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Research Article

Korean Red Ginseng extract ameliorates demyelination by inhibiting infiltration and activation of immune cells in cuprizone-administrated mice

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ABSTRACT

Background: Korean Red Ginseng (KRG), the steamed root of *Panax ginseng*, has pharmacological activities for immunological and neurodegenerative disorders. But, the role of KRGE in multiple sclerosis (MS) remains unclear.
Purpose: To determine whether KRG extract (KRGE) could inhibit demyelination in corpus callosum (CC) of cuprizone (CPZ)-induced murine model of MS
Methods: Male adult mice were fed with a standard chow diet or a chow diet supplemented with 0.2% (w/w) CPZ *ad libitum* for six weeks to induce demyelination while were simultaneously administered with distilled water (DW) alone or KRGE-DW (0.004%, 0.02 and 0.1% of KRGE) by drinking.
Results: Administration with KRGE-DW alleviated demyelination and oligodendrocyte degeneration associated with inhibition of infiltration and activation of resident microglia and monocyte-derived macrophages as well as downregulation of proinflammatory mediators in the CC of CPZ-fed mice. KRGE-DW also attenuated the level of infiltration of Th1 and Th17) cells, in line with inhibited mRNA expression of IFN-γ and IL-17, respectively, in the CC. These positive effects of KRGE-DW mitigated behavioral dysfunction based on elevated plus maze and the rotarod tests.
Conclusion: The results strongly suggest that KRGE-DW may inhibit CPZ-induced demyelination due to its oligodendroglial protective and anti-inflammatory activities by inhibiting infiltration/activation of immune cells. Thus, KRGE might have potential in therapeutic intervention for MS.

1. Introduction

Multiple sclerosis (MS), anautoimmune-mediated degenerative disorder of the central nervous system (CNS) that affects young adults, is characterized by various neurologic dysfunctions due to progressive demyelination, oligodendrocyte death, and diffuse neurodegeneration [1–3]. Although the etiology of MS remains elusive, the pathogenesis of MS engages aberrant overactivation of immune responses according to results of immunological, genetic, and histopathology studies on patients with MS [4–7]. Although a better understanding of MS has accumulated our knowledge of the etiology and mechanism of the disease, exploration for its therapeutics is still challenging [4–7].

Anti-inflammatory and immune-modulating drugs currently prescribed for MS extain interferon-beta (IFN-β), glatiramer acetate, alemyuzumab, FTY720 (also known as fingolimod), and natalizumab. They have little evidence of effectiveness for stimulating remyelination with several adverse effects, including flu-like symptoms, nausea, progressive multifocal leukoencephalopathy, and cardiovascular events [4,6,8–10].

Administration of these medicines is primarily aimed at minimizing relapses or delaying the onset of disability [4,6,8–10]. To date, no MS therapeutic has displayed any significant actions to

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enhance functioning of patients with fixed disability. Thus, the development of fundamental medication that can delay the onset of MS or forestall its progression is crucial.

Currently, natural product-derived foods and drugs are becoming increasingly more popular because of their safe and effective effects for improving physical strength and/or disease [11]. *Panax (P.) ginseng*, also known as Korean ginseng, is a popular medicinal herb known as an energizing herb that can enhance strength and stamina to elderly individuals in Asian countries for thousands of years [12–14]. Active pharmacological ingredients of *P. ginseng* are known to include main ginsenosides (triterpene saponins) such as -Rb1, -Rb2, -Rc, -Rd, -Re, and -Rg1 and minor ginsenosides [15,16]. However, the multifaceted pharmacological activities of *P. ginseng* are not caused by ginsenosides alone, but by the complex action of its various components including ginsenosides, polysaccharides, peptides, alkaloids, polyacetylene, and phenolic compounds [15,16]. The steamed root of *P. ginseng*, known as Korean Red Ginseng (KRG), includes ginsenosides-Rg2, -Rg6, -F4, -20(E)-F4, -Rh1, -Rh4, -Rk3, -Rg3, -Rg5, -Rz1, -Rk1, -Rg9, and -Rg10, which are converted from major ginsenosides [17,18]. KRG has *in vivo* pharmacological effects on neurological disorders such as Alzheimer's, Parkinson's, and Huntington's diseases [19–21]. Lately, we have studied that KRG extract (KRGE) and ginsenoside-Rb1/Rg1 can mitigate motor disability and alleviate spinal demyelination by downregulating Th1 and Th17 cells and stimulating Treg cells in a myelin oligodendrocyte glycoprotein (MOG) peptide-immunized EAE model, a murine model for MS [22]. The KRGE's positive effects against demyelination are also mediated by inhibiting p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kB signaling pathways [23]. These studies propose that KRGE has potential as multi-target interventions to cure MS. But, its roles in the MS, an autoimmune-mediated disorder of the CNS, are presently not known in details. Thus, the objective of this study was to investigate effects of KRGE on cuprizone (CPZ)-induced demyelination in mouse corpus callosum (CC). The CPZ model is used to explore demyelination/remyelination because of its unique pathological mechanisms. CPZ can play pivotal roles of myelin-forming oligodendrocytes [24,25]. The CPZ-induced demyelination is accompanied by immune response including activated microglia and astrocytes, decreased tight junction protein zonula occludens-1, and infiltrated $CD4^+/IFN\gamma^+$ (Th1) and $CD4^+/IL\text{-}17^+$ (Th17) cells [26]. The studies proposed that KRGE might exert therapeutic possibilities for autoimmune demyelinating diseases such as MS by infiltrating microglia, macrophages, and T cells (Th1 and Th17).

2. Materials and methods

2.1. Ethical approval

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Kyung Hee University (KHUASP-17-065). Proper randomization of laboratory animals and handling of data were performed in a blinded manner as previously described [27].

2.2. Animal and husbandry

Male adult C57BL/6NTac mice (7- to 9-week-old) were purchased from Narabiotec Co., Ltd. (Pyeongtaek, Republic of Korea). Male mice were used to reduce the effect of the menstrual cycle on the results. The mice were maintained at a constant temperature of 23 ± 2°C and relative humidity of 50 ± 15% with a 12-hour light-dark cycle (light on 07:30 to 19:30) and air exhaustion cycle of 15 minutes/hour, and fed food and water *ad libitum*. Five mice were bred cage. The mice were allowed to habituate to the housing facilities for 1 week before the experiments.

