltration was assessed according to the following scoring system described by Okuda et al. [23]: 0 = no infiltration; 1 = slightly cellular infiltrates were around meninges; 2 = mild cellular infiltrates in parenchyma (1–10/section); 3 = cellular infiltrates in parenchyma (11–100/section); and 4 = severe cellular infiltrates in parenchyma (>100/section). The cerebral cortices of mice were sectioned and examined by LFB staining to evaluate the severity of demyelination. The LFB color intensities were recorded by Image J system and normalized to CFA-control group.

Isolation of mononuclear cells from the CNS and splenocytes

Fresh brain and spinal cord tissues were removed from mice and rinsed in ice-cold PBS. The brain and remaining spinal cord were cut into small pieces, using the back of a sterile 1 ml syringe plunger press each piece of organ filtered through a 100mm wire mesh in a 10cm petri dish containing 10ml of ice-cold PBS. The tissues were dispersed into a single cell suspension and inflammatory cells were recovered from the CNS, then centrifuged at 1, 500 rpm for 5 min at 4°C to resuspend the cells in 30% Percoll and then layered over 70% Percoll. The cells were collected from the 30%/70% interface after centrifugation at 600 g for 20 min at room temperature and washed twice with PBS. Spleens were removed from the same mice and placed in PBS. Tissues were forced through 100-mesh stainless steel screens to give a single cell suspension. Red blood cells in spleen cell preparations were lysed by red blood cell lysis buffer, and the cells were washed and resuspended in PBS. The total number of cells derived from each mouse was counted by a hemocytometer for further staining.

Fluorescence-activated cell sorting analysis

Spleen or CNS-derived inflammatory cells (0.5-1 × 106) were washed with PBS, then incubated for 30 min at room temperature in the dark with specified antibodies such as anti-CD4-FITC, anti-CD3-APC, anti-CD8-PE or anti-B220-PE-cy5.5. Nonspecific staining was determined by incubating cells with labeled isotype-matched. Staining kit were purchased from Thermo Fisher Scientific Inc. and used according to the manufacturer’s instructions. Data collection was performed using a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, USA), and analyzed with Cell Quest.

Semi-quantitative RT-PCR

Total RNA was isolated from the brains of the mice with Trizol (Invitrogen, USA) in accordance with the manufacturer's guidelines. The total RNAs were reverse-transcribed (RT) to cDNA using a Gene Amp RNA PCR kit with oligo dT primer. PCR amplification of each cDNA target (IFN-γ, IL-10 and TGF-β) was performed from the same RT reaction according to the manual:

IFN-γ	forward 5'TCCTGCAGAGCCAGATTATCTCT 3' reverse 5'ACCTCAAACTTGGCAATACTCATG 3'	169bp
TGF-β	forward 5'-TGGACCGCAACAACGCCATCTATGAGAAAACC-3' reverse 5'- TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT-3'	525bp
IL-10	forward 5'-GCTCTTACTGACTGGCATGAG3 reverse 5'-CGCAGCTCTAGGAGCATGTG3'	105bp

Amplification conditions including annealing temperatures, number of cycles, and extension times were optimized for each target. PCR products were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium

bromide, and were visualized under UV light. The density of the band was quantitated using a Digital Imaging System.

Statistics analysis

Data were analyzed by the SPSS statistical program. Quantitative results were expressed as mean ±standard deviation. Data comparison between two groups was analyzed by Student's unpaired 2-tailed t test. One-way ANOVA was utilized for multiple group comparison followed by post hoc analysis with Dunnett's test to identify significant groups. A P value less than 0.05 was taken as a significant difference for all statistical analyses.

