

The Protective Effect of *Ginkgolide K* on Astrocytes Exposed to Myelin Debris *in vitro* and Demyelination *in vivo*

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Abstract

Background: *Ginkgolide K* (GK) has a protective effect on neurons and myelin sheath, and the function of astrocytes in phagocytizing myelin debris has attracted extensive attention in remyelination.

Objectives: This study focuses on the impact of GK on the phagocytosis of myelin debris by astrocytes and explores the possibility of treating demyelination.

Materials and Methods: Male C57BL/6 mice were used to establish a cuprizone (CPZ)-induced demyelination model. After being fed a normal or CPZ diet for 4 weeks, mice were intraperitoneally injected with PEG400 or GK for 14 consecutive days. GL261 and primary astrocytes were exposed to myelin debris. Immunohistochemistry/immunocytochemistry staining, western blot, RT-PCR, and other methods were used to detect the relevant indicators.

Results: Astrocytes engulfed myelin debris, leading to astrocyte reactivity with increased p-NF-κB/p65 and ATF6 expression and decreased nuclear factor erythroid 2-related factor 2 (Nrf2) expression, which was reversed by GK. Subsequently, astrocytes stimulated by myelin debris underwent self-apoptosis with increased expression of caspase-3 and BCL2-associated X (Bax), which was inhibited by GK. Simultaneously, GK efficiently promoted astrocytes to increase the production of neurotrophic ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF), speculating that increased CNTF/bFGF, decreased p-NF-κB/p65/ATF6, and up-regulated Nrf2 should participate in the protection of astrocyte apoptosis and build a beneficial microenvironment for myelin regeneration.

Conclusion: The results suggest that GK may have the potential to treat demyelination by promoting debris clearance and improving the brain microenvironment. However, further studies are needed to understand the physiological and pathological consequences of astrocytic phagocytosis and to investigate the possibility of using GK as a therapeutic application.

Keywords

Ginkgolide K, astrocytes, phagocytosis, cuprizone-induced demyelination

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that is associated with prominent demyelination (Attfield et al., 2022). The current immunomodulatory treatment has been proven to be effective in treating the relapse or remission of MS but has failed to prevent disease progression (Lassmann et al., 2012; Piehl, 2021). Remyelination is a natural regeneration against demyelination, but the reasons for the failure or incompleteness of remyelination during MS have not been fully understood (Motavaf et al., 2017). Obviously, remyelinating therapy has become a new direction and challenge in treating MS (Plemel et al., 2017). However, the presence of myelin debris around the lesion site further exacerbated the remyelination. Therefore, removing myelin debris is crucial for effective remyelination after injury (Sen et al., 2022).

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Traditionally, microglia are considered the major phagocytes. Recent studies have reported that astrocytes also have a phagocytic function and may play beneficial and detrimental roles during lesion development (Lee et al., 2021; Ponath et al., 2017). Astrocytes maintain the homeostasis of the brain through phagocytizing synapses, neuronal debris, axons, mitochondria, and pathological protein aggregates (Lee & Chung, 2021). Therefore, the phagocytosis of astrocytes may contribute to removing the cell debris or other wastes produced by brain tissue injury, further ameliorating neuroinflammation and neurological impairments after traumatic brain injury (Zhou et al., 2021), and improving the brain microenvironment after transient brain ischemia (Morizawa et al., 2017). Although accumulating evidence suggests that astrocytes participate in debris clearance in the brain, the reactivity of astrocytes to myelin debris during phagocytosis is still poorly understood.

Ginkgolide K (1,10-dihydroxy-3,14-didehydroginkgolide, *GK*) is a diterpene lactone extracted from the leaves of *Ginkgo biloba*. *GK* inhibited platelet activating factor (PAF)-induced platelet aggregation by antagonizing the PAF receptor, reversed the apoptosis induced by oxygen and glucose deprivation/reoxygenation (OGD/R) injury, and improved the neurobehavioral score and cerebral infarction volume after cerebral ischemia-reperfusion injury in a dose-dependent manner (Tao et al., 2017). Previous studies also revealed that *GK* exerted anti-inflammation, anti-oxidative stress, and neuroprotective effects in Parkinson's disease (Miao et al., 2022) and experimental autoimmune encephalomyelitis (EAE). Based on the reactivity of astrocytes and the plasticity of *GK* targeting astrocytes, we tried to explore whether *GK* promotes astrocytes to phagocytize myelin debris, thus affecting the brain microenvironment and myelin regeneration (Yu et al., 2019).