2.3. Experimental groups

To explore application conditions of KRGE-diluted water (KRGE-DW), mice were randomly divided into the following experimental groups (n = 5 each group): 1) Sham [distilled water (DW), per oral (p.o.); without DW-deprivation for 12 hours], 2) DW-D [DW, p.o.; DW-deprivation for 12 hours], 3) KRGE-0.05 [0.05% of KRGE, p.o.; KRGE- deprivation for 12 hours], and 4) KRGE-0.1 [0.1% of KRGE, p.o.; KRGE-deprivation for 12 hours] (Fig. 1A). To test the effect of KRGE against demyelination in CPZ-induced model, mice were randomly divided into the following experimental groups (n = 5 each group): 1) sham [standard diet, p.o. + DW, p.o.], 2) CPZ [CPZ-enriched diet, p.o.+ DW, p.o.], 3) CPZ + KRGE-0.004 [CPZ-enriched diet, p.o. + 0.004% of KRGE-DW, p.o.], 4) CPZ + KRGE-0.02 [CPZ-enriched diet, p.o. + 0.02% of KRGE-DW, p.o.], 5) CPZ + KRGE-0.1

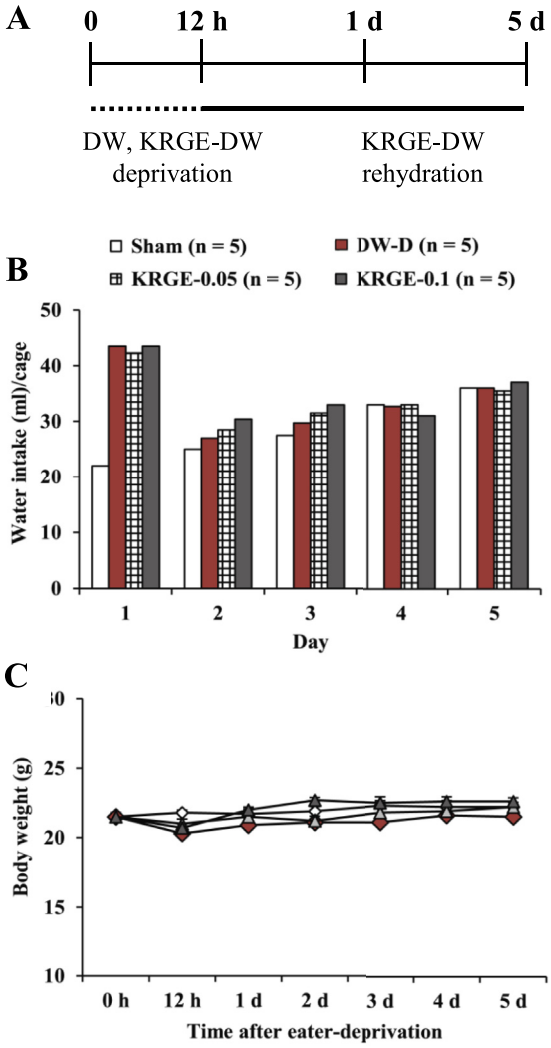

Fig. 1. KRGE-diluted water does not significantly affect body weight or total water intake of mice.
(A-C) Mice were deprived of drinking DW for 12 hours and rehydrated by drinking KRGE-DW (KRGE-0.05 and KRGE-0.1) (A). Body weights (B) and water intake (C) were measured daily. Data are expressed as mean ± standard error of the mean (SEM) (One-way ANOVA test with post hoc; $*p < 0.05$ and $**p < 0.01$ versus normal group; $\dagger p < 0.05$ and $\dagger\dagger p < 0.01$ versus control group).

[CPZ-enriched diet, p.o. + 0.1% of KRGE-DW, p.o.], and 6) KRGE-0.1 alone group [standard diet, p.o. + 0.1% of KRGE-DW, p.o.] (Fig. 2A).

2.4. CPZ model of demyelination and remyelination

Mice were fed with standard chow diet (Purina rodent chow, Purina Korea, Seoul, Republic of Korea) or chow diet supplemented with 0.2% (w/w) bis(cyclohexanone)oxaldihydrazone (CPZ; Sigma-Aldrich, Inc., St. Louis, MO, USA) *ad libitum* for six weeks to induce experimental demyelination. The CPZ chow diet was changed every other day. After six-week, all CPZ diet feedings were discontinued and mice were sacrificed for various analyses of demyelination.