Results

2-BFI attenuated neurological deficits of EAE mice

In this study, immunization with MOG35-55 induced 100% mice (8/8) in the EAE-control group developed typical clinical signs of EAE after 14 days from immunization, while 50% mice (4/8) in the EAE-2BFI group showed clear EAE symptoms within 14 days of 2-BFI treatment, indicating strong neuroprotective properties of 2-BFI against EAE. In all the EAE mice, the EAE-control group developed symptoms of neurological deficits around 13 days (13.38 ± 2.13) post immunization (d.p.i), whereas the latency seemed to be prolonged to 14 days (14.50 ± 2.65, P = 0.44) with 2-BFI treatment. The symptoms gradually aggravated and reached a peak around 17 d.p.i (17.13 ± 1.13) in EAE-control group and 18 d.p.i (18 ± 2.65) in the EAE-2BFI group, indicating that 2-BFI may extend the time to peak clinical signs (3.50 ± 1.00 vs 3.00 ± 0.93, P = 0.40). Meanwhile, the max clinical scores of the EAE-2BFI group were significantly reduced compared with those of the EAE-control group (5.75 ± 1.71 vs 9.38 ± 1.92, P = 0.0098) (Table 1).

Table 1
Comparison of clinical data of EAE mice with or without 2BFI treatment.

Group	Incidence (%)	Latency (d)	Time to peak clinical signs (d)	Max clinical score
EAE-control	100 (8/8)	13.38±2.13	3.00±0.93	9.38±1.92
EAE-2BFI	50 (4/8) *	14.50±2.65	3.50±1.00	5.75±1.71 **
$^{*}P < 0.05$, $^{**}P < 0.01$ compared with EAE-control group.				

We noticed that mice in the EAE-control group developed ascending flaccid paralysis with the appearance of a flaccid tail around 11 to 13 d.p.i, and quickly deteriorated to hind leg paralysis and eventually complete paralysis. EAE-suffering mice display less activity, less feeding behavior as clinical signs became severe accompanied with the loss of body weight. The mobility deficits and severity of mice in the EAE-2BFI group were ameliorated, and the mean clinical score was significantly downregulated from 15 d.p.i to the day they were euthanized (Fig. 1). These data confirmed that 2-BFI improved the progression of EAE by attenuation of neurological deficits of mice.

2-BFI ameliorated the infiltration of inflammatory cells and demyelination in the CNS of EAE mice

We further used H&E staining to evaluate the inflammatory cell infiltration in the spinal cord of the mice. In the CFA (complete Freund's adjuvant)-control group, no detectable inflammatory cell infiltration showed in the tissues. Meanwhile, serious inflammatory cell inflammation and perivascular cuffing were observed in the lumbar spinal cords, whereas the pathological changes of 2-BFI-treated mice were significantly alleviated with mild inflammatory invasion observed under the spinal meninges (Fig. 2A). To evaluate the severity of demyelination, LFB staining was adopted to detect demyelination in the cerebral cortex sections of the mice. As expected, the EAE-control group showed typical signs of loose myelin while demyelination was reduced in the EAE-2BFI group based on LFB staining when compared with the EAE-control group (Fig. 2B). Consistently, the average pathological scores were markedly reduced in the EAE-2BFI group (1.75 ± 0.96 vs 3.25 ± 0.89, $P = 0.022$, Fig. 2C). The color intensities of LFB were elevated in the EAE-2BFI group compared those of the with EAE-control group (0.75 ± 0.13 vs 0.38 ± 0.06 normalized to the CFA-control group, $P < 0.001$, Fig. 2D). The amelioration and reduction of pathological lesions were consistent with the attenuation of clinical morbidity with 2-BFI treatment.

2-BFI regulated lymphocyte subsets in the spleen and CNS of EAE mice

To explore the cell-mediated immunity mechanisms underlying the neuroprotective effects of 2-BFI, we used flow cytometry to examine the expression of lymphocyte subsets in the periphery and CNS. Spleen and CNS lymphocytes were isolated separately from the same immunized mice as described above. As shown in Fig. 3A, the splenic CD4+, CD8 + T cells and CD28 + lymphocytes were decreased both in the EAE-control group and EAE-2BFI group compared with the CFA-control group. Moreover, quantification analysis confirmed that the percentages of CD28 + lymphocytes in the spleen were reduced in the EAE-2BFI group compared with the EAE-control group (10.92 ± 6.04% vs 19.43 ± 7.1%, $P = 0.022$, Fig. 3C). In contrast to the T cells, in the spleen of EAE mice, the percentages of B cells and CD39 + lymphocytes were increased compared with the CFA-control group, while 2-BFI treatment dramatically downregulated the ratio of B cells compared with that of the EAE-control group (31.22 ± 10.05% vs 54.35 ± 10.6%, $P = 0.007$, Fig. 3C).