Materials and Methods

Animals and Drugs

Male C57BL/6 mice (10–12 weeks old) were purchased from Vital River Experimental Animal Technology Co. Ltd. (Beijing, China). The study was performed under the animal use protocol number: AWE202209006 and approved by the Ethics Committee of the Shanxi University of Chinese Medicine, Jinzhong, China. The experimental procedure was carried out in accordance with the guidelines of the International Council for Laboratory Animal Science. Mice were housed on a 12-h light/dark cycle with access to food and water *ad libitum* under clean conventional housing conditions. *GK* was purchased from Nanjing Dawdorf Biological Co.

Cuprizone-induced Demyelination Model

For the cuprizone (CPZ)-induced demyelination model, mice were fed with 0.2% (w/w) CPZ (Sigma-Aldrich,

USA) in a chow diet *ad libitum* for 6 weeks. As an experimental design, mice were randomly divided into three groups ($n = 8$ /per group) as follows: (1) control group = normal diet + solvent; (2) CPZ group = CPZ diet + solvent; and (3) CPZ + *GK* group = CPZ diet + *GK*. After being fed a regular or CPZ diet for 4 weeks, mice were intraperitoneally injected with PEG400 or *GK* (20 mg/kg, 200 μ L/mouse) for 14 consecutive days. *GK* was dissolved with PEG400 ultrasound at a 10 mg/mL concentration for 5 min and then diluted with saline.

GL261 Cells

Mouse glioblastoma cells were cultured in complete medium (Dulbecco's modified eagle medium (DMEM), 4500 mg/L glucose, 4 mM glutamine, and 1 mM sodium pyruvate) containing 1% streptomycin (10,000 μ g/mL) and penicillin (10,000 units/mL) in a humidified incubator with 37°C and 5% CO₂.

Primary Astrocytes

Primary astrocytes were obtained from postnatal (1 day of age) C57BL6 mice. Under sterile conditions, the cerebral cortex was dissected and placed in a complete medium (Thermo Science) containing 1% streptomycin-penicillin. Cells were dissociated into a single-cell suspension and then seeded in a 75-cm flask culture plate (Corning, USA) in a humidified incubator at 37°C and 5% CO₂. When mixed glial cultures reached 90% confluence, microglia were repeatedly removed by constant shaking for 12 h at 180 rpm. The purity was close to 100% by glial fibrillary acidic protein (GFAP) immunocytochemistry staining.

Myelin Debris Coupled with carboxyfluorescein succinimide ester

Myelin debris was resuspended with sterile phosphate buffered saline (PBS) and labeled in a 50 M carboxyfluorescein succinimide ester (CFSE) solution for 30 min at room temperature (RT). After centrifugation at 14,000g for 10 min, the pellet was washed three times with PBS. CFSE-labeled myelin debris was resuspended with sterile PBS. Protein concentration was determined by wet weight (mg/mL, w/v).

Myelin Debris Coupled with pHrodo

Phrodo coupling of myelin debris was carried out according to the operation instructions (pHrodo™ Red, Invitrogen P36600). Briefly, myelin debris was mixed with sterile Phrodo and gently stirred at 30–40 rpm for 1–2 h RT. After washing with PBS, the tube was centrifuged at 20,000g for 1–2 min. After repeated washing, the pellet was resuspended with PBS and 5% dimethyl sulfoxide (DMSO). Protein concentration was determined by wet weight (mg/mL, w/v).

***In vitro* Phagocytosis of Myelin Debris by Astrocytes**

GL261 and primary astrocytes (1×10^5 /well) were plated in 12-well plates and respectively incubated with CFSE-conjugated myelin debris (5 mg/mL) or pHrodo-coupled myelin debris in the presence and absence of *GK* (50 μ g/mL) for 24 h at 37°C with 5% CO₂. Unphagocytosed myelin debris was washed out, and phagocytosis capacity was measured under fluorescence microscopy and by multifunctional Synergy H1 Hybrid Reader (BioTek, USA) using fluorescence 485 excited light.