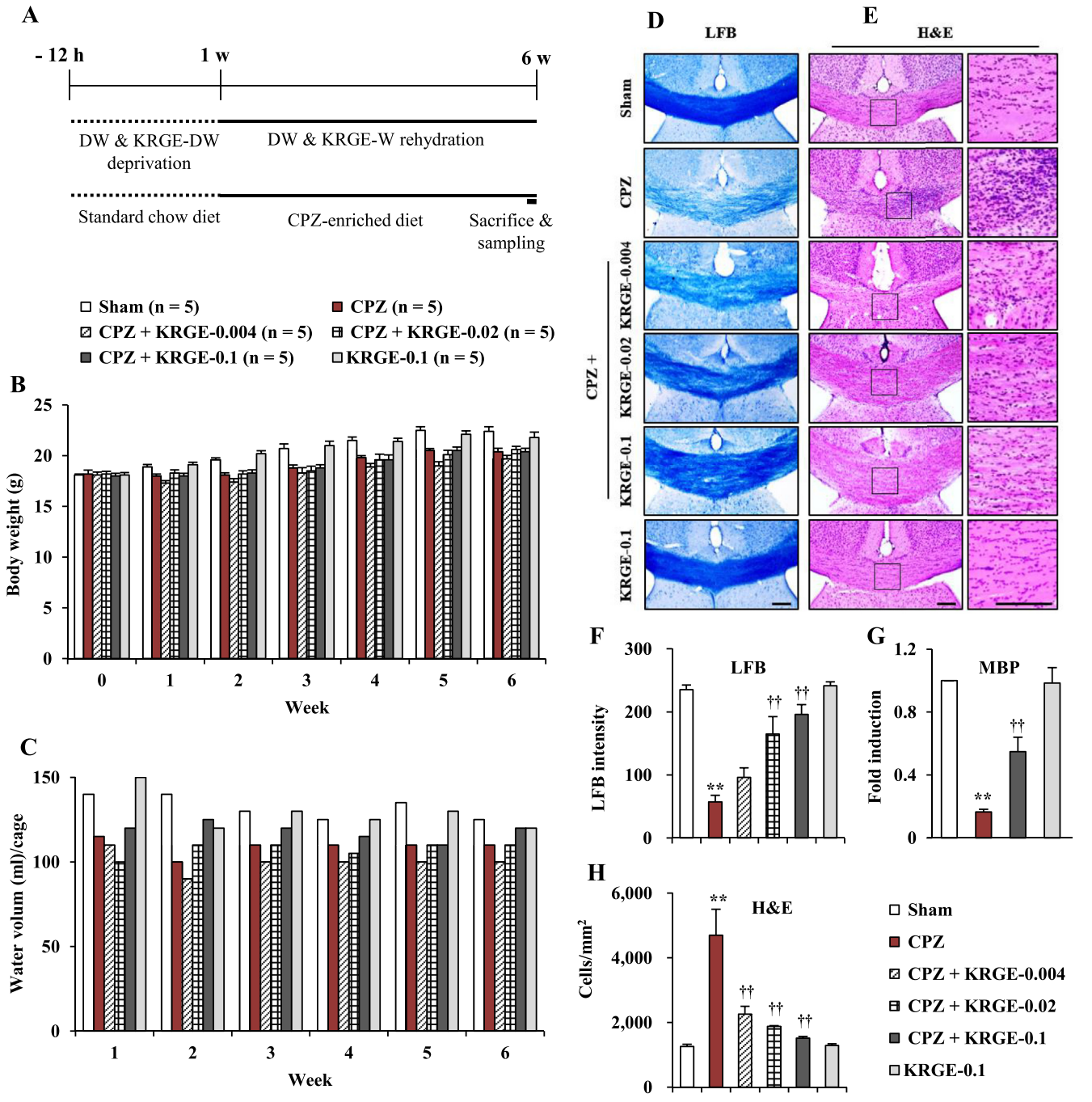

Fig. 2. KRGE-DW alleviates demyelination in the CC of CPZ-induced mice
(A) Mice were deprived of drinking DW for 12 hours, followed by administration with CPZ-enriched diet and KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1). They were then sacrificed and sampled for various assays (A). **(B and C)** Body weights (B) and water intake (C) were measured weekly for 6 weeks. **(D-F and H)** At 6 weeks after CPZ-feeding, cryosections (n = 3 per brain) with CC from all groups (n = 5 per group) were subjected to Luxol fast blue dye staining to investigate levels of demyelination (D) and hematoxylin-eosin staining to test the degree of recruitment/infiltration of immune cells (E), followed by quantification (F and H). Bars = 100 μm. Squares in B were enlarged at the right side. (G) At 6 weeks after CPZ-feeding, lysates CCs were obtained from all groups (n = 5 per group) and analyzed by real-time PCR to investigate mRNA expression levels of MBP (G). Data are expressed as mean expressive value (the ratio of each value against GAPDH for each sample) ± SEM (One-way ANOVA with post hoc; *$p < 0.05$ and **$p < 0.01$ versus sham group; †$p < 0.05$ and ††$p < 0.01$ versus CPZ group).

2.5. KRGE-DW administration

According to the dose used in clinic and preclinical studies using mice [22,28], KRGE (Hong Sam Jung Plus™; Korea Ginseng Corporation, Daejeon, Republic of Korea) was diluted with drinking DW (KRGE-DW). Composition of KRGE was described in our previous study [22]. To establish application conditions of drinking KRGE-DW, mice in drinking DW-deprived groups were DW-deprived for 12 hours followed by drinking KRGE-DW [0.05% (KRGE-0.05) and 0.1% KRGE (KRGE-0.1)] or drinking DW without KRGE (control group) every day for 5 days (Fig. 1A). Mice in the normal group were administrated with drinking DW without KRGE instead of KRGE-DW. Body weight and total water intake were measured every day for mice in all groups. To measure the effect of KRGE on demyelination, drinking DW and KRGE-DW were deprived from mice for 12 hours and followed by administration with CPZ-enriched diet and drinking KRGE-DW [0.004% (KRGE-0.004), 0.02% (KRGE-0.02), and 0.1% KRGE (KRGE-0.1)] every day until termination of the experiment (Fig. 2A). Drinking DW and KRGE-DW were freshly changed every day.

2.6. Histopathological assessment of corpus callosum

Six weeks following CPZ induction, mice were anesthetized by isoflurane (1-2%) and perfused intracardially with iced physiological saline, followed by treatment with iced 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and sequential coronal sections (30 μm thickness) including CC were prepared as previously described [29–32]. Cryo-sections with CC were stained with luxol fast blue (LFB) dye to investigate levels of demyelination (n = 5) as well as hematoxylin-eosin (H&E) dye to determine levels of recruitment/infiltration of immune cells (n = 5). The intensity of LFB-stained myelin and the number of H&E-stained cells in the CC were quantified by automatic cell counting using the ImageJ program (http://rsb.info.nih.gov/ij/).

2.7. Immunohistochemical assessment

Six weeks following CPZ induction, cryo-sections including CC in each group (n = 5 per group) were conducted by immunohistochemical staining as previously described [29–32], using rabbit anti-Iba-1 (1:2,000; WAKO, Osaka, Japan), rabbit anti-glial fibrillary acidic protein (GFAP) (1:1,500; Millipore, Bedford, MA, USA), rabbit anti-oligodendrocyte transcription factor 2 (Olig2) (1:1,000; Millipore), and mouse anti-CC1 (1:200; Millipore) antibodies. Regions of interest in immunostained sections were captured using a DP70 digital camera system (Olympus Co.). Intensities of Iba-1- and GFAP-immunoreactivity and numbers of Oligo2- and CC1-immunoreactive cells in the CC were quantified using the ImageJ program.

2.8. Flow cytometry

Six weeks following CPZ induction, mice were anesthetized by isoflurane (1-2%) and perfused intracardially with saline. CCs were then carefully cropped. To test cell population, single-cell suspensions refined from CCs were prepared as previously described [29–33]. Cells were fluorescently stained as previously described [29–33]

2.9. Polymerase chain reaction (PCR) analyses

Six weeks following CPZ induction, the mice were anesthetized by isoflurane (1-2%), perfused intracardially with saline, and then CC carefully dissected. Real-time PCR assay was conducted as previously described [20,29–31,34]. The primer sequences were described in previous studies [20,29–31,34].

2.10. Behavioral evaluation

Elevated plus maze: Plus maze test is used to measure anxiety-like behavior by avoiding open arms of the plus maze [35] and conducted as previously described [36]. Briefly, each mouse was positioned in the center area facing an open arm at first and allowed to move at liberty during the testing. The mouse's entry into any of the four arms was calculated manually and evaluated when all four paws crossed from the central region into an arm. The number of total arm entries and the amount of time spent on the open arms during a 10-minute testing period were recorded by video. *Rotarod performance test:* Rotarod performance test was used to investigate motor function, coordination and balance [37] and performed as previous described [38]. Briefly, the mice were placed on a rotating cylinder (4 cm in diameter) with a coarse surface for firm grip and tested for three trials with an accelerating speed of 16 rpm/s. A cut-off time of 5 min and an intertrial interval of 15 min were used. Latency on the rod before falling was measured.