In the CNS, very few inflammatory lymphocytes were detectable in the CFA-control group. CD4+, CD8 + T cells and CD28 + lymphocytes of the CNS infiltrates were decreased with 2-BFI treatment (Fig. 3B), but only the level of CD4 + T cells showed statistical significance compared with the EAE-control group (10.56 ± 10.33% vs 25.67 ± 12.73%, $P = 0.021$ Fig. 3D). Correspondingly, 2BFI significantly up-regulated the ratio of activated cells in the B cells (74.04 ± 22.29% vs 45.01 ± 18.29%, $P = 0.01$, Fig. 3D) and CD39 + lymphocytes (77.79 ± 13.96% vs 62.69 ± 13.7%, $P = 0.049$, Fig. 3D) in the CNS of EAE mice. These results suggested that the regulation of lymphocyte subsets may be the key mechanism of the therapeutic effects of 2-BFI against EAE.

2-BFI inhibited proinflammatory cytokines expression while promoted anti-inflammatory cytokines in the CNS of EAE mice

As we had proven that 2-BFI could regulate the expression of lymphocyte subsets in the CNS of EAE mice, it was necessary to observe how the related inflammatory cytokines affected the immune responses. RT-PCR was employed to detect the mRNA expression of the proinflammatory and anti-inflammatory cytokines (including IFN-γ, IL-10 and TGF-β) in the brains of the mice. RNA extraction, cDNA synthesis, imaging and semi-quantitation were performed as described in the Methods (Fig. 4A). We found that the mRNA level of proinflammatory cytokine IFN-γ which was previously reported as the key inflammatory factor in EAE-2BFI group were dramatically decreased compared with the EAE-control group (1.23 ± 0.49 vs1.87 ± 0.27, $P = 0.006$). Meanwhile, 2-BFI treatment significantly enhanced the mRNA expression of IL-10 (1.36 ± 0.51 vs 0.87 ± 0.27, $P = 0.028$) and TGF-β (1.91 ± 0.55 vs 1.04 ± 0.43, $P = 0.0034$) compared with the EAE-control group (Fig. 4B). The imbalance of expression of proinflammatory and anti-inflammatory cytokines was consistent with the lymphocyte subsets in the CNS of EAE mice regulated by 2-BFI.

Discussion

EAE is mediated by the imbalance of lymphocytes

EAE is an animal model for T cell-mediated autoimmune disease, which mimics the pathogenesis of MS. In MOG-induced EAE, activated T cells cross the BBB and bind to the antigen presenting cells, which further promotes the activation of B cells and macrophages by stimulating the secretion of a large number of chemokines and cytokines. Peripheral T cells, monocytes and B cells are recruited to access the brain parenchyma, and result in inflammation and demyelination of the CNS [14]. Current studies have suggested that the migration of autoreactive myelin-specific T cells from peripheral blood into the CNS is an important initiating factor for the onset of EAE. After peripheral T cells were activated, only a small number of lymphocytes crossed the BBB and infiltrated the CNS, causing mild neurological deficit. With the enhanced permeability of the BBB, the CNS was invaded by millions of pro-inflammatory lymphocytes and macrophages in the second wave within hours, which was consistent with the clinical manifestations of MS, further suggesting that T cell migration plays a central role in the pathogenesis of MS [24, 25]. In recent years, attention has been given to the migration of CD8 + T cells into the CNS during this process. It has been reported that in the pathogenesis of MS, CD8 + T cells reacted directly with demyelination and damage of axons by releasing granzyme B [26]. In EAE, the number of CD4 + T and CD8 + T cells that infiltrated the CNS were considered to be directly correlated with the progression and severity of EAE [27]. In the acute phase of EAE, B cell recruitment to the infiltrating areas of the CNS was also observed. Recent studies have demonstrated the functions of B cells in the immune system, including secreting immunoglobulin and cytokines, antigen presentation, and regulating effector T-cell differentiation [28]. It was generally considered that B cells play a key role in the regulation of EAE. In the process of interaction between T and B cells, self-reactive B cells act as efficient antigen presenting cells, and enhance Th2 polarization of MOG-specific T cells rather than Th1 [29]. Studies confirmed that B cells regulate the recovery of EAE by secreting IL -10, TGF- β and IL-35, and block the activation of T cells and macrophages [30, 31]. B cell deficiency is associated with numerous immunological abnormalities.

