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Protective and therapeutic role of Bilobalide in cuprizone-induced demyelination

Ruo-Xuan Sui[a], Qiang Miao[a], Jing Wang[b], Qing Wang[a], Li-Juan Song[a], Jing-Wen Yu[c], Liang Cao[d], Wei Xiao[d], Bao-Guo Xiao[e,*], Cun-Gen Ma[a,b,c,**]

[a] The Key Research Laboratory of Benefiting Qi for Acting Blood Circulation Method to Treat Multiple Sclerosis of State Administration of Traditional Chinese Medicine, Shanxi University of Chinese Medicine, Taiyuan 030024, China
[b] The First Clinical College, Shanxi Medical University, Taiyuan 030001, China
[c] Institute of Brain Science, Shanxi Datong University, Datong 037009, China
[d] Key Laboratory of New-tech for Chinese Medicine Pharmaceutical Process, Lianyungang, China
[e] Institute of Neurology, Huashan Hospital, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200025, China

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ABSTRACT

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system characterized by recurrent and progressive demyelination, neuroinflammation and oligodendrocyte loss. The cuprizone (CPZ) model is characterized by primary and reversible demyelination, accompanied by oligodendrocyte loss and neuroinflammation. In the current study, we explored the efficiency of Bilobalide in the demyelination and remyelination. The results demonstrate that Bilobalide improved behavioral abnormality and promoted remyelination in the corpus callosum by using Luxol Fast Blue, Black Gold II and myelin basic protein (MBP) staining. We for the first time found that CPZ caused the splenic atrophy and induced the formation of myelin oligodendrocyte glycoprotein (MOG) antibody, which was attenuated by Bilobalide. Thus, Bilobalide decreased the loss of O4+ oligodendrocytes possibly through MOG antibody-dependent cell cytotoxicity. Bilobalide also prevented the infiltration of $CD4^+$ T cells, $CD68^+$ macrophages and $B220^+$ B cells within the brain, and reduced the inflammatory microenvironment mediated with $Iba1^+iNOS^+$ and $Iba1^+NF\text{-}kB^+$ microglia after CPZ challenge, accompanied by the inhibition of IL-1β and IL-6 in the brain. These results identify a potent therapeutic efficiency for Bilobalide and highlight clear pleiotropic effects of the compound beyond specific autoantibody and inflammatory microenvironment in CPZ-mediated demyelination.

1. Introduction

Multiple sclerosis (MS) is T cell-mediated autoimmune disease in the central nervous system (CNS), characterized by demyelination, inflammatory infiltration, oligodendrocyte loss and axonal damage [1]. Because the causative agent or trigger factor of MS is not fully understood, the therapeutic effect is still challenging. The current treatment of MS with immuno-modulating drugs mainly includes interferon beta (IFN-β), glatiramer acetate, natalizumab and fingolimod, but it is still not clear whether immune dysregulation is responsible for the formation of new lesions [2]. Although a variety of FDA-approved drugs are available for MS, we are still far from cure of MS.

Cuprizone (CPZ)-induced demyelination is often used to mimic the pathology of human MS, which is characterized by apoptosis of primary oligodendrocytes and demyelinating lesions, particularly in the corpus callosum (CC) [3]. Primary oligodendrocyte apoptosis and microglia activation are the major histopathological features of the CPZ model. Remarkably, some aspects of the histopathology in CPZ model are similar to those found in MS [4]. CPZ exposure also causes behavioral changes, damages motor skills and affects mood, as that observed in clinical demyelinating disorders. The damage of myelin sheath induced with CPZ was predictable and reached peak demyelination in the CC after 4 weeks of consecutive CPZ exposure [5]. Therefore, CPZ-induced demyelination is a good experimental model for reproducing some key features of demyelinating diseases, including MS. At the same time, the CPZ model is also an appropriate pharmacological model to explore

* Corresponding author at: The Key Research Laboratory of Benefiting Qi for Acting Blood Circulation Method to Treat Multiple Sclerosis of State Administration of Traditional Chinese Medicine, Shanxi University of Chinese Medicine, Taiyuan 030024, China.
** Correspondence to: C.-G. Ma, The Key Research Laboratory of Benefiting Qi for Acting Blood Circulation Method to Treat Multiple Sclerosis of State Administration of Traditional Chinese Medicine, Shanxi University of Chinese Medicine, Taiyuan 030024, China.
E-mail addresses: bgxiao@shmu.edu.cn (B.-G. Xiao), macungen2001@163.com (C.-G. Ma).

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myelin protection and regeneration after drug intervention [6].

Extract of *Ginkgo biloba* leaves, a traditional Chinese herbal medicine, has been demonstrated to have therapeutic effects in several cardiovascular and neurological disorders, including ischemic stroke and Alzheimer's disease dementia [7,8]. Bilobalide (BB) is a predominant sesquiterpene triactone constituent that possesses many beneficial effects, such as neuroprotective, anti-inflammatory, antioxidative and anti-apoptotic effects in several experimental models [9–12]. Previously, Bilobalide has emerged as attractive candidates to reduce infarct volume and edema formation in experimental focal cerebral ischemia [10,13], antagonize neuronal damage [14], and accelerate the regeneration of motor neurons in cell culture [15]. Recent studies have indicated that Bilobalide attenuated inflammatory microenvironment and neuronal apoptosis in the frontal cortex and hippocampus of Alzheimer's model [16], declined ischemia-mediated glutamate release in both core and penumbral regions [17], enhanced hippocampal neuronal proliferation and synaptogenesis, and protected against β-amyloid-oligomer-induced synaptic loss [18].

This study aimed to observe the efficiency of Bilobalide in demyelination and remyelination, characterize peripheral cellular and humoral immune responses, and explore the possible cellular and molecular mechanisms for myelin protection and regeneration in CPZ-induced demyelination.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice (10–12 weeks) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Animals were housed in pathogen-free conditions at the experimental center of Shanxi University of Chinese Medicine for 1 week prior to experimental manipulation. The care and use of laboratory animal were in accordance with the guidelines of International Council for Laboratory Animal Science. All experimental protocols were approved by the Ethics Committee of Shanxi University of Chinese medicine, Taiyuan, China.

2.2. CPZ -induced demyelination model

To induce demyelination, mice were fed with 0.2% (w/w) CPZ (Sigma-Aldrich, USA) in chow diet ad libitum for a total of 6 weeks. For CPZ-containing diet intake, both body weight and food consumption were recorded daily [19]. Bilobalide was extracted and separated from ginkgo leaf, and the purity was > 98% by recrystallization and high-performance liquid chromatography (HPLC) separation. After CPZ feeding for 4 weeks, mice were randomly divided into three groups (n = 8 per group) as follows: (i) normal mice fed with a normal diet (Normal); (ii) CPZ mice injected with saline during CPZ feeding (CPZ) and; (iii) Bilobalide-treated mice that were intraperitoneally (i.p.) injected with Bilobalide (40 mg/kg/day) for consecutive 2 weeks during CPZ feeding (CPZ + BB).

2.3. Behavioral tests

Elevated plus maze test (EPM) is an avoidance test based on animal unconditioned fear to open spaces/heights, proclivity toward enclosed/dark spaces and motivation to explore novel environments [20]. Briefly, the EPM consists of a 4-armed wooden platform (50 cm in length, 10 cm in width) in the shape of a plus sign, which is raised 50 cm above the floor. Two arms facing away from each other are enclosed/dark spaces, whereas the remaining two arms keep open. The mice moved freely for 5 min at the start of each experiment. The percentages of time spend in the open arm were used to measure anxiety [21].

Pole test also detect behavioral disorders in mice. Briefly, mice were placed tenderly the pole of a vertical round wood (diameter: 1 cm; height: 50 cm) with gauze-wrapped rough surface enabling mice to grab [22]. The touch-down time (time descending to the bottom of round wood) was recorded. Mice conducted three consecutive trials and the average time was obtained for statistical analysis [23].

2.4. Tissue preparation

Half of the mice in each group (n = 4) were perfused intracardially with saline, followed by 4% paraformaldehyde in PBS. Brains were removed and immersed in 30% sucrose solution. Coronal sections (10 μm) were cut using a cryostat microtome (Leica CM1850) and stored at 4 °C for histopathologic and immunohistochemical staining. Another half of the mice in each group (n = 4) were perfused intracardially with saline only. Brains were removed and quickly stored at −80 °C for the preparation of brain homogenate.