Immunohistochemistry and Immunocytochemistry Staining

Briefly, the slides of the brain (10 μ m) and cultured astrocytes were blocked with 1% BSA at RT for 30 min. The immunostaining was performed with the following antibodies: anti-ABCA1 (Abcam, USA), anti-GFAP (Abcam), anti-Iba1 (Abcam), anti-DMBP (Abcam), anti-bFGF (Abcam), anti-CNTF (Abcam), anti-Nrf2 (Abcam), anti-NF- κ B (Abcam), anti-Caspases3 (Cell Signaling Technology, USA), anti-Bax (Abcam), and anti-ATF-6 (Millipore, USA), followed by corresponding secondary antibodies at RT for 2 h. Results were visualized under fluorescent microscopy (BX60, Olympus Imaging America Inc., USA) in a blinded fashion. Quantification was performed by Image-Pro Plus 6.0 software.

Western Blot

The proteins isolated from the brain and astrocytes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane (Millipore). After blocking with 5% milk for 1 h at RT, the membranes were incubated with anti-ABCA-1, anti-CNTF, anti-bFGF, anti-NF- κ B, anti-Nrf2, and anti- β -tubulin (Cell Signaling Technology) at 4°C for overnight, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) 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 -tubulin was used as an internal reference.

Quantitative Real-Time PCR

The total RNA of mice from the corpus callosum was isolated using a total RNA kit (Mei5bio, China). The reverse transcription of total RNA was achieved using a Prime Script TMRT reagent kit (Takara, Japan) to obtain cDNA. Quantitative PCR with SYBR Green Dye Gene Expression Assays was applied to determine gene expression using the ABI7500 system (Applied Biosystems, Carlsbad, CA, USA).

The required primers were synthesized by Beijing Qingke Biological Engineering Technology Company (Beijing, China). The required primer sequences were as follows: TLR4 (forward: 5'-AGCTTCTCCAATTTTTCAGAACTTC; reverse: 5'-TGAGAGGTGGTGTAAAGCCATGC), iNOS (forward: 5'-GAGACAGG GAAGTCTGAAGCAC; reverse: 5'-CCAGCAGTAG TTGCTCCTCTTC), and β -Actin (forward: 5'-CCTCACTGTCCACCTTCC; reverse: 5'-GGGTGTAAAACGCAGCTC). The cycle threshold was determined for each sample as the initial increase in fluorescence above the background. β -actin was used as an internal control for normalization.

ELISA Assay

The concentrations of IL-1 β , IL-6, and TNF- α were measured by sandwich ELISA kits (R&D System, USA) following the manufacturer's instructions. Determinations were performed in duplicate, and the results were expressed as pg/mL.

Statistical Analysis

Data were presented as the mean $\pm$ standard error of the mean. The raw data were analyzed using a one-way analysis of variance with Tukey's *post hoc* test using the GraphPad Prism 5.0 package (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value < 0.05 was considered to indicate a statistically significant difference.

Results

GK Promotes Astrocytes to Phagocytize Myelin Debris

In recent years, the clinical significance of astrocytes phagocytizing myelin debris has attracted extensive attention. In the CPZ model, degraded myelin basic protein (DMBP) was co-localized with GFAP⁺ astrocytes, which were elevated by *GK* as compared to CPZ model mice (Figure 1A, *p* < 0.0001). Then, to further verify whether *GK* can promote the phagocytosis of myelin debris by astrocytes, we investigated the phagocytosis capacity of astrocytes exposed to myelin debris *in vitro*. The results showed that *GK* promoted GL261 astrocytes to phagocytize myelin debris coupled with CFSE, as compared with myelin debris alone (Figure 1B, *p* < 0.001). Using a fluorescence microplate reader, the fluorescence intensity in the myelin debris + *GK* group was significantly higher than that in the myelin debris group (Figure 1C, *p* < 0.0001). Furthermore, primary astrocytes were exposed to myelin debris labeled with pHrodo that could be red in a lysosomal acid environment. GFAP⁺ astrocytes co-localizing pHrodo were significantly elevated after *GK* addition (Figure 1D, *p* < 0.0001). Taken together, astrocytes can phagocytize myelin debris, while *GK* can further promote the phagocytic ability of myelin debris by astrocytes.