Therefore, B cells are considered to have potential therapeutic effects for a variety of autoimmune diseases [32].

2-BFI regulated the lymphocytes subsets in the CNS of mice with EAE

2-BFI, a high affinity ligand for I2R, is mainly distributed on the outer membrane of the mitochondrial of astrocytes, which are important components of the BBB [33]. In the early phase of EAE, it was noticed that activated astrocytes lose the perivascular end-feet, resulting in increased BBB permeability [34]. The appropriate dose of 2-BFI exhibits protective properties against astrocyte damage in an ischemic stroke [35, 36]. It was reported that 2-BFI can enhance the intercellular tight junction *via* the downregulation of MMP-9 expression levels, and suppress vascular endothelial permeability to preserve cerebral microvascular endothelial integrity[37, 38]. Our previous studies demonstrated that 2-BFI reduced the disruption of the BBB and alleviated oxidative stress in the CNS of EAE mice [20]. In this study, 2-BFI was proven to effectively reduce the incidence and alleviate the symptoms of neurological deficit in EAE mice. The infiltration of inflammatory cells and demyelination in the CNS of EAE mice were also ameliorated by 2-BFI treatment. Using flow cytometry to analyze the subsets of lymphocytes in the spleen and CNS of EAE mice, we found that the ratio of CD4 + T and CD8 + T cells in the spleen were reduced compared with that of the CFA-control group, and these proinflammatory lymphocytes were only detected in the CNS of the EAE mice, indicating that the MOG35-55 stimulated T cell migration from the periphery to the CNS during the pathogenesis of EAE. We further discovered that the percentage of CD4 + T cells in the CNS of EAE mice treated with 2BFI were significantly reduced than that in the EAE-control group, and the ratio of CD8 + T lymphocytes also tend to decreased, suggesting that 2-BFI inhibited the migration of inflammatory lymphocytes into the CNS. It is worth noting that 2BFI did not increase the CD4 + T and CD8 + T cells in the spleen. We want to further explore the effect of 2BFI on the proliferation and differentiation of peripheral T cells. CD28 is recognized as an important component of the B7/CD28 family, the classical costimulatory signal during naive T-cell activation [39, 40]. It was confirmed that CD28 coordinated T cell proliferation, differentiation and survival, and was considered to play an key role in the progression of MS [41, 42]. We found that with the intervention of 2-BFI, the peripheral CD28 expression of EAE mice was markedly downregulated, while the CD28 + lymphocytes in the CNS were not significantly decreased. These results indicate that 2-BFI may directly affect the proliferation and differentiation of peripheral inflammatory T cells and delay the progression of EAE by reducing the expression of CD28. Moreover, this study indicated that the percentage of B cells in the CNS of the EAE-2BFI group was significantly upregulated as well as obviously decreased in the spleen compared with those in the EAE-control group. The activation and migration of B cells depend on the stimulation signals provided by T cells. CD39 is mainly expressed on B cells, dendritic cells and Treg cells, and is considered to be a marker of activation of B cells [43]. We discovered that the ratio of CD39-labeled lymphocytes in the CNS of EAE mice was also markedly increased by 2-BFI intervention. Previous studies have confirmed the significant role of immune regulation of CD39 by clearing the pro-inflammatory stimuli [44]. Therefore, it is speculated that 2BFI promotes the migration of B cells to the CNS of EAE mice, increases the ratio of activated B cells and exerts immunomodulatory effects against EAE.