2.5. Myelin staining and analysis

Luxol Fast Blue (LFB) staining: Section was stained in LFB at 56 °C for overnight and washed in 95% ethanol and distilled water to remove excess blue stain [24]. The colour was then differentiated (until white matter was easily distinguishable from gray matter) in lithium carbonate solution for 15 s, followed by further washing in distilled water. Finally, the section was dehydrated and fixed.

Black Gold II staining: Section was incubated in a 0.3% solution of Black-Gold II dissolved in 0.9% saline and heated to 60 °C for 20 min. If myelin impregnation was not complete under microscope, section was returned to the staining solution until all myelinated fibers were darkly stained. After further washing in distilled water, section was fixed in 1% sodium thiosulfate for 3 min.

Myelin basic protein (MBP) immunohistochemistry staining: Section was blocked with 1% BSA/PBS at room temperature (RT) for 30 min, and stained with anti-MBP (1:500, Abcam, USA) at 4 °C for overnight, followed by corresponding secondary antibody at RT for 2 h. Negative controls were treated similarly, but the primary antibodies were omitted. Captured images were analyzed under gray-scale with different gray intensity range, depending on the strength of immunohistochemical signals. Digital background subtraction was done and intensity was inverted in order to achieve positive correlation of staining intensity and brightness as a gray intensity. Mean gray values were measured on three separate slides per animal, in 4 animals/group by Image-Pro Plus software in a blinded fashion.

2.6. Preparation of splenocytes

In the aseptic state, spleen was weighed and photographed after taking out. Suspensions of splenocytes were prepared by grinding the organ through a 40 μm nylon mesh in medium. Erythrocytes were osmotically lysed. After washing with PBS, splenocytes were re-suspended in the complete medium, and adjusted to 5×10^6/ml. Cells were incubated in the presence or absence of $MOG_{35–55}$ (10 μg/ml) for 48 h.

2.7. Flow cytometry analysis

Splenocytes were stained for 20 min at RT in1% BSA-PBS or in 0.3% saponin/1% BSA-PBS buffer with the following panel of antibodies: FITC-CD4 and PE-IFN-γ, PE-IL-17 (eBioscience), and FITC-B220 (eBioscience). Dead cells were excluded and lymphocytes were gated on the basis of forward and side scatter. Background values obtained with fluorochrome conjugate isotype controls were subtracted. At least 10,000 events were collected using flow cytometer (BD Biosciences, USA) and data were analyzed using CellQuest software.

2.8. Myelin oligodendrocyte glycoprotein (MOG) antibody assay

$MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) and a-synuclein ($a\text{-}syn_{123–140}$ (EAYEMPSEEGYQDYEPEA) were produced in an automatic

synthesizer (CL. Bio-Scientific Company, Shanghai, China). The purity was > 95% by high-performance liquid chromatography.

ELISA method: The blood was collected from the orbit of mice and centrifuged at 3000 rpm 4 °C for 10 min. Splenocytes were incubated in the presence of MOG (10 μg/ml) for 48 h. Brains were homogenized and centrifuged at 12,000*g* for 10 min. Serum, supernatant of cultured splenocytes and extract of brain homogenate were collected and stored for −80 °C. $MOG_{35–55}$ (10μg/ml) dissolved in PBS (pH 7.4) was coated in 96 wells at RT for overnight. After washing with PBST, the wells were blocked with 1% BSA/PBS at RT for 1 h. Diluted samples (serum = 1:50 and 1:200, extract of brain homogenate = 1:50) were added and remained at RT for 2 h. After washing with PBST, HRP-conjugated anti-mouse IgG (Abcam, USA) was added at RT for 1 h, and OD value (at 450 nm) was read by multi-scan spectrum.

Dot blot method: $MOG_{35–55}$ and a-$syn_{123–140}$(1 μg/10 μl) dissolved in PBS (pH 7.4) were coated onto a nitrocellulose membrane (Millipore) at RT for 30 min. After washing with PBST, the membrane was blocked with 1% BSA/PBS at RT for 1 h. The serum and supernatant of splenocytes (20 μl) were added and remained at RT for 2 h. After washing with PBST, HRP-conjugated anti-mouse IgG was added at RT for 1 h. Immunoblots were developed with an enhanced chemiluminescence system (GE Healthcare Life Sciences) and measured using Quantity Software (Bio-Rad, Hercules, CA, USA).

2.9. Immunohistochemistry

Brains sections were blocked with 1% BSA/PBS at RT for 1 h, and incubated with anti-O4 (R and D System, USA), anti-CD4 (Abcam, USA), anti-CD68 (Abcam, USA), anti-Iba-1 (BD Bioscience), anti-iNOS (BD Bioscience) and anti-p-NF-kB/p65 (Bio world, USA) at 4 °C for overnight, followed by corresponding secondary antibodies at RT for 2 h. Additional sections were treated similarly, but the primary antibodies were omitted for negative control. Results were observed under fluorescence microscopy in a blinded fashion. Analysis and quantification were performed on three sections per mouse by Image-Pro Plus software.

2.10. Western blot analysis

Brains were homogenized with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, PR China) supplemented with protease inhibitors. After centrifugation at 4 °C 12,000*g* for 20 min, protein concentration in supernatant was measured by BCA kit (Beyotime Institute of Biotechnology, PR China). Total protein (30 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred electrophoretically to nitrocellulose membrane (Millipore). After blocking with 5% skimmed milk at RT for 1 h, the membranes were incubated with anti-MBP (Abcam, USA) and anti-β-actin (Cell Signaling Technology, USA) at 4 °C for overnight, followed by HRP-conjugated secondary antibodies (Abcam, USA) at RT for 1 h. Immunoblots were developed with an enhanced chemiluminescence system (GE Healthcare Life Sciences) and measured using Quantity Software (Bio-Rad, Hercules, CA, USA). β-actin was used as internal reference.

2.11. Cytokine ELISA assay

IFN-γ, IL-10 and IL-17 in supernatant of cultured splenocytes and IL-1β, IL-6 and TNF-α in both supernatant of cultured splenocytes and extract of brain homogenate were measured by a sandwich ELISA kits following the manufacturer's instructions. Determinations were performed in duplicate. The results were expressed as pg/ml.

2.12. Statistical analysis

All statistical analyses were performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test using GraphPad Prism 5 software (Cabit Information Technology Co., Ltd., Shanghai, China). Results are expressed as the mean ± SEM. *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Bilobalide improved behavioral performance and promoted remyelination

Induction of acute demyelination is typically performed by feeding adult mice a diet of 0.2% CPZ. CPZ intoxication is known to induce selective loss of oligodendrocytes, which peaks around 2–3 weeks of exposure followed by massive loss of myelin at 4–5 weeks. At the first week after CPZ feeding, the weight of mice was significantly declined and maintained at a stable but lower weight in subsequent three weeks, as compared to mice with normal diet, which was consistent with previous investigations (data not shown). To determine the demyelination induced by CPZ, we assessed the Black Gold II staining in the corpus callosum of brain after CPZ feeding for 4 weeks. The intensity of Black Gold II in the corpus callosum was obviously decreased in the CPZ-fed mice, as compared to normal diet-fed mice (Fig. 1b), demonstrating a demyelinating response to CPZ at time point for Bilobalide treatment.

To investigate whether Bilobalide can improve demyelination, mice were fed with CPZ diet for 4 weeks and randomized into CPZ and CPZ + BB groups. Mice were i.p. injected with saline or Bilobalide (400 μg/200 μl) daily for 2 weeks when CPZ feeding continued (Fig. 1a). The results show that CPZ diet significantly increased touchdown time and decreased the percentage of time in the open arm compared to normal mice by both Pole and EPM tests (Fig. 1a, $p < 0.001$ and $p < 0.05$, respectively). By contrast, the administration of Bilobalide significantly improved behavioral performance (Fig. 1a, $p < 0.05$ respectively).