2.11. Statistical analyses

Data was analyzed by using SPSS Statistics for Windows, Version 25.0 (SPSS Inc, Chicago, USA) for Windows. The data were accomplished using one-way ANOVA with Tukey post hoc test for comparison of multiple groups. Data are expressed as mean ± standard error of the mean (SEM). *P* values were considered significant when $p < 0.05$.

3. Results

3.1. KRGE-diluted water does not significantly affect body weight or total waterintake of mice

Since KRGE-DW has its own unique scent and bitterness [39], mouse may not prefer to drink it when it first experiences this. To solve this problem, mice were deprived of drinking DW for 12 hours. They were then rehydrated with drinking KRGE-DW (KRGE-0.05 and KRGE-0.1) (Fig. 1A). Body weights of mice from DW-D and KRGE-DW groups were slightly reduced at 12 hours after drinking DW deprivation, but recovered quickly (day 1) after rehydration, showing no significant changes afterwards (Fig. 1B). Total volumes of drinking DW and KRGE-DW intake were remarkably increased at 12 hours after rehydration (1 day), decreased on the second or third day, and similar in all groups from fourth and fifth days (Fig. 1C).

3.2. KRGE ameliorates demyelination and cellular recruitment/infiltration in the CC of CPZ-induced mice model

First, body weight gain was measured every week. Mean body weight of mice in the CPZ group was reduced between 1 − 6 weeks compared to that of the Sham group, although the mean body weight was not affected by administration of KRGE-DW (KRGE-0.05 and KRGE-0.1) (Fig. 2B). Mean water intake of mice in the CPZ group was also reduced between 1 − 6 weeks compared to that of the Sham group, although the mean water intake was not affected by administration of KRGE-DW (KRGE-0.05 and KRGE-0.1) (Fig. 2C). These results suggest that reduced body weight might be related to the administration of CPZ rather than the administration of KRGE-DW. Since CNS demyelination is an unique pathological event of MS patients and the event is clearly seen in white matters including CC in this CPZ model [40,41], we first examined the effect of KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1) on the level of

demyelination in the CC of CPZ mice (Fig. 2A). At 6 weeks following CPZ induction, demyelination (pale area) by staining with LFB dye was clearly increased in the CC of CPZ mice, whereas its level in CPZ mice was significantly mitigated by KRGE-DW in a dose-dependent manner (Fig. 2D and F). The results were in accordance with the pattern seen in mRNA expression of MBP by immunoblot analyses (Fig. 2G). Since demyelination and inflammation are closely related to each other, hematoxylin and eosin staining was performed to examine whether mitigating positive actions of KRGE on demyelination in the CC of CPZ mice were associated with an inhibition of inflammatory/immune cell infiltration in the CC. Results are shown in Fig. 2E and H. The level of cellular recruitment/infiltration was clearly increased in the CC of the CPZ group compared to that in the sham group, while its degree was significantly lower in KRGE-DW-administrated CPZ mice (Fig. 2E and H). The findings indicate that KRGE may block CNS demyelination in CPZ mice.

3.3. KRGE inhibits recruitment/activation of microglia and macrophages as well as mRNA expression of proinflammatory agents in the CC of CPZ mice

In early demyelination and inflammation, increased cellular recruitment/infiltration can stimulate MS deterioration by accelerating recruitment/infiltration/activation of resident microglia and monocyte-derived macrophages into demyelinating sites [42,43]. Thus, we explored the regulatory role of KRGE-DW (KRGE-0.1) on recruitment of microglia and macrophage in the CC of CPZ mice. The degree of Iba-1 immunoreactivity was remarkably increased in the CC of the CPZ group compared to that of the sham group, whereas its degree was significantly alleviated in the CPZ group compared to that of the CPZ group (Fig. 3A and B), corresponding to altered mRNA level of CD11b, another marker of microglia and macrophage (Fig. 3C). However, Iba-1 and CD11b antisera might bind to microglia and macrophages in the CNS [44]. Thus, we further differentiated their populations by flow cytometry (Fig. 3D and E). The percentage of $CD11b^{+}/CD45^{+(low)}$ cells (R7 rectangle in Fig. 3D and E) representing brain-resident microglia [22,30,31,45] was increased to 2.48 ± 0.2% in the CC of the CPZ group, whereas it was lowered to only 1.94 ± 0.2% in the CC of the CPZ + KRGE-0.1 group (Fig. 3D and E). The percentage of $CD11b^{+}/CD45^{+(high)}$ cells (R8 rectangle in Fig. 3D and E) representing macrophages [22,30,31,45] was also increased to 4.94 ± 0.2% in the CC of the CPZ group, while it was reduced to 1.79 ± 0.6% in the CC of the CPZ + KRGE-0.1 group (Fig. 3D and E). These results suggest that KRGE administration might reduce infiltration of microglia and macrophages in the CC of CPZ-induced mice. We subsequently