2-BFI regulated the expression of cytokines in the CNS of mice with EAE

To validate the aforementioned results, we further examined the expression of cytokines in the CNS of the mice. We found that in the EAE-2BFI group, the level of IFN- γ, which is mainly produced by Th1 cells, decreased significantly, while the expression of anti-inflammatory cytokines such as IL-10 and TGF- β secreted by activated B cells dramatically increased. It has been reported that IL-10 inhibits the progression of EAE mediated by Th1 cells [45]. Evans et al. confirmed that B cells alleviate the inflammatory reaction by producing IL-10 to inhibit the differentiation of CD4 + T cell and the activity of effector T cell in animal models of collagen-induced arthritis [46]. A study of a mouse model of systemic lupus erythematosus suggested that anti-inflammatory cytokines secreted by B cells were found to inhibit the proliferation of T cell and the differentiation into Th1 cells [47]. In other animal models including type I diabetes, systemic lupus erythematosus, rheumatoid arthritis and contact dermatitis, B cells were confirmed to play negative regulatory roles during immune responses, and that the transfer of IL-10-secreting B cells can effectively decrease inflammation [48]. It has also been reported that the anti-inflammatory effect of regulatory B cells indirectly affects the proliferation of T cells by inhibiting the antigen presentation of dendritic cells and downregulating the expression of cytokines secreted by CD4 + T cells [30]. Our results further confirmed that 2BFI decreased the proliferation and activation of CD4 + T cells, and elevated the ratio of B cells in the CNS of EAE mice, suggesting that 2-BFI can regulate the lymphocyte subset rates to ameliorate the severity of the disease.

Conclusions

In this study, we demonstrated that the neuroprotective effect of 2-BFI against EAE was mainly achieved by inhibiting the activation and proliferation of pathogenic T lymphocytes and upregulating the ratio of B cells to reverse the immune imbalance induced by EAE. Further studies on I2R receptor ligands are expected to clarify the specific mechanism and to provide a new idea in the treatment for neuroimmune diseases.

Abbreviations

BBB: Blood-brain barrier; CFA: Complete Freund’s adjuvant; CNS: Central nervous system; H&E: Hematoxylin-Eosin staining; IFA: Incomplete Freund’s adjuvant; IFN-γ: Interferon-γ; IL-10: Interleukin-10; I2R: Imidazoline 2 receptor; LFB: Luxol Fast Blue staining; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; PBS: Phosphate-buffered saline; PT: Pertussis toxin; RT-PCR: Reverse Transcription–Polymerase Chain Reaction; Treg: Regulatory T cell; 2-BFI: 2-(-2-benzofuranyl)-2-imidazoline

Declarations

Ethics Approval and consent to participate

Animal experiments were conducted in accordance with the ARRIVE guidelines, and were approved by the Ethics Committee of Animal Experiments at Wenzhou Medical University.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this manuscript.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This study was supported by Youth Program of National Natural Science Foundation of China [grant number 81701058], Shandong Academy of Sciences [grant number ZR2017PH027] and China Postdoctoral Science Foundation [grant number 2017M612288].

Authors' contributions

Xiaofeng Shang and Nana Xi analyzed the expression of lymphocytes subsets in the CNS and spleen of mice with EAE. Tan Wang performed the histological examination and was a major contributor in writing the manuscript. Xiaofeng Shang and Tan Wang contributed equally to this work. All authors read and approved the final manuscript.

Acknowledgments

All animal experiments were supported by Animal Experiments Center of Wenzhou Medical University.