CPZ intoxication induced a profound demyelination in the corpus callosum of mice as evidenced by a marked decrease in the intensity of LFB, Black Gold II and MBP staining (Fig. 1c, $p < 0.001$, 0.01 and 0.05 respectively). Because the myelin fibers are formed by oligodendrocytes, we further performed immunofluorescence staining using anti-O4 antibody, a marker for mature oligodendrocytes. The MBP expression and $O4^+$ oligodendrocytes were reduced in the bran of CPZ-fed mice, as compared with normal mice (Fig. 1d and e, $p < 0.05$ and $p < 0.01$ respectively). Taken together, CPZ-fed mice used in this study exhibits an obvious demyelination.

Bilobalide treatment increased the intensity of LFB and Black Gold II staining in the corpus callosum compared to CPZ-fed mice (Fig. 1c, $p < 0.05$, respectively). In addition, the extent of MBP immunohistochemistry staining was also enhanced in identical brain region of mice treated with Bilobalide (Fig. 1c, $p < 0.05$). Simultaneously; there was an increase of MBP expression and $O4^+$ oligodendrocytes after Bilobalide treatment compared to CPZ-fed mice (Fig. 1d and e, $p < 0.05$ respectively), which was consistent with the results from MBP immunohistochemistry staining (Fig. 1c). These results indicated that CPZ-fed mice showed severe demyelination, which was significantly improved by Bilobalide treatment in CPZ-induced demyelinating model.

3.2. Bilobalide regained the volume of the spleen

Unexpectedly, CPZ feeding caused splenic atrophy (Fig. 2a), and reduced splenic weight compared to normal mice (Fig. 2b, $p < 0.001$). Bilobalide treatment regained the volume of spleen (Fig. 2a), and increased the weight of spleen compared to CPZ-fed mice (Fig. 2b, $p < 0.05$).

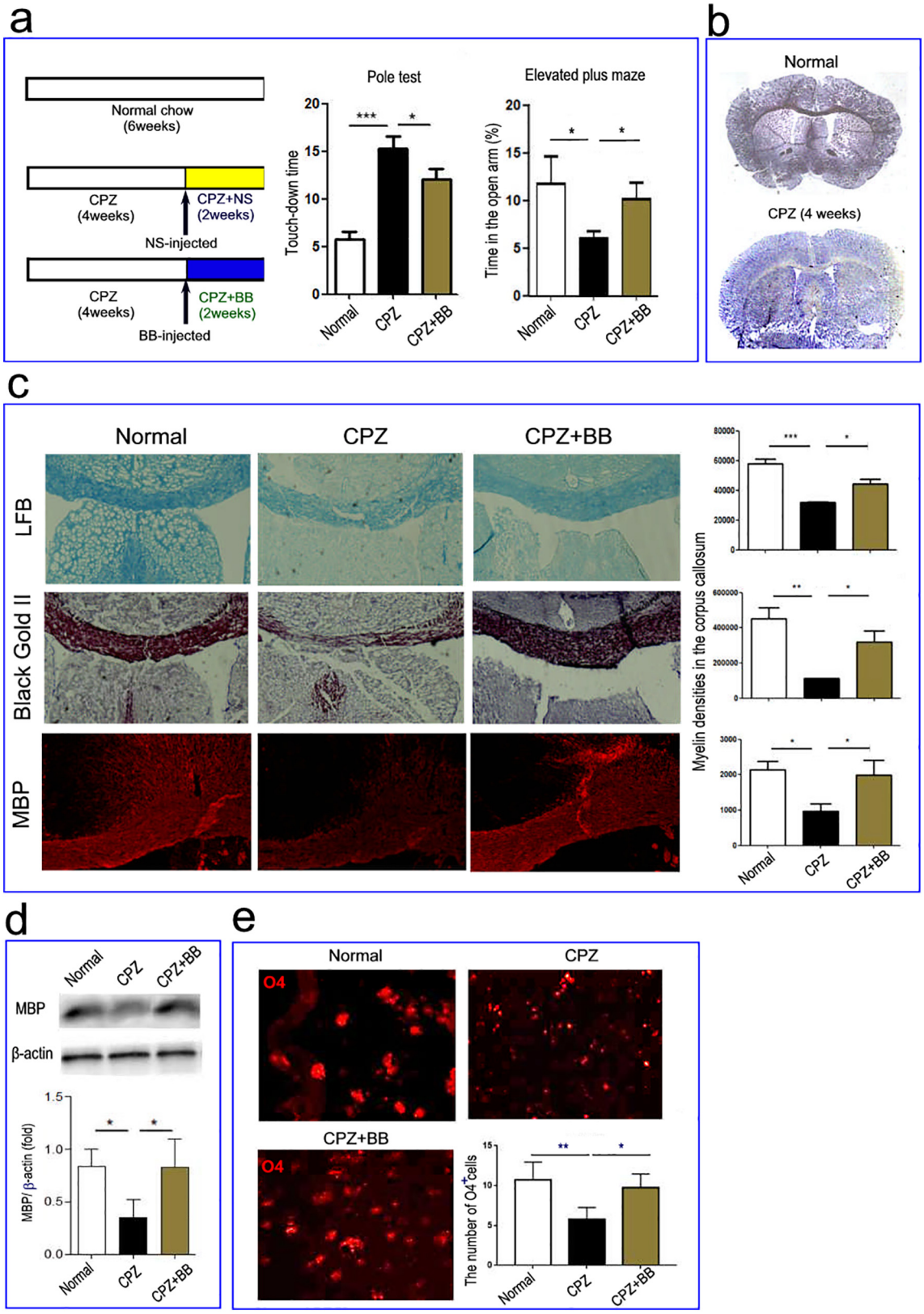

Fig. 1. The improvement in the behaviourology and pathology of CPZ-fed mice by bilobalide treatment. (a) After 4 weeks of CPZ feeding, Bilobalide was treated for 2 weeks without CPZ withdrawal. Pole test and Elevated Plus Maze were used to measure behavioral performance. (b) Pathological changes of demyelination were assessed by Black Gold II staining after 4 weeks of CPZ feeding. (c) Pathological changes of demyelination were detected by Black Gold II staining, LFB staining and MBP immunohistochemistry staining in the corpus callosum of brain after 6 weeks of CPZ feeding or CPZ + Bilobalide treatment. (d) The expression of MBP in the brain was detected by Western Blot. (e) Transcription factor O4+ oligodendrocytes in the striatum were detected by immunohistochemistry staining. The results represent the mean ± S.E.M. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Normal vs. CPZ and CPZ vs. CPZ + BB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

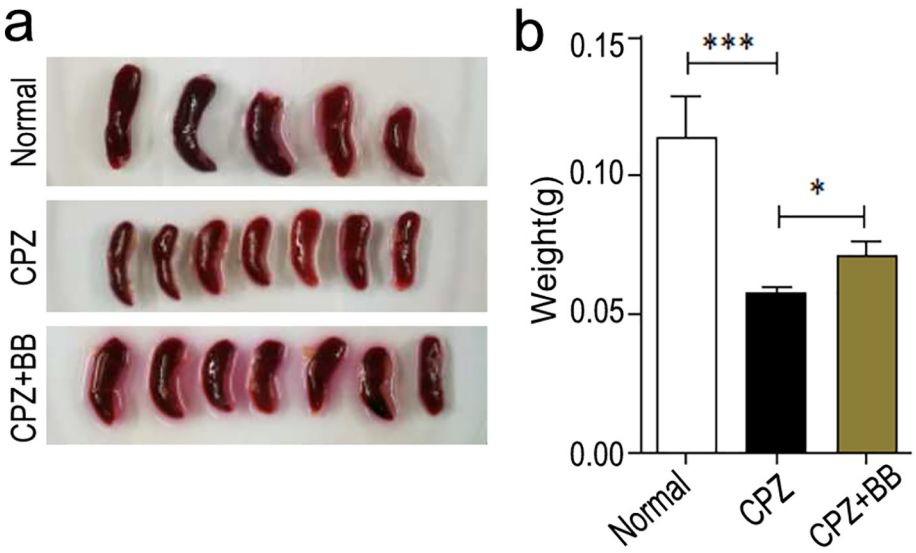

Fig. 2. Bilobalide regained the volume (a) and weight (b) of the spleen in CPZ-fed mice. The results represent the mean ± S.E.M. (*p < 0.05, ***p < 0.001. Normal vs. CPZ and CPZ vs. CPZ + BB).