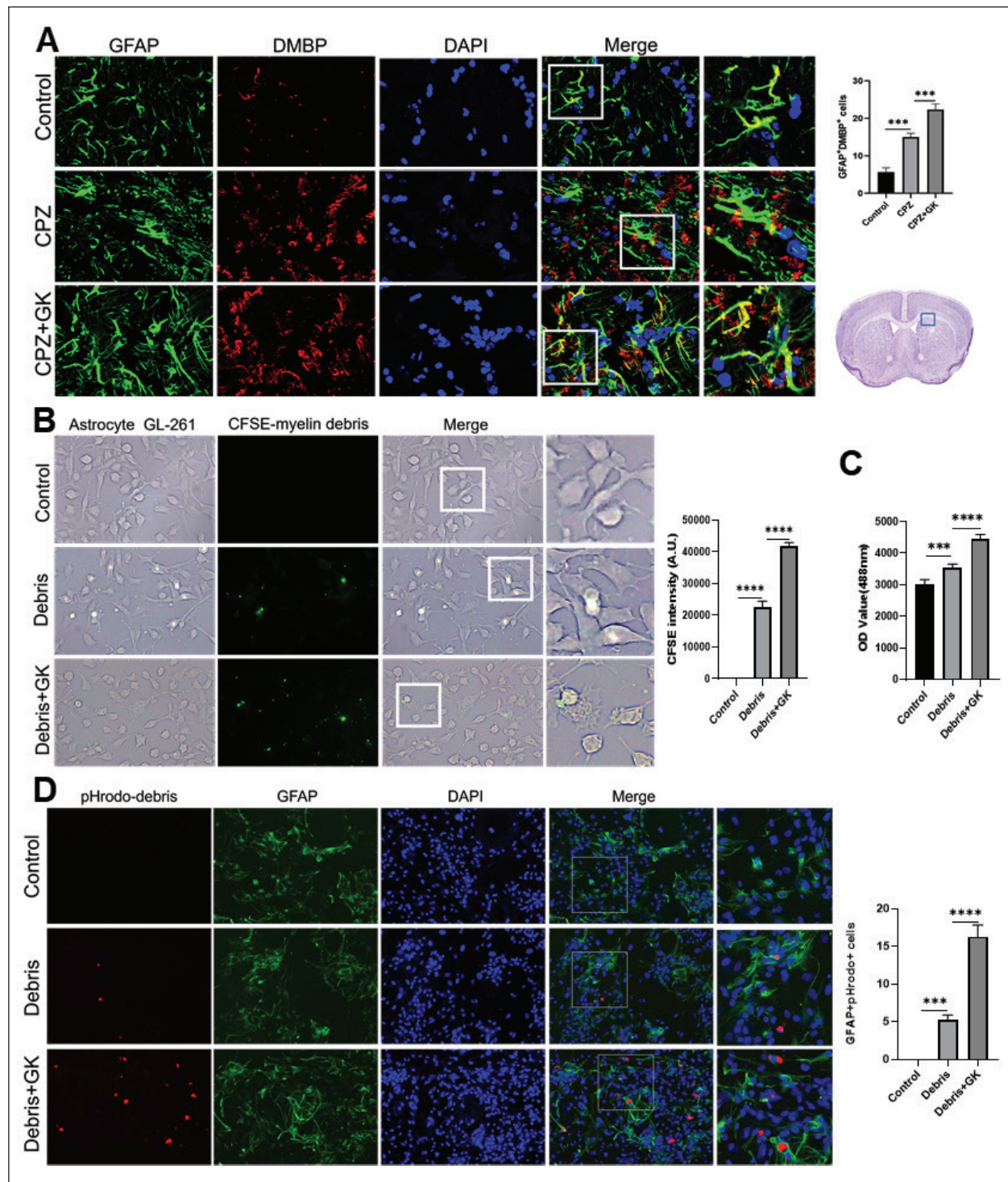


Figure 1. GK Promotes Astrocytes to Phagocytize Myelin Debris *in vitro* and *in vivo*. (A) *In Vivo* Experiments, Double Immunohistochemical Staining with Anti-GFAP and Anti-DMBP in the Corpus Callosum. (B) *In vitro* Experiments, after 3 h of GK Treatment, Astrocyte GL261 Cells Were Exposed to CFSE-Labeled Myelin Debris (4 mg/mL) for 24 h and Observed Under Fluorescence and Bright Field. (C) The Intensity of Fluorescence Was Recorded by Microplate Reader. (D) After 3 h of GK Treatment, Primary Astrocytes Were Exposed to Phrodo-labeled Myelin Debris (2 mg/mL) for 24 h and Observed under Fluorescence Microscope.