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Figures

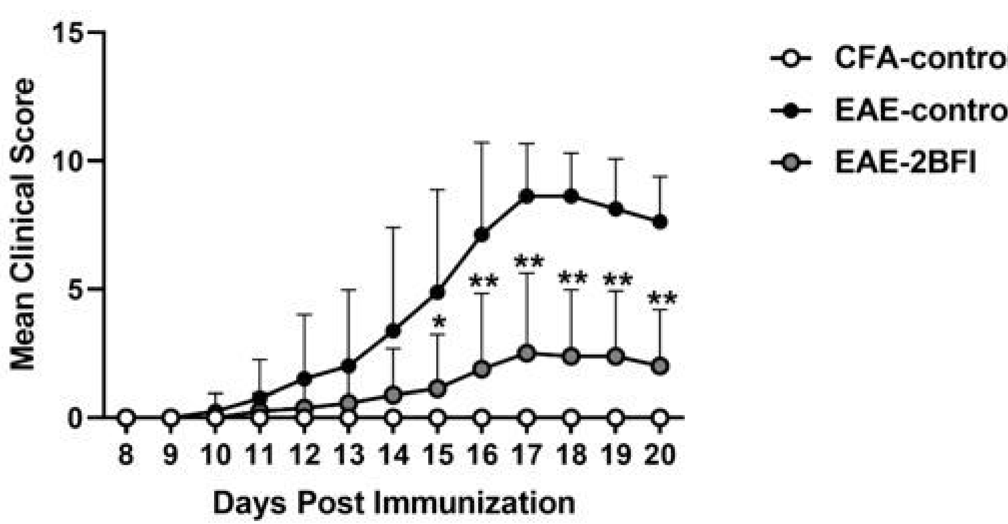

Figure 1

2BFI treatment reduced the daily mean clinical score of EAE mice by attenuation of neurological deficits. * indicates P < 0.05 compared with the EAE-control group. ** indicates P < 0.01 compared with the EAE-control group.