3.3. Bilobalide inhibited the production of MOG specific antibody

It has been noted that the presence of serum autoantibodies to central nervous system-specific proteins was observed in a group of neural or demyelinating injury [25]. Because oligodendrocytes are the main target cells in CPZ-fed model, we detected $MOG_{35–55}$ specific antibody in serum, supernatant of cultured splenocytes and extract of brain homogenate. As shown in Fig. 3a, compared to normal mice, $MOG_{35–55}$ specific antibody was elevated in serum, supernatant of cultured splenocytes and extract of brain homogenate from CPZ-fed mice ($p < 0.001$ and $p < 0.01$ respectively). The results from dot blot also indicated that CPZ feeding induced the formation of $MOG_{35–55}$ specific antibody in serum (Fig. 3b, $p < 0.001$ respectively). Although the $MOG_{35–55}$ antibody with low titer appears to exist in serum, it was negative in supernatant of culture splenocytes (Fig. 3a) and by dot blot assay (Fig. 3b), revealing that low antibody level in serum should be non-specific. We further tested the specificity of $MOG_{35–55}$ antibody, with $MOG_{35–55}$ and a-$syn_{123–140}$ for coating antigen. The results show that MOG antibody was positive, while a-$syn_{123–140}$ antibody was negative in CPZ-fed mice (Fig. 3c, $p < 0.001$), proving that CPZ-fed mice had specific antibody against oligodendrocyte components.

Bilobalide treatment effectively reduced the titer of MOG antibody in serum, supernatant of cultured splenocytes and extract of brain homogenate by both ELISA and dot blot (Fig. 3a, b and c, $p < 0.001$ and $p < 0.05$ respectively). These results indicate that CPZ feeding had myelin damage, resulting in the production of MOG specific antibody that can penetrate into the brain. In CPZ-fed mice, levels of IL-1β and IL-6 in supernatant of cultured splenocytes were elevated compared with normal mice (Fig. 3d, $p < 0.001$ respectively), which was significantly reduced by Bilobalide treatment (Fig. 3d, $p < 0.001$ respectively).

As compared with normal mice, the percentage of splenic $B220^+$ B cells was significantly increased in CPZ-fed mice (Fig. 3e, $p < 0.001$), which was reduced by Bilobalide treatment (Fig. 3e, $p < 0.001$). No alteration in the percentage of $CD4^+$ T cells was detected among three groups.

To understand whether MOG antibody participates in ongoing cascades of CNS demyelination, we first stained the myelin protein by MBP immunohistochemistry (Fig. 4a). We next evaluated the binding of MOG-IgG to $O4^+$ oligodendrocytes by adding MOG antibody$^+$ serum to brain slices from normal mice. As expected, the overlapping of O4 and IgG immunostaining was observed in both striatum and anterior commissure (Fig. 4b), suggesting that there was a specific binding of MOG antibody on oligodendrocytes. By using anti-mouse IgG secondary antibody, we further detected IgG binding to $O4^+$ oligodendrocytes in brain sections from normal, CPZ and CPZ + BB mice. The results demonstrated that compared with normal mice, $O4^+$ oligodendrocytes were reduced in CPZ-fed mice (Fig. 4c, $p < 0.01$), which was elevated by Bilobalide treatment (Fig. 4c, $p < 0.05$). On the contrary, IgG staining in brain sections from CPZ and CPZ + BB mice was increased on $O4^+$ oligodendrocyte (Fig. 4c, $p < 0.01$ and $p < 0.05$ respectively), indicating that MOG antibody can penetrate into the brain to form specific binding on oligodendrocytes and cause the damage of myelin sheath. There was no significant difference between CPZ and CPZ + BB, possibly because of the loss of $O4^+$ oligodendrocytes in CPZ-fed mice, which affected the binding of MOG-IgG.

3.4. Bilobalide did not alter T cell responses

Due to the splenic atrophy and MOG antibody, we further analyzed the changes of T cell subsets among three groups. The results show that there was no significant difference in the percentage of CD4 + IFN + γ + and $CD4^+IL\text{-}17^+T$ cell subsets in the absence or presence of MOG stimulation (Fig. 5a). The concentration of IFN-γ, IL-10 and IL-17 was detected, but no difference was observed among three groups (Fig. 5b). These results suggest that CPZ feeding or Bilobalide treatment did not change T cell subsets. The results from immunohistochemistry show that a few of CD4+ T cells, CD68+ macrophages and B220+ B cells invaded the surrounding brain parenchyma, which was decreased by Bilobalide treatment (Fig. 5c, $p < 0.05$ and $p < 0.001$ respectively).

3.5. Bilobalide attenuated microglia-associated neuroinflammation

The cuprizone model is characterized by primary and reversible demyelination, due to peripheral immune system-independent myelin injury [26]. Many studies have demonstrated that neuroinflammation exacerbates the severity of demyelinating lesion. We explored the effect of Bilobalide on microglia-associated neuroinflammation, using immunohistochemistry staining of a microglial marker (Iba-1) in the brain. Compared to normal mice, Iba-1^+ microglia were increased in CPZ-fed mice, which was attenuated by Bilobalide treatment (Fig. 6a, $p < 0.01$ and $p < 0.05$ respectively).