Note: The results were obtained from two independent cell experiments *in vitro* and four mice/each group *in vivo*. Micrographs are representative of slices in each sample with similar results. The results are represented as the mean $\pm$ SEM. *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: CFSE, carboxyfluorescein succinimide ester; GK, *Ginkgolide K*; DMBP, degraded myelin basic protein; GFAP, glial fibrillary acidic protein.

Previous investigations strongly support the hypothesis that ABCA1 is essential for astrocytic phagocytic capacity. Therefore, we detected the expression of ABCA1 on astrocytes after *GK* intervention in the CPZ mouse model. As shown in Figure 2A, *GK* significantly increased the numbers of GFAP⁺ astrocytes expressing ABCA1 by immunohistochemistry staining, as compared with CPZ model mice ($p < 0.0001$). The expression of ABCA1 protein in brain homogenate of mice treated with *GK* was also significantly higher than that of CPZ model mice by western blot (Figure 2B, $p < 0.0001$). To further test whether *GK* can induce ABCA1 expression on astrocytes exposed to myelin debris, we established an astrocyte model stimulated by myelin debris *in vitro*. The results showed that the expression of ABCA1 on primary astrocytes after *GK* treatment was higher than without *GK* (Figure 2C, $p < 0.001$). These results indicate that *GK* can promote astrocytes to upregulate ABCA1 expression, which should be related to the increased phagocytosis of myelin debris by astrocytes.

GK Regulates the Rebalance of Inflammatory and Oxidative Responses by Astrocytes Exposed to Myelin Debris

In the CPZ model, the expression of p-NF- κ B increased while the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) decreased in astrocytes as compared with control mice (Figure 3A; $p < 0.001$ and $p < 0.01$, respectively). Similar results also appeared in brain homogenate by western blot (Figure 3B; $p < 0.01$ and $p < 0.0001$, respectively). IL-6 and TNF- α protein as well as iNOS and TLR4 mRNA in brain homogenate of CPZ model mice were also higher than those of control mice (Figure 3C and D; $p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively). On the contrary, *GK* decreased astrocytes expressing p-NF- κ B and increased astrocytes expressing Nrf2 (Figure 3A; $p < 0.01$ and $p < 0.001$, respectively), which was consistent with that from brain homogenate by Western blot (Figure 3B; $p < 0.001$ and $p < 0.0001$, respectively). Similarly, *GK* inhibited the production