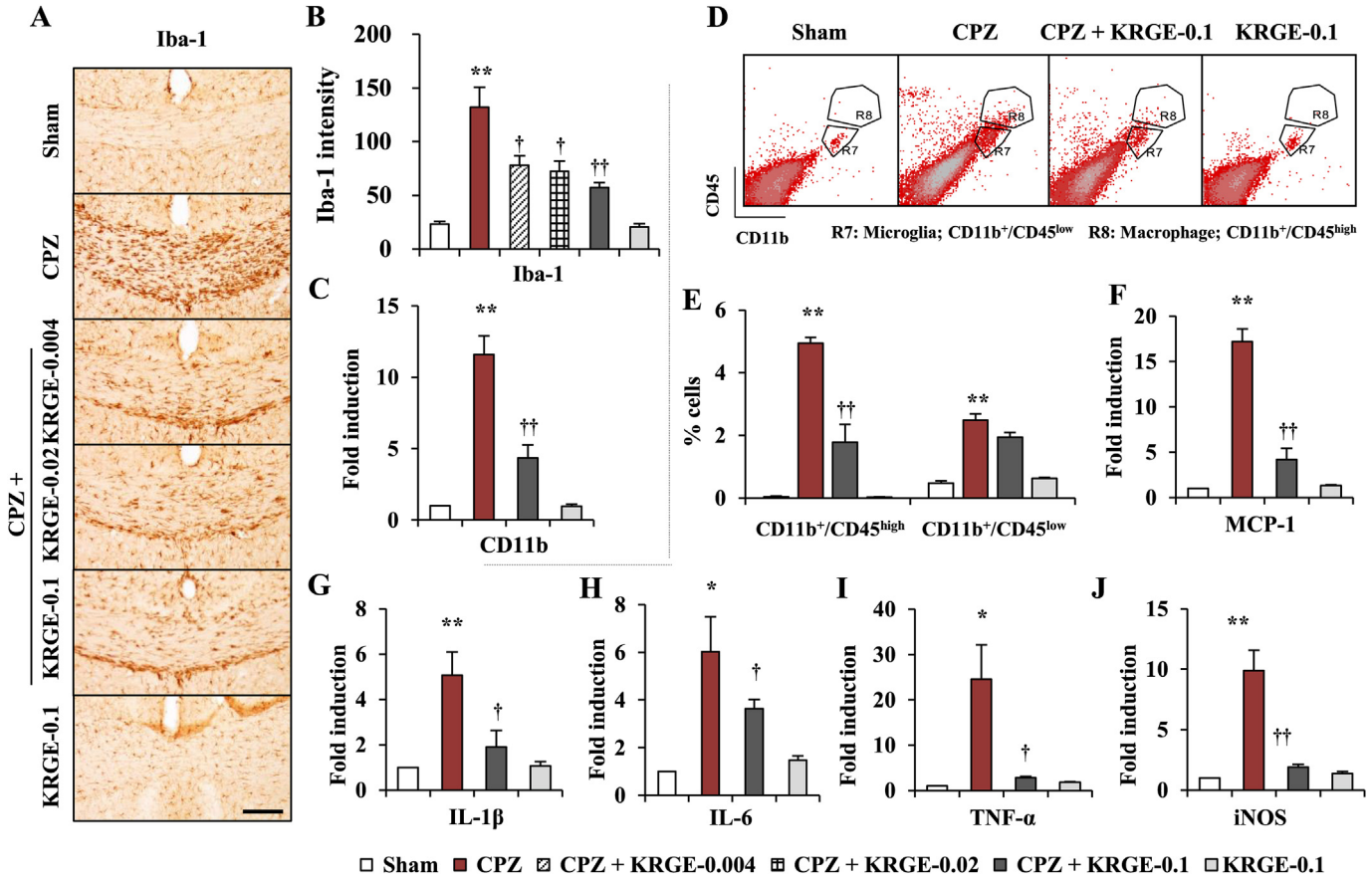

Fig. 3. KRGE-DW blocks infiltration/activation of microglia/macrophages in CC of CPZ mice.
CCs (n = 5 per group) were obtained from the Sham, CPZ, CPZ + KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1), and KRGE-DW (KRGE-0.1) groups at 6 weeks after CPZ-feeding. **(A-C)** Cryosections (n = 3 per brain) of brains including CC (n = 5) from each group were conducted to immunohistochemical staining with an anti-Iba-1 antiserum (A), followed by quantification (B). Bars = 100 μm. **(D and E)** CCs (n = 5 per group) were sampled at 6 weeks days after CPZ-feeding to investigate levels of infiltration of microglia and macrophages using flow cytometry. A total of 1×10^4 cells was acquired within the combined gate based on forward scatter and side scatter. $CD11b^{+}$ cells were divided into R7 ($CD11b^{+}/CD45^{+low}$ microglia) and R8 ($CD11b^{+}/CD45^{+high}$ macrophage) areas (D) and populations of both cell types were displayed in graphs (E). **(C and F-J)** CC lysates (n = 3 per group) were assayed by real-time PCR to measure mRNA expression levels of CD11b (C), MCP-1 (F), IL-1β (G), IL-6 (H), TNF-α (I), and iNOS (J). Data are expressed as the mean ratio of each value against GAPDH for each sample ± standard error of the mean (SEM) (One-way ANOVA with post hoc; *$p < 0.05$ and **$p < 0.01$ versus sham group; †$p < 0.05$ and ††$p < 0.01$ versus CPZ group).

evaluated whether decreases of infiltration and activation of microglia and macrophages due to KRGE-0.1 (Fig. 3A–E) were associated with alteration in the expression of proinflammatory mediators in the CC by real-time PCR assay (Fig. 3F–J). Results revealed that mRNA expression levels of representative proinflammatory chemokines (MCP-1/CCL2), cytokines [interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α), and enzymes inducible nitric oxide synthase (iNOS) were clearly increased in the CC from the CPZ group compared to those of sham group. But, upregulation of their mRNA expression was significantly blocked in the KRGE-DW (0.1% KRGE)-administrated group (Fig. 3F–J). These findings imply that KRGE might reduce CPZ-induced demyelination by reducing the expression of inflammatory factors in the CC.

3.4. KRGE inhibits immunoreactivities of astrocytes and oligodendrocytes in the CC of CPZ mice

CPZ can induce oligodendrocyte death that lead to a demyelination and activation of astrocytes in white matter regions [46,47]. Thus, the status of oligodendrocytes was confirmed by immunohistochemistry (Fig. 4). The number of oligodendrocyte transcription factor 2 (Olig2, a master regulator for oligodendrocyte lineage specification)- and immature/mature oligodendrocyte marker CC1-immunopositive cells was decreased in the CC of the CPZ-ingested group, while the reduction in the number was inhibited by KRGE-DW administration in a dose-dependent manner (Fig. 4A and D). Since astrocytes play a major role in demyelination with consecutive axon degeneration and impaired remyelination in MS patients and CPZ model [46,47], we also examine the effect of KRGE on the expression of astrocytes in the CC (Fig. 4B and E). GFAP (a specific marker of astrocytes)-positive immunoreactivity was clearly enhanced in the CC of CPZ-ingested mice, whereas its immunoreactivity was inhibited in CPZ + KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1) ingested mice (Fig. 4C and F). The findings propose that KRGE may diminish demyelination by reducing degeneration of oligodendrocytes in the CC of CPZ model.

3.5. KRGE inhibits recruitment/infiltration of $CD4^+$ T, Th1, and Th17 cells in the CC of CPZ mice

Since recruitment and activation of T cells are major immunological events in MS and CPZ [2,26], we explored the degree of T cell infiltration into the CC of mice in the CPZ group by real time PCR assay. Results of showed that mRNA expression of CD3, a marker for T cells, was upregulated by 5.56-fold in the CC of CPZ group mice at 6 weeks after CPZ induction, while its expression degree was significantly decreased by 2.88-fold (48.2%) in the CC CPZ + KRGE-0.1 group of mice (Fig. 5A). According to flow cytometry assay, the percentage of $CD4^+$ T cells as key cells of the adaptive immune system was clearly increased in the CC after CPZ induction (2.85 ± 0.4%), whereas the percentage of $CD4^+$ T cells was decreased in the CC in the CPZ + KRGE-0.1 group (0.85 ± 0.1%) compared to that in the CPZ group (Fig. 5B and C). However, the percentage of $CD8^+$ cytotoxic T cells as critical components of the adaptive immune system was not critically affected by CPZ induction or KRGE-0.1 administration (Fig. 5B and C).