iNOS is an important marker of inflammatory M1 microglia, while

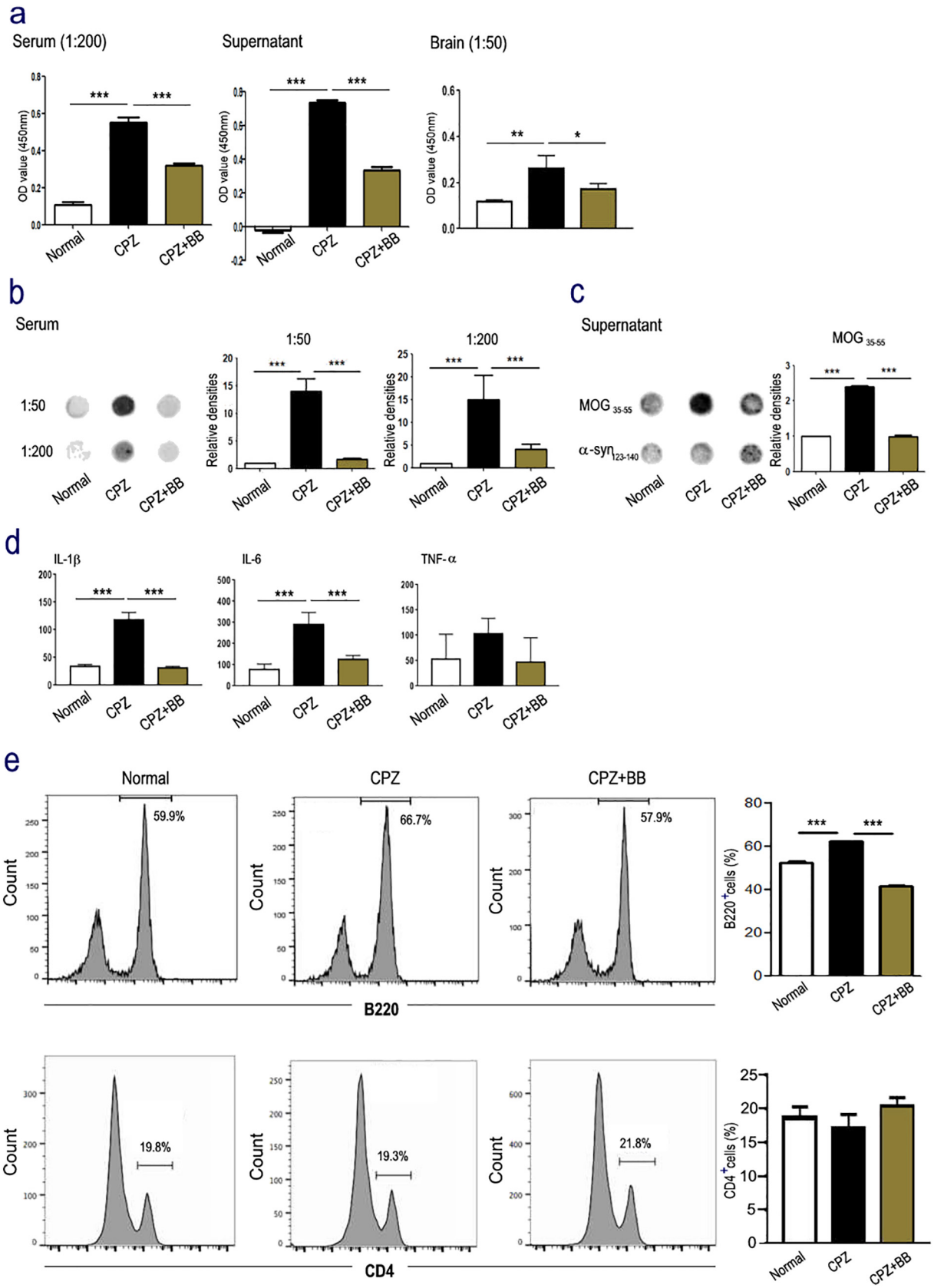

Fig. 3. Bilobalide inhibited the production of MOG antibody in CPZ-fed mice. (a) the titers of $MOG_{35–55}$ antibody in serum, culture supernatant and brain homogenate were determined by ELISA. (b and c) the titers and specificity of $MOG_{35–55}$ antibody were detected by dot blot. (d) the concentration of IL-1β, IL-6 and TNF-α in culture supernatant was measured by ELISA. (e) the percentages of B220+ B cells and CD4+ T cells in lymphocytes of splenocytes was determined by flow cytometry. The results represent the mean ± S.E.M. (*$p < 0.05$, ***$p < 0.001$. Normal vs. CPZ and CPZ vs. CPZ + BB).

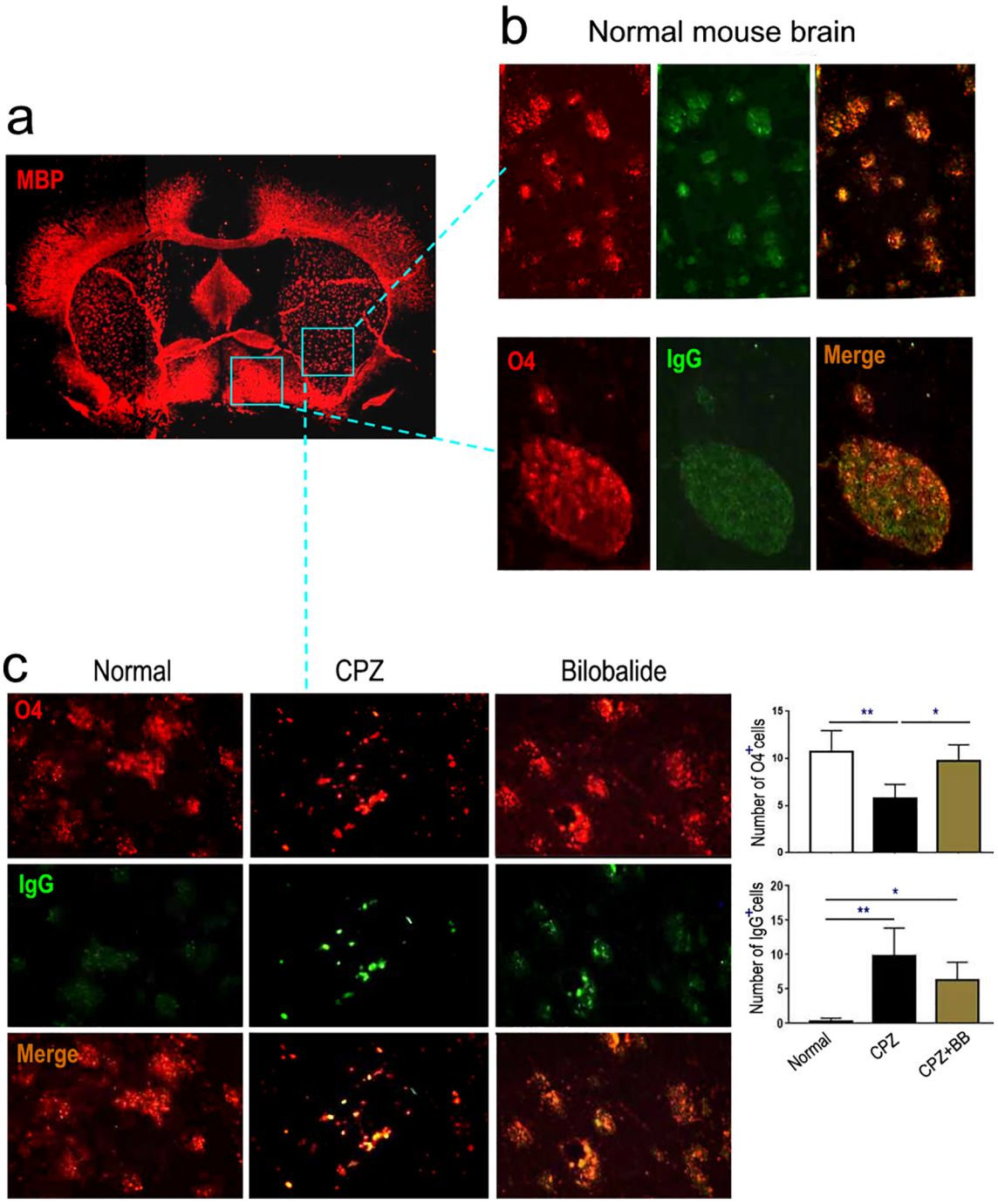

Fig. 4. MOG antibody binds to O4+ oligodendrocytes, which may be related with the damage of oligodendrocytes. (a) computer mapping of MBP immunohistochemistry staining in mouse brain. Blue squares link immunohistochemistry sites in b and c. (b) in normal mouse brain, the binding of MOG IgG to O4+ oligodendrocytes in the striatum and ventral pallidum of brain was detected by using MOG antibody+ serum from CPZ-fed mice. (c) in brain sections from normal, CPZ and CPZ+ BB mice, IgG binding to O4+ oligodendrocyte was detected in the striatum of brain. Micrograph are representative of three independent experiments with similar results. The results represent the mean ± S.E.M. (*$p < 0.05$, ***$p < 0.01$. Normal vs. CPZ and CPZ vs. CPZ + BB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NF-κB is the major inflammatory signaling pathway in microglia. Our results show that in CPZ-fed mice, the number of $Iba1^+iNOS^+$ and $Iba1^+NF\text{-}\kappa B^+$ microglia in the brain was significantly higher than that of normal mice (Fig. 6b and c, $p < 0.001$ respectively). Bilobalide treatment effectively reduced the numbers of $Iba1^+iNOS^+$ and $Iba1^+NF\text{-}\kappa B^+$ microglia (Fig. 6b and c, $p < 0.01$ respectively), revealing that the anti-inflammatory effect of Bilobalide was confirmed by inhibiting the activation of microglia or M1-polarization in CPZ-induced demyelination.

Activated microglia or M1-polarized microglia release inflammatory and cytotoxic media, leading to cell death and neurological dysfunction [27,28]. To confirm the association between the microglia phenotype and inflammatory cytokines, we measured the proinflammatory cytokines IL-1β, IL-6 and TNF-α in the brain. As shown in Fig. 6d, compared to normal mice, the levels of cytokines IL-1β, IL-6 and TNF-α increased in the brain of CPZ-fed mice ($p < 0.001$ and $p < 0.05$ respectively), while Bilobalide treatment inhibited the production of IL-1β and IL-6 ($p < 0.001$ respectively). These results indicated that CPZ-induced