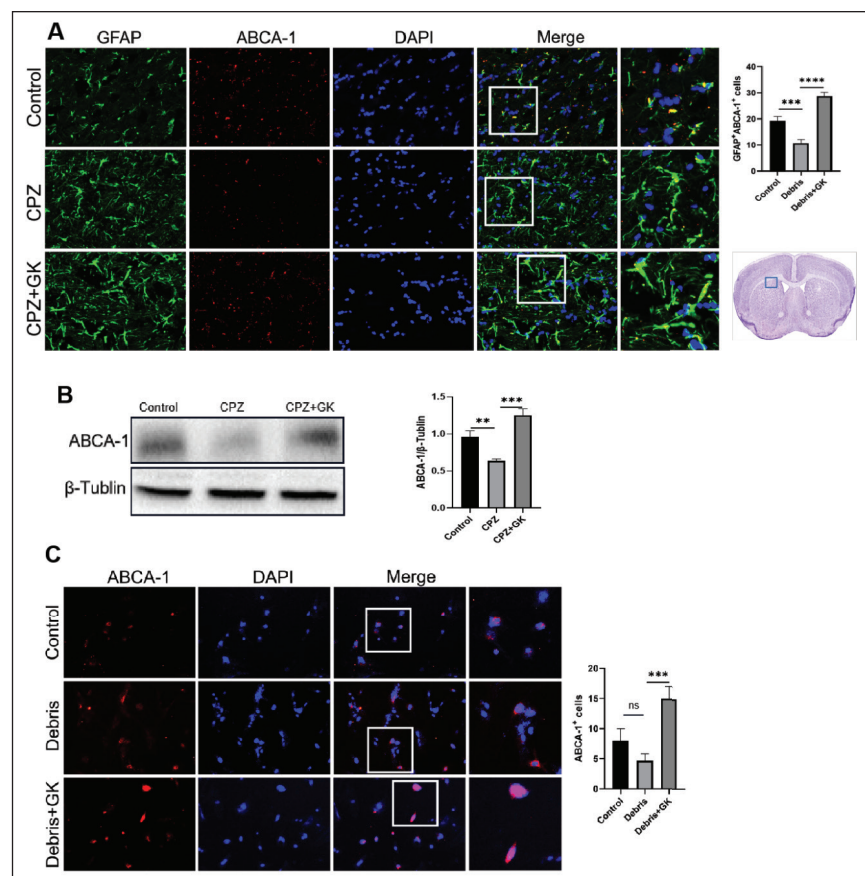


Figure 2. *GK* Upregulated the Expression of ABCA1 by Astrocytes Exposed to Myelin Debris *in vitro* and *in vivo*. (A) GFAP + ABCA1 + Cells in the Corpus Callosum. (B) The Expression of ABCA1 in Brain Homogenate. (C) The Expression of ABCA1 in Primary Astrocytes.

Note: The results were obtained from two independent cell experiments *in vitro* and four mice/each group *in vivo*. Micrographs are representative of slices in each sample with similar results. The results were represented as the mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: *GK*, Ginkgolide K; GFAP, glial fibrillary acidic protein; ABCA1, ATP binding cassette transporter A1.

of IL-6 and TNF- α (Figure 3C; $p < 0.0001$ and $p < 0.01$, respectively) and the expression of iNOS and TLR4 mRNA (Figure 3D; $p < 0.01$ and $p < 0.0001$, respectively). However, inconsistent with expectation, when primary astrocytes were exposed to myelin debris *in vitro*, myelin debris did not affect the expression of p-NF-kB and Nrf2 and the production of IL-1 β , IL-6, and TNF- α (Figure 4A and B). These results suggest that the phagocytosis of myelin debris is not enough to affect astrocytes' inflammatory and oxidative responses *in vitro*, and the inflammatory and oxidative responses in the brain of CPZ model mice may be more related to the microglial responses or astrocyte-microglia interaction.

GK Prevents Apoptosis by Astrocytes Exposed to Myelin Debris

We further observed the outcome of astrocytes phagocytizing myelin debris *in vitro*. As shown in Figure 5A, after primary astrocytes were exposed to myelin debris, cell death increased by lactate dehydrogenase (LDH) assay ($p < 0.0001$), while GK effectively inhibited cell death ($p < 0.001$). Further observation showed that GL261

astrocytes exposed to myelin debris could cause nuclear pyknosis under a microscope bright field. At the same time, GK significantly prevented nuclear pyknosis (Figure 5B). Similarly, primary astrocytes exposed to myelin debris showed uneven nuclear staining and small fragments in nuclei. GK can prevent this phenomenon under a fluorescence microscope (Figure 5C). These results reveal that astrocytes exposed to myelin debris should lead to self-apoptosis, which can be inhibited by GK.

Next, we detected the apoptosis-related molecules and cell apoptosis. As expected, the exposure to myelin debris induced astrocytes to upregulate caspase-3 and Bax and increase TUNEL+ apoptotic cells (Figure 5D and E; $p < 0.0001$). GK inhibited the expression of caspase-3 and Bax and the apoptosis of astrocytes (Figure 5D and E; $p < 0.0001$). Subsequently, based on a literature study, we detected apoptosis-related molecules in ER stress. The results showed that the expression of ATF6 increased in astrocytes exposed to myelin debris, which was also inhibited by GK (Figure 5F, $p < 0.0001$), suggesting that the signal pathway in the endoplasmic reticulum may be involved in the apoptosis of astrocytes exposed to myelin debris.