Since activated CD4 T cells may differentiate into subtype T cells during T cell receptor activation in a specific cytokine milieu engaged in autoimmune response and release pro-inflammatory cytokines including interferon (IFN)-γ, IL-17, and transforming

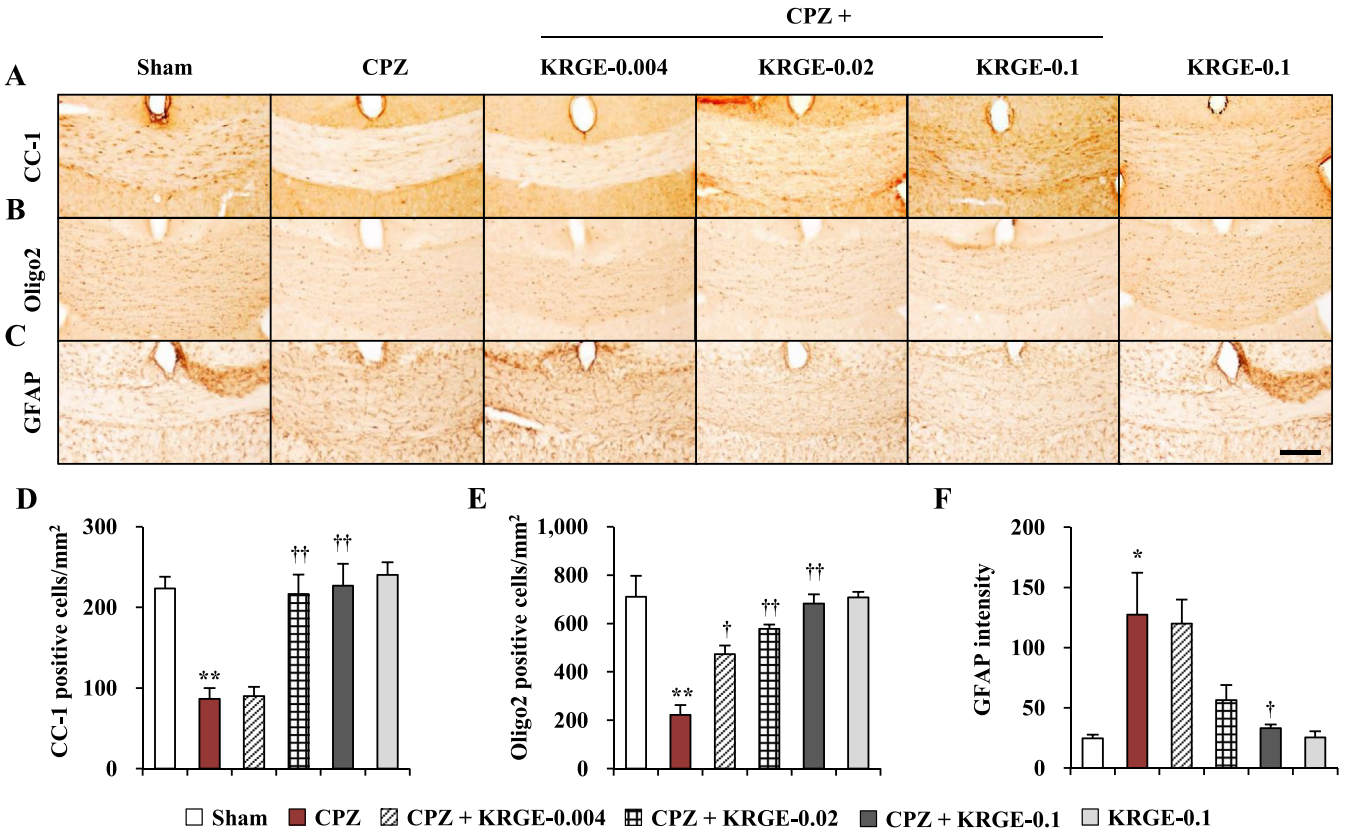

Fig. 4. KRGE-DW inhibits astrocyte activation and oligodendrocyte degeneration in CC of CPZ mice.
(A-F) CCs (n = 5 per group) were obtained from the Sham, CPZ, CPZ + KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1), and KRGE-DW (KRGE-0.1) groups at 6 weeks after CPZ-feeding. Cryosections (n = 3 per brain) of brains including CC (n = 5) from each group were conducted to immunohistochemical staining with anti-GFAP, ant-oligo-1, and CC1 antibodies (A-C), followed by quantification (D-F) (One-way ANOVA test with post hoc; *$p < 0.05$ and **$p < 0.01$ versus sham group; †$p < 0.05$ and ††$p < 0.01$ versus CPZ group).