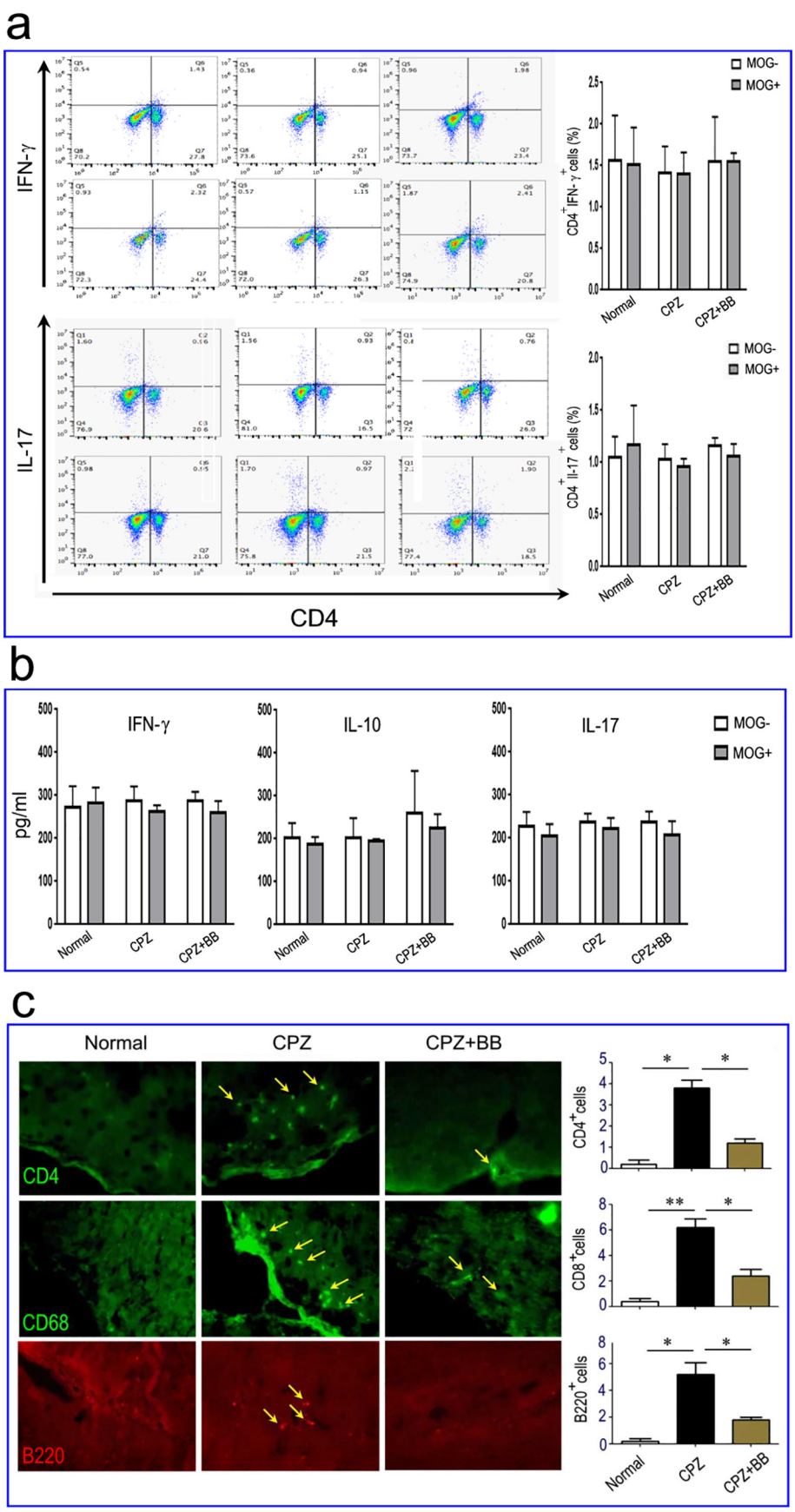

Fig. 5. Bilobalide did not affect T cell response in CPZ-wed mice. (a) the population of CD4 + IFN-γ + and CD4 + IL-17 + T cells in splenic lymphocytes was determined by flow cytometry. (b) the level of IFN-γ, IL-10 and IL-17 in the supernatant of splenocytes was measured by ELISA. (c) the infiltration of CD4 + T cells CD68 + macrophages and B220 + B cells in the brain was measured by immunohistochemistry. The results represent the mean ± S.E.M. (*$p < 0.05$, **$p < 0.01$. Normal vs. CPZ and CPZ vs. CPZ + BB).

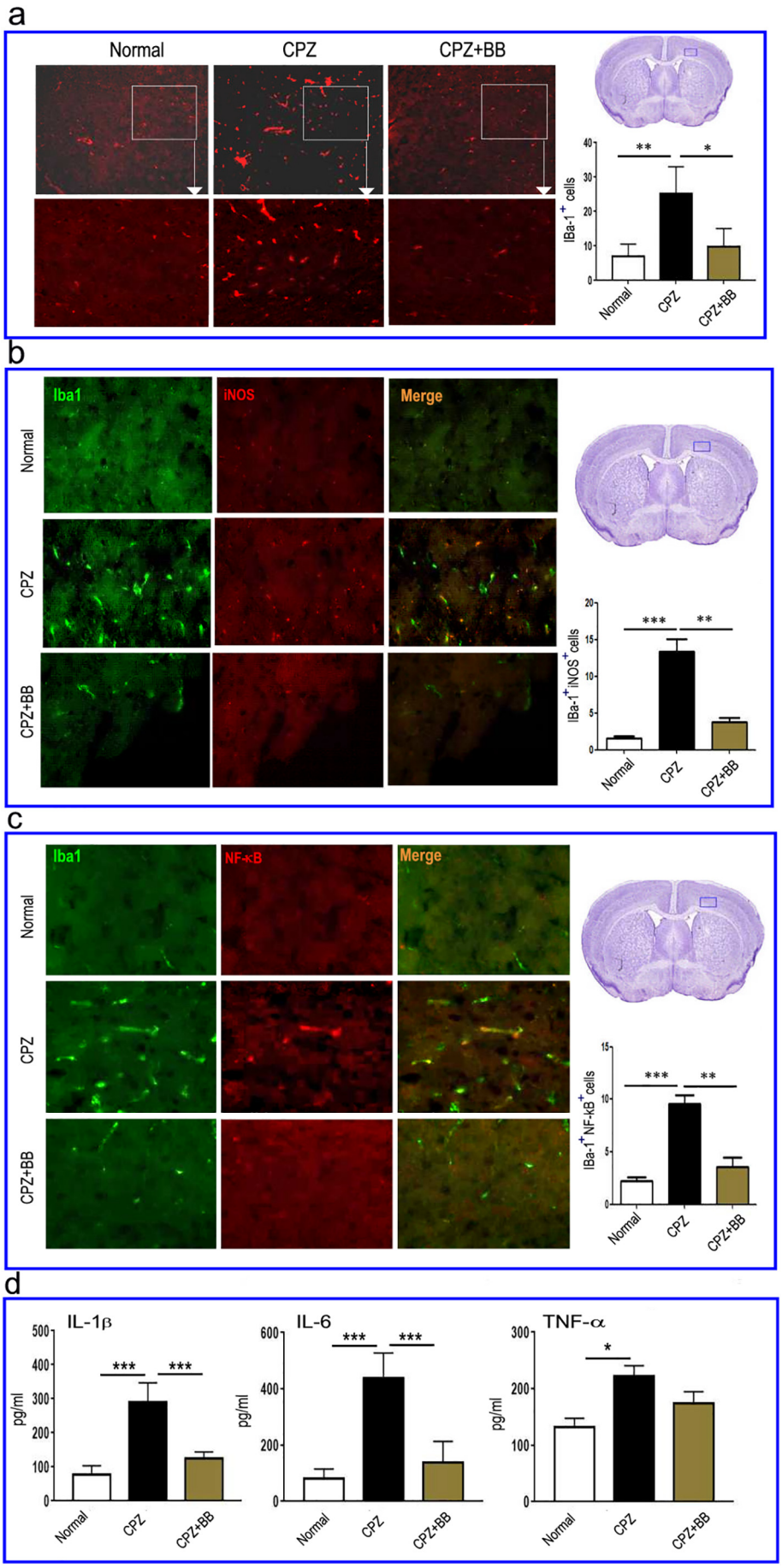

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Fig. 6. Bilobalide attenuated microglia-associated neuroinflammation. (a) Iba-1 + activated microglia was observed in the brain. (b) Iba-1 + NF-κB + microglia and (c) Iba-1 + iNOS + microglia were measured in the brain by double immunofluorescence. (d) the level of IL-1β, IL-6 and TNF-α in brain homogenate was measured by ELISA. The results represent the mean ± S.E.M. (*p < 0.05, **p < 0.01, ***p < 0.001. Normal vs. CPZ and CPZ vs. CPZ + BB).