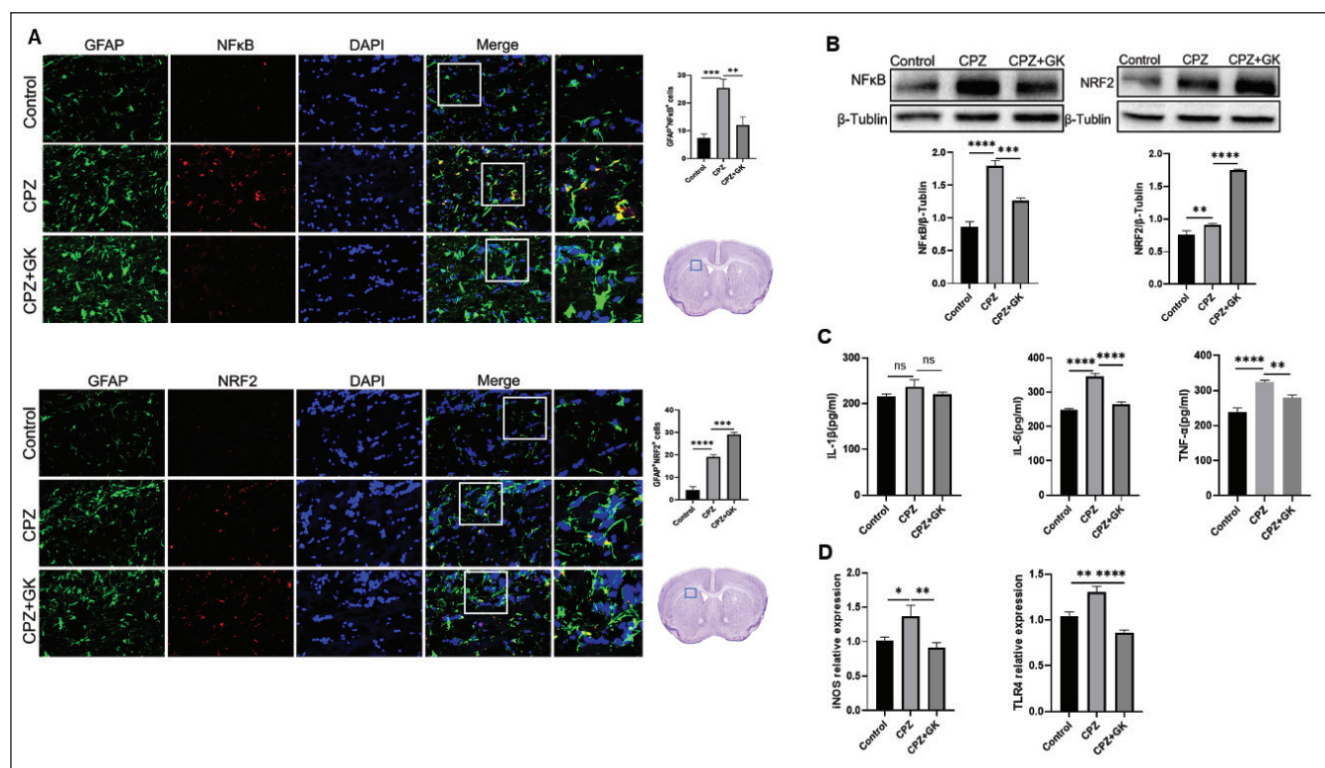


Figure 3. GK Regulated the Rebalance of Inflammatory and Oxidative Responses by Astrocytes *in vivo* Demyelination. (A) GFAP + NF-kB + or GFAP + Nrf2 + Cells in the Corpus Callosum. (B) The Expression of NF-kB and Nrf2 in Brain Homogenate. (C) The Level of IL-1 β , IL-6, and TNF- α in Brain Homogenate. (D) The Expression of iNOS and TLR4 mRNA in Brain Homogenate.

Note: The results were obtained from four mice/each group *in vivo*. Micrographs are representative of slices in each sample with similar results. The results are represented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

Abbreviations: GK, Ginkgolide K; GFAP, glial fibrillary acidic protein; Nrf2, nuclear factor erythroid 2-related factor 2; NF-kB, noncanonical nuclear factor-kB; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; TNF- α , tumor necrosis factor- α .