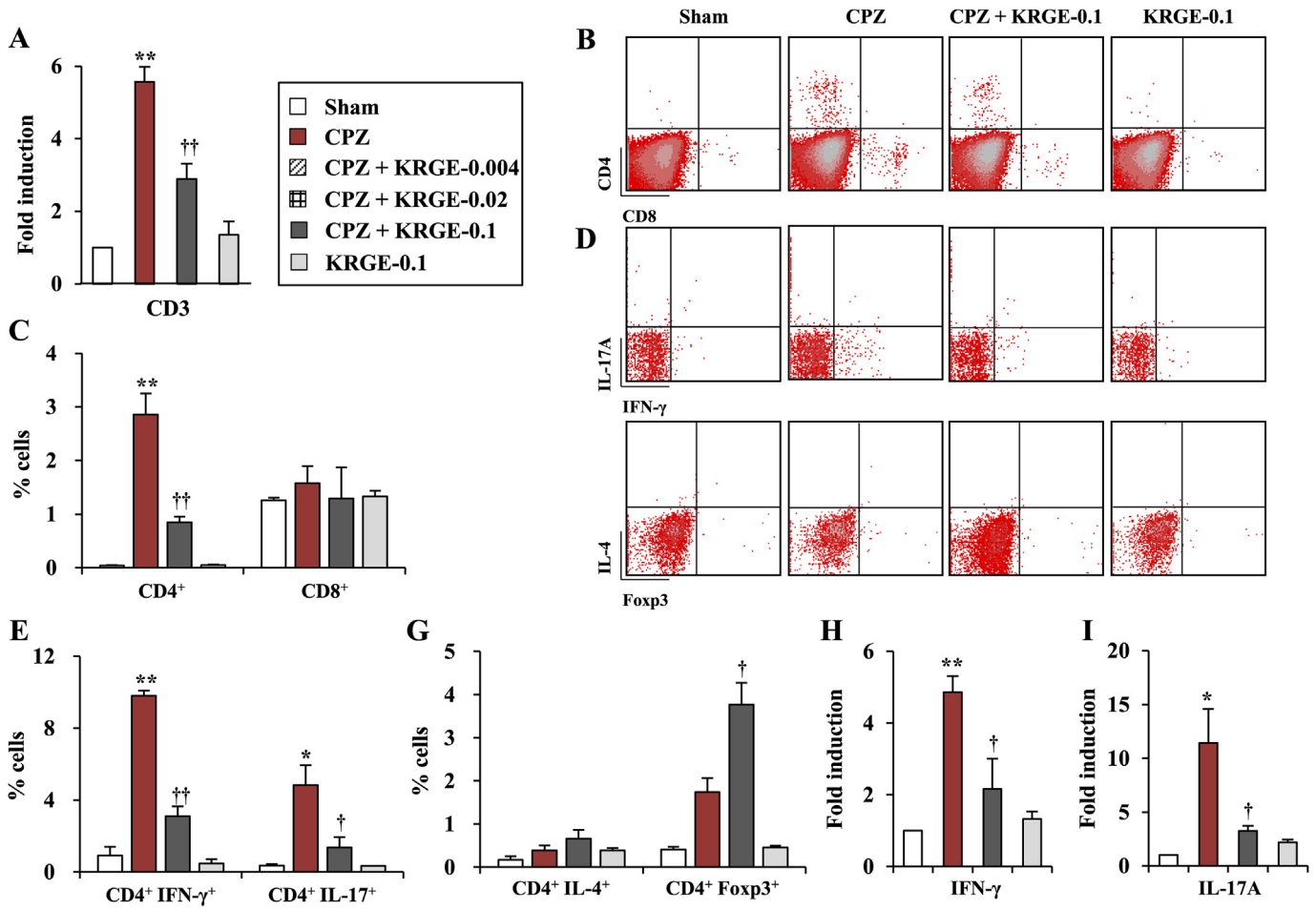

Fig. 5. KRGE-DW inhibits infiltration and activation of $CD4^+$ T, Th1, and Th17 cells into CC of CPZ mice.
(B and C) CCs (n = 3 per group) were sampled from Sham, CPZ, CPZ + KRGE-DW (KRGE-0.004, KRGE-0.02, and KRGE-0.1), and KRGE-DW (KRGE-0.1) groups at 6 weeks after CPZ-feeding. These CCs were subjected to measurement of populations of $CD4^+$ and $CD8^+$ T cells through flow-cytometric analysis. Populations of $CD4^+$ and $CD8^+$ T cells were displayed in dot plot diagram (B) and graphs (C). **(A and C)** To determine populations of Th1 and Th17 cells through flow-cytometric analysis, $CD4^+$ T cells (1×10^4) were first gated using forward scatter and side scatter properties from the CC from each group. $CD4^+$ T cells were used to assay populations of Th1 ($CD4^+$/IFN-γ^+), Th17 ($CD4^+$/IL-$17A^+$), Th1 ($CD4^+$/IL-4^+), and Treg ($CD4^+$/$CD25^+$/$Foxp3^+$) cells. Populations of Th1 and Th17 cells were displayed in dot plot diagram (D and F) and graphs (E and G). **(D-F)** Lysates of CCs (n = 3 per group) from all groups were assayed using real-time PCR to determine mRNA expression levels of IFN-γ (D) and IL-17 (E). Quantified data are expressed as mean % cells or fold induction ± SEM (One-way ANOVA test with post hoc; *$p < 0.05$ and **$p < 0.01$ versus sham group; †$p < 0.05$ and ††$p < 0.01$ versus CPZ group).

growth factor (TGF)-β [2,48], we performed the subtype assay of $CD4^+$ T cells in CPZ-induced CC tissue (Fig. 5D–G). In the CPZ group, percentages of Th1 ($CD4^+$/IFN-γ^+) cells and Th17 ($CD4^+$/IL-17^+) cells in the CC were enhanced to 9.80 ± 0.3% and 4.84 ± 1.1%, respectively (Fig. 5D–G). However, in the CC of the CPZ + KRGE-0.1 group, their percentages were enhanced to only 3.11 ± 0.5% and 1.36 ± 0.6%, respectively (Fig. 5D–G). In line with these findings, expression degrees of IFN-γ (a cytokine released by Th1 cells; Fig. 5H) and IL-17A (a cytokine released by Th17 cells; Fig. 5I) in the CC of the CPZ group were clearly enhanced by 4.8-fold and 11.4-fold, respectively, compared to those in the sham group, whereas their expressions degrees were almost completely inhibited in the CC of the CPZ + KRGE-0.1 group (Fig. 5H and I). However, percentages of Th2 ($CD4^+$/IL-4^+) and Treg ($CD4^+$/$CD25^+$/$Foxp3^+$) cells in the CC were not significantly altered by CPZ induction or KRGE-0.1 administration (Fig. 5H and J).

3.6. KRGE mitigates behavioral impairments of CPZ-induced mice model

Behavioral deficits induced by CPZ treatment associated well with the degree of white matter demyelination in the murine model [49]. Therefore, behavioral testing might serve as a valuable surrogate marker for demyelination [41]. Thus, we assessed whether KRGE-DW (KRGE-0.1) might have beneficial effects on behavioral impairments at 6 weeks after CPZ induction using a range of tests, including the functional observation battery. In the elevated plus maze test, the mean time spent in open arms was remarkedly reduced in the CPZ group (28.7 ± 10.6 s) compared to that of the sham group (123.5 ± 13.1 s), while the reduction was reduced in the CPZ + KRGE-0.1 group (116.0 ± 12.7 s) compared with that in the CPZ group (Fig. 1A). In the rotarod test, the mean latency to fall from cylinder was remarkedly reduced in the CPZ group (167.5 ± 31.3 s), whereas such reduction was significantly prevented in the CPZ + KRGE-0.1 group (268.1 ± 13.2 s) compared with that in the CPZ group (Fig. 1B).