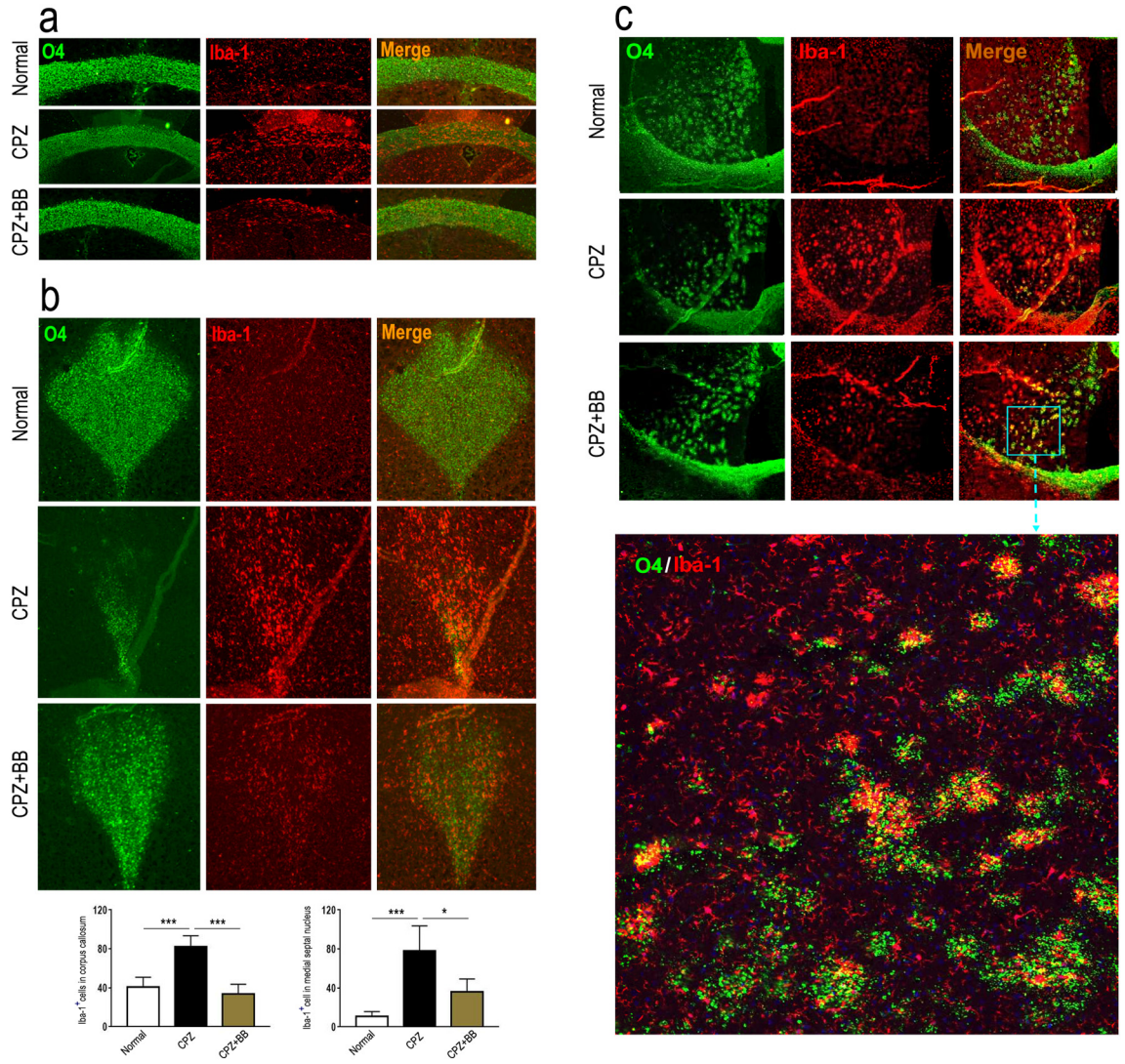

Fig. 7. Bilobalide prevented the migration and enrichment of Iba-1$^+$ microglia that caused the damage of O4$^+$ oligodendrocytes. (a) double immunohistochemistry of O4 (green) and Iba-1 (red) in the corpus callosum; (b) double immunohistochemistry of O4 (green) and Iba-1 (red) in the medial septal nucleus, and (c) double immunohistochemistry of O4 (green) and Iba-1 (red) in the striatum. The enlarged photograph revealed that Iba-1$^+$ microglia migrated and aggregated to O4$^+$ oligodendrocytes. The results represent the mean ± S.E.M. (*p < 0.05, ***p < 0.001. Normal vs. CPZ and CPZ vs. CPZ + BB). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

demyelination is associated with neuroinflammation caused by the activation of M1 microglia, which was attenuated by Bilobalide treatment.

Next, we observed the migration and enrichment of Iba-1$^+$ microglia in different region of myelin sheath. The results showed that compared with normal mice, CPZ-fed mice represented the migration and enrichment of Iba-1$^+$ microglia toward corpus callosum, striatum and medial septal nucleus (Fig. 7a-c). In the striatum, the migration and enrichment of Iba-1$^+$ microglia shifted from edge to middle and exhibited a negative correlation between O4$^+$ oligodendrocytes and Iba-1$^+$ microglia (Fig. 7c), suggesting that the migration and enrichment of Iba-1$^+$ microglia may be closely related to the damage of O4$^+$ oligodendrocytes. Bilobalide treatment can obviously reduce the migration and accumulation of Iba-1$^+$ microglia toward myelin sheath (Fig. 7a-c), which should be beneficial to resist myelin damage mediated by inflammatory microglia.

4. Discussion

Bilobalide is a predominant sesquiterpene trilactone constituent that accounts for 2.9% of the standardized *Ginkgo biloba* extract EGb 761, which has been widely used to treat a variety of neurological disorders involving cerebral ischemia and neurodegeneration [8,29]. The experimental evidence has indicated that Bilobalide possesses many beneficial effects, such as neuroprotective, anti-inflammatory, anti-apoptotic, and anti- oxidative effects in various models [9,10]. In experimental focal cerebral ischemia and Alzheimer's models, Bilobalide exhibited neuroprotective effects [30], which may be related with anti-inflammatory and anti-oxidative efficiency. Here, in the non-immunized demyelinating model, we observed the effect of Bilobalide in the protection and regeneration on the myelin sheath. Whereas the majority of studies were carried out prior to the onset of demyelination, in our present approach Bilobalide intervention was installed when

brain demyelination is definitively established. Therefore, it is possible to explore the myelin regenerative efficacy in CPZ-induced demyelination model.

Histopathological examination shows that mice were more severely demyelinated after CPZ feedings for 4 weeks. At the time point, mice were injected with Bilobalide for consecutive 2 weeks without CPZ withdrawal. We found that Bilobalide treatment significantly improved the anxious state and motor coordination ability in CPZ-induced mice. The three pathological stains were as follows: 1) histology data of Black Gold II and LFB staining clearly indicated that Bilobalide treatment improved demyelination; 2) immunohistochemistry and western blot results showed that Bilobalide treatment enhanced MBP expression in the corpus callosum of brain; 3) immunohistochemistry results also confirmed that Bilobalide treatment reduced oligodendrocyte loss or damage. These data clearly demonstrate that Bilobalide is effective for the myelin protection and/or regeneration in CPZ-induced demyelinating mice.

In this study, one major finding is that CPZ feeding reduced the volume and weight of spleen and induced the formation of $MOG_{35–55}$ specific antibody. At present, there is still no direct evidence to elucidate the cellular and molecular mechanisms of splenic atrophy and MOG antibody in CPZ-fed mice. Previous studies showed that by magnetic resonance imaging, the spleen of patients with ischemic stroke (< 24 h) was significantly atrophy [31]. Other investigators found that the size of spleen decreased following transient middle cerebral artery occlusion (MCAO) in mice [32,33], possibly due to cell apoptosis and loss of functional centers within spleen [32]. Further observation revealed that in MCAO model, catecholamine activated α1-adrenergic receptors on splenic capsule, causing a contraction of smooth muscles in the capsule [34]. Blocking the α1-adrenergic receptors prevented splenic atrophy, revealing that catecholamine regulates splenic response to MCAO through the activation of α-adrenergic receptors [35]. In mice with progressive experimental autoimmune encephalomyelitis (EAE), splenic atrophy was observed and apoptosis of splenocytes was confirmed by TUNEL staining, indicating that splenic atrophy may be related cell apoptosis in spleen [36]. However, certain cell types are relatively resistant to apoptosis in mice with progressive EAE, such as plasma cells [36]. These findings are consistent with our results: 1) The number of B cells increased; 2) the titer of MOG antibody increased, whether the presence of splenic atrophy in patients with MS has not been reported. Therefore, it is necessary to further explore the reasonable explanation of CPZ-induced splenic atrophy.