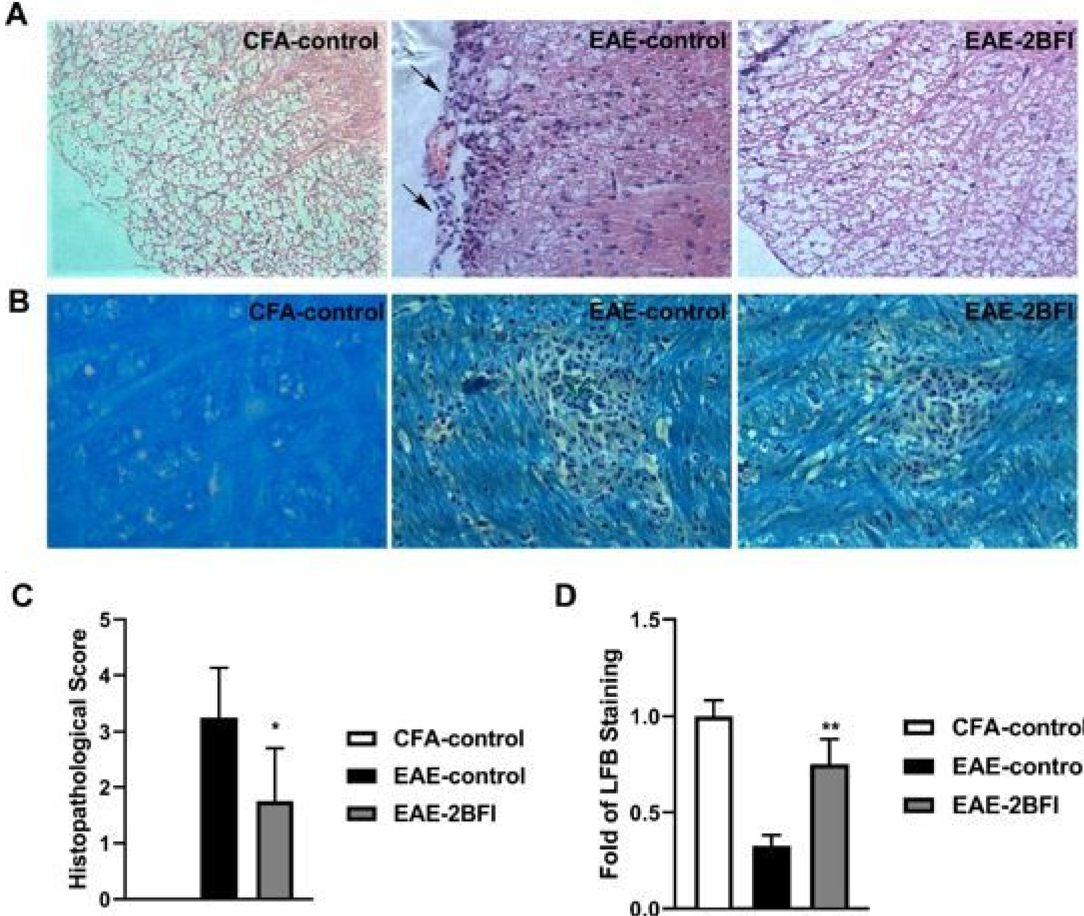

Figure 2

2BFI treatment ameliorated the infiltration of inflammatory cells in the spinal cords and demyelination in the brain of EAE mice. (A) The lumbar spinal cords of mice were sectioned and examined using H&E staining. EAE-control showed exacerbated tissue damage while 2BFI reduced EAE pathology. Arrows indicates the inflammatory cell infiltration. (B) LFB staining showed that the demyelination of cerebral cortex was more palliative in 2BFI group compared with EAE-control group. (C) 2BFI treatment significantly down-regulated the histopathological score of inflammatory in the EAE mice. (D) The LFB color intensities were up-regulated with 2BFI treatment, normalized to CFA-control group. * indicates $P < 0.05$ compared with the EAE-control group. ** indicates $P < 0.01$ compared with the EAE-control group.