4. Discussion

Results of this study revealed that KRGE could alleviate demyelination in the CC caused by CPZ induction. KRGE-induced reductions in demyelination (Fig. 2) were associated with infiltration/activation of microglia and macrophages (Fig. 3), expression of pro-inflammatory cytokines, chemokines, and enzymes (Fig. 3),

immunoreactivities of astrocytes and oligodendrocytes (Fig. 4), recruitment/infiltration of $CD4^+$ T, Th1, and Th17 cells (Fig. 5), and behavioral impairments (Fig. 6) in the CC after CPZ induction. To the best of our knowledge, this is the first study to demonstrate that KRGE may inhibit demyelination and behavioral dysfunction in CPZ model by upregulation anti-inflammatory and immune regulatory actions. Our results suggest that KRGE might be used to design new functional foods and medications for MS based on its anti-inflammatory and immune regulatory actions. Further studies using MS patient-derived samples will be necessary to reconfirm these findings.

The alteration of body weight in CPZ model was measured. The weight of the CPZ group was clearly lower than that of the normal control group at 6 weeks following CPZ-feeding (Fig. 6C). This result may be associated with the increase of basal metabolic rate and the reduction of food intake [50]. Interestingly, administration with KRGE-DW (200 mg/kg) prevented the reduction in the body weight by CPZ-feeding.

The degree of permanent neurological dysfunction in MS patients and CPZ models is associated with the degree of demyelination in the CNS, which is significantly related to the level of infiltration of resident microglia and peripheral macrophages within or around lesions [51–53]. Thus, various herb-derived drugs have been discovered to overcome inflammatory responses in MS [54]. Currently, no innovative drugs have been reported for controlling this inflammatory response. Thus, finding completely effective and safe drugs is still ongoing. Medicinal plants such as Oenothera biennis and *Hypericum perforatum* and their derivatives have been reported to be safe with reliable therapeutic effects in MS patients by inhibiting demyelination and anti-inflammatory activity [54]. Here, administration with KRGE-DW significantly reduced levels of demyelination based on results of LFB staining and H&E staining (Fig. 2) as well as flow cytometry assay results on the level of infiltration of residential microglia and monocyte-derived macrophages (Fig. 3) in the CC of CPZ mice. Additionally, KRGE-DW inhibited the enhancement of mRNA expression of representative pro-inflammatory chemokine (MCP-1), cytokines (IL-1β, IL-6, and TNF-α), and enzymes (iNOS) (Fig. 3). These results suggest that the anti-demyelination activity of KRGE may be related to its anti-inflammatory role.

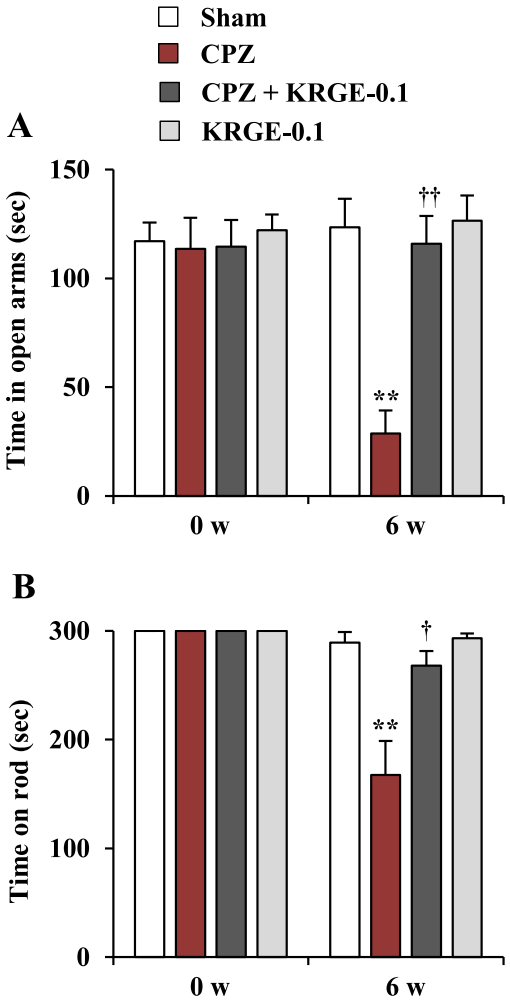

Fig. 6. KRGE-DW mitigates behavioral impairments of CPZ-induced mice.
(A and B) At 6 weeks after CPZ-feeding, mice of Sham, CPZ, CPZ + KRGE-DW (KRGE-0.1), and KRGE-DW (KRGE-0.1) groups were subjected to evaluation of behavioral impairments by plus maze (A) and rotarod tests (B). Mean time spent in open arms (A) and mean latency to fall from cylinder (B) are presented as mean ± SEM. (One-way ANOVA test with post hoc; $*p < 0.05$ and $**p < 0.01$ versus sham group; $\dagger p < 0.05$ and $\dagger\dagger p < 0.01$ versus CPZ group).

Proinflammatory $CD4^+$ T, Th1, and Th17 cells are considered to play a major role in neuroinflammatory processes in MS [2,48,55]. By stimulation of IFN-γ and IL-12, Th1 cells can release cytokines IFN-γ, IL-2, and TNF-α. By stimulation of IL-1β, IL-6, IL-23, and TNF-α, Th17 cells can secrete IL-17A, IL-17F, and IL-21. These cytokines released from Th1 and Th17 play critical immunological and pathological roles in T cell-mediated autoimmune diseases such as MS [2,48]. Th1 and Th17 cells and associated cytokines can be used to discriminate clinically isolated syndrome and MS phenotypes [56]. Transfer of MOG-specific CD4 T or Th17 cells can significantly reduce the amount of myelin protein and induce demyelination in the CC of CPZ mice [55]. Thus, targeting the redox (oxidation-reduction) state of proinflammatory CD4 T, Th1, and Th17 cells might be a novel therapeutic for MS accompanying infiltration of proinflammatory CD4 T cells, although their exact functions are still poorly challenged. Here, administration of KRGE-DW significantly alleviated the enhancement in the population of Th1 and Th17 cells and the mRNA expression of IFN-γ and IL-17A in the CC of CPZ mice, whereas KRGE-DW administration did not significantly affect the population of Th2 or Treg cells in the CC (Fig. 4). These findings suggest that KRGE may reduce demyelination via preventing Th1 and Th17 cell infiltration/activity in the CC of CPZ mice. These results strongly suggest that KRGE might exert a therapeutic function by down-regulating Th1 and Th17 cells without causing significant response of Treg cells in the CC of CPZ mice.

5. Conclusions

Innovative treatments to overcome MS have not been developed to date. Here, KRGE attenuated demyelination in the CC of CPZ mouse model. Its positive effect was closely related to its protective action against oligodendrocyte degeneration and its anti-inflammatory activity by controlling infiltration of microglia, macrophages, Th1, and Th17 cells. These findings suggest that KRGE might have potential for therapeutic intervention of MS.

Declaration of competing interest

All the authors of this manuscript have no conflict of interest in this subject.

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