The next question is how we explain the formation of $MOG_{35–55}$ specific antibody resulting from CPZ feeding. To answer this question, we need to understand whether the blood-brain barrier (BBB) is complete in CPZ-induced demyelinating model. Previous results are inconsistent. Although extensive demyelination was noted and macrophage infiltration was pronounced in the brain, BBB remained intact [37]. Recent study indicated that BBB hyperpermeability precedes demyelination in CPZ-induced demyelinating model [38]. Whether the BBB is intact or not, the other route between the brain and the periphery may also exist. Some studies found that the debris from damaged cells in the nervous system can subsequently reach the cervical lymph nodes and palatine tonsils by passing through the cribriform plate and travelling along basement membranes in the walls of capillaries and cerebral arteries and activate the innate and adaptive arms of the immune system and further stimulate the inflammatory cascade [39]. Therefore, the myelin debris produced from the destructed myelin sheath can enter the blood circulation and cause splenic T and B cell response to myelin debris. Coincide with this hypothesis, other data suggest that after traumatic CNS injury, T-dependent and/or T-independent self-antigens elicit adaptive immune responses with important functional consequences [25,40]. In addition, it was reported that mice with progressive EAE had large numbers of apoptotic cells in certain lymphoid organs. Uptake of apoptotic cells by macrophages and dendritic cells has been shown to induce a variety of immune responses, including altered cytokine profiles [41] and an increase in antibody production [42].

We found that MOG antibody was obviously elevated in the supernatant of cultured splenocytes, indicating that the production of MOG antibody was derived from peripheral immune cells. Our results also showed that anti α-syn antibody was negative, pointing out at least two points: 1) anti MOG antibody is a myelin protein specific antibody; and 2) there is no evidence that CPZ feeding can also cause the damage to dopamine neurons, because MPTP-induced the damage of dopamine neurons can produce anti a-syn antibody (data not shown). Here, we tried to explore whether MOG antibody contributes to the damage of oligodendrocyte damage. In vitro experiments demonstrated that MOG antibody$^+$ serum obtained from CPZ-fed mice can specifically bind to $O4^+$ oligodendrocytes of normal mice. In vivo experiments, IgG was detected on $O4^+$ oligodendrocytes in the brain of CPZ and CPZ + BB mice, which may cause the damage of $O4^+$ oligodendrocytes possibly through inhibiting MOG antibody-dependent cell cytotoxicity. Therefore, our results suggest that MOG antibody can enter the brain and should be pathogenic. Previous studies show that antibodies to GalC and MOG play a major role in destabilizing myelin through MBP breakdown [43]. In the current study, MOG-specific antibodies from MS patients can contribute to EAE exacerbation in a transfer model [44]. Autoantibodies against myelin sheath are also associated with cognitive dysfunction in patients with rheumatoid arthritis, indicating that these autoantibodies may lead to pathological damage in the CNS [45]. Therefore, it is reasonable to explore the inhibition of B cell function as a therapeutic option. Antibody to MOG has been reported in serum and brain biopsy in a small percentage of patients with MS [46,47]. Human antibody to MOG belonging to the complement-activating IgG1 subclass can cause complement-dependent demyelination in both the organotypic brain slice and in EAE [48], indicating that at least in part of MS patients, the MOG antibody may be related to demyelination. Therefore, MOG has received renewed attention as an antibody target in childhood MS and in a small subgroup of adult patients with MS [49]. However, the majority of patients with MS do not have detectable levels of antibodies to MOG, the investigation for other antibody targets needs to be explored.

The next question is by which Bilobalide inhibited the production of MOG specific antibody. A fundamental function of IL-6 is to promote B cell maturation into Ig secreting cells in an autocrine/paracrine way [50]. In murine lupus and humans with SLE, IL-6 plays an important role in sustaining B cell over-activity and auto-antibody production and has a direct role in mediating tissue damage [51]. In this study, we speculate that the inhibition of MOG antibody may be related to the decrease of IL-6 production. In fact, direct evidence on IL-6 in regulating B cell function and MOG antibody production remain to be investigated.

In the cuprizone model, the demyelination coincides with microglial activation. Activated microglia contribute to the death of oligodendrocytes through secretion of pro-inflammatory cytokines. In the present study, we also observed that Iba-1^+ microglia were increased in brain, indicating that cuprizone induced microglia activation. It has reported that activated microglia can cause neurodegenerative diseases through a variety of cytotoxic molecules, especially when microglia are polarized into M1 microglia [52]. iNOS is an important marker of inflammatory M1 microglia, and NF-κB is the major inflammatory signaling pathway in microglia. The activation of inflammatory microglia expressing iNOS and NF-κB will contribute to the formation of inflammatory microenvironment. Our results support the fact that demyelinating events can trigger the recruitment of peripheral immune cells into the brain, which is in line with previous study that CPZ feeding resulted in the recruitment of peripheral leukocytes and the formation of an inflammatory, demyelinating lesion [53]. In vitro oxygen–glucose deprivation and reoxygenation, Bilobalide inhibited the secretion of inflammatory factors in BV2 microglia by inhibiting the

TLRs/MyD88/NF-κB signaling pathways [54]. In vivo cerebral ischemia and reperfusion, Bilobalide decreased the production of inflammatory cytokines by down-regulating p-JNK1/2, and p-p38 MAPK expression [55]. These results accord with the explanation that Bilobalide inhibited microglial activation and inflammatory response in CPZ model by acting on upstream signal pathways of inflammatory molecules. Therefore, Bilobalide treatment reduced myelin damage that may be related with the inhibition of M1 microglia-mediated inflammatory microenvironment. Here, Bilobalide attenuated the production of inflammatory cytokines IL-1β and IL-6, which contributes to the myelin protection. However, it needs further study to elucidate whether the inhibition of microglial inflammatory microenvironment coincided with an improvement in demyelination.

In this study, we were surprised to find that in the brain of CPZ mice, Iba-1 + microglia migrated and enriched toward corpus callosum, medial septal nucleus and striatum, and moved from the edge to the middle line. Simultaneously, the number of Iba-1 + microglia is inversely proportional to O4 + oligodendrocytes, suggesting that the enrichment of inflammatory microglia in these regions is closely related to myelin depletion. However, Bilobalide treatment can significantly inhibit the migration and attachment of Iba-1 microglia toward myelin sheath, which may be related to myelin protection of Bilobalide. Further studies are needed to understand the triggering factors for the migration and accumulation of Iba-1 + microglia, as well as the cellular and molecular mechanisms for Bilobalide-mediated inhibition of microglial migration and accumulation.

5. Conclusion

Unexpectedly, we first found that the spleen size and weight in CPZ-mice were reduced, and that MOG antibodies were detected in the serum, spleen and brain of mice fed with CPZ. Bilobalide improved CPZ-induced demyelination, inhibited the production of MOG specific antibodies, and declined the inflammatory microenvironment of M1 microglia in the brain. Accordingly, our data provide a potential therapeutic strategy to alleviate demyelination or other related diseases. Future studies should determine whether MOG autoantibodies can cause demyelination lesion and how Bilobalide reduce the production of MOG antibody in CPZ-demyelinating mice.

Author contribution

Cun-Gen Ma and Bao-Guo Xiao designed the experiments and checked all experimental data and analysis results; Ruo-Xuan Sui, Jing Wang and Li-Juan Song are involved in the establishment of animal model and intervention of drug; Qiang Miao carried out Western blot; Qing Wang and Jing-Wen Yu carried out immunohistopathology and immunohistochemistry; Liang Cao and Wei Xiao have prepared and identified Bilobalide used in this experiment.

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Conflict of interest

None of the authors has any potential financial and non-financial conflict of interest related to this manuscript.

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