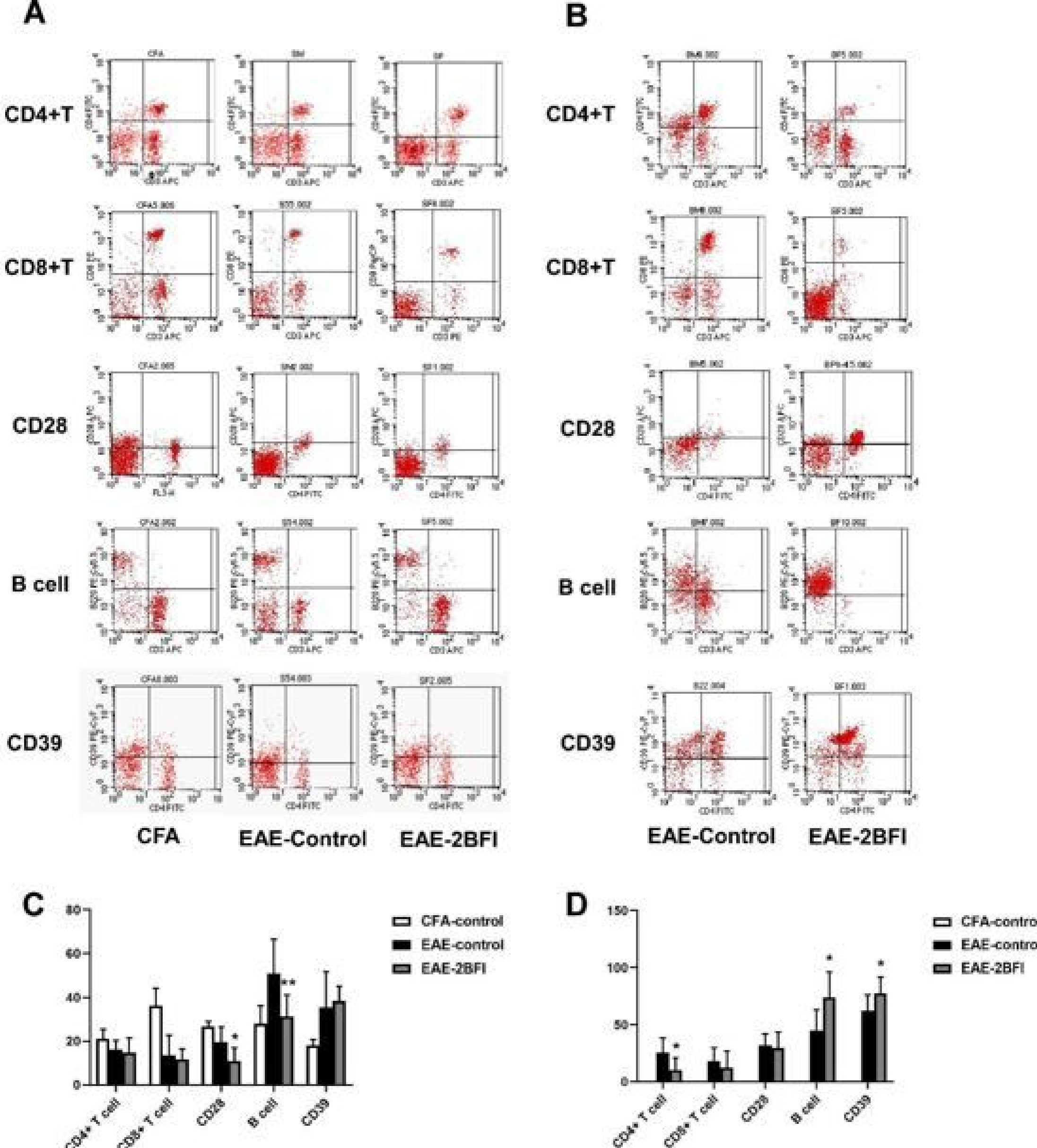

Figure 3

2BFI regulated lymphocytes subsets in the spleen and CNS of EAE mice. (A) Proportion of lymphocyte subsets in the spleen of mice determined by flow cytometry. B-cell numbers were calculated from the percentages of total cells which stained as B220+. (B) Proportion of lymphocyte subsets in the CNS of mice determined by flow cytometry. No lymphocytes were not detected in the CNS of CFA-control group. (C) Quantification of lymphocytes in the spleen of mice by flow cytometry. (D) Quantification of lymphocytes the CNS of mice by flow cytometry. * indicates $P < 0.05$ compared with the EAE-control group. ** indicates $P < 0.01$ compared with the EAE-control group.

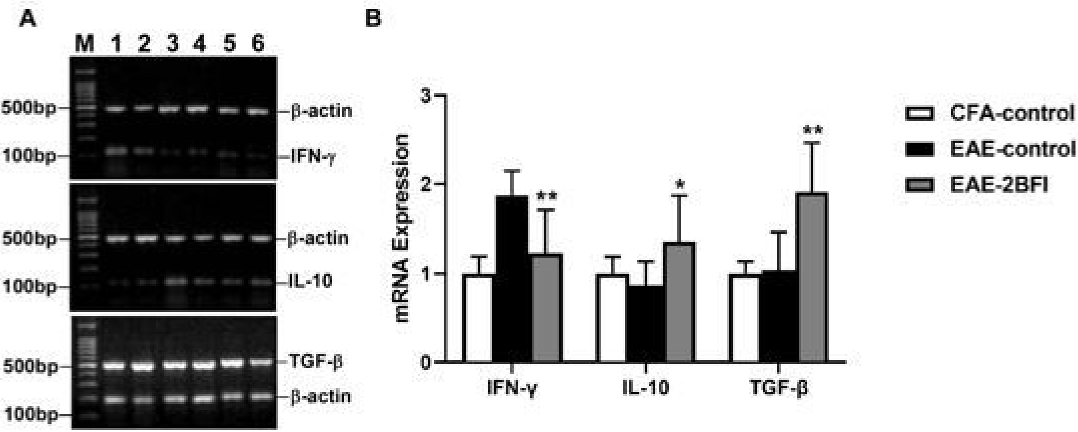

Figure 4

2BFI treatment inhibited the mRNA expression of proinflammatory cytokines while promoted the expression of anti-inflammatory cytokines in the CNS of EAE mice. (A) RT-PCR was used to detect the mRNA expression of cytokines in the cerebral cortex of mice. M: Marker; 1, 2: EAE-control group; 3, 4: EAE-2BFI group; 5, 6: CFA-control group. (B) Semi-quantitatively of the expression levels of mRNA, normalized to CFA-control group. * means P < 0.05 compared with the EAE-control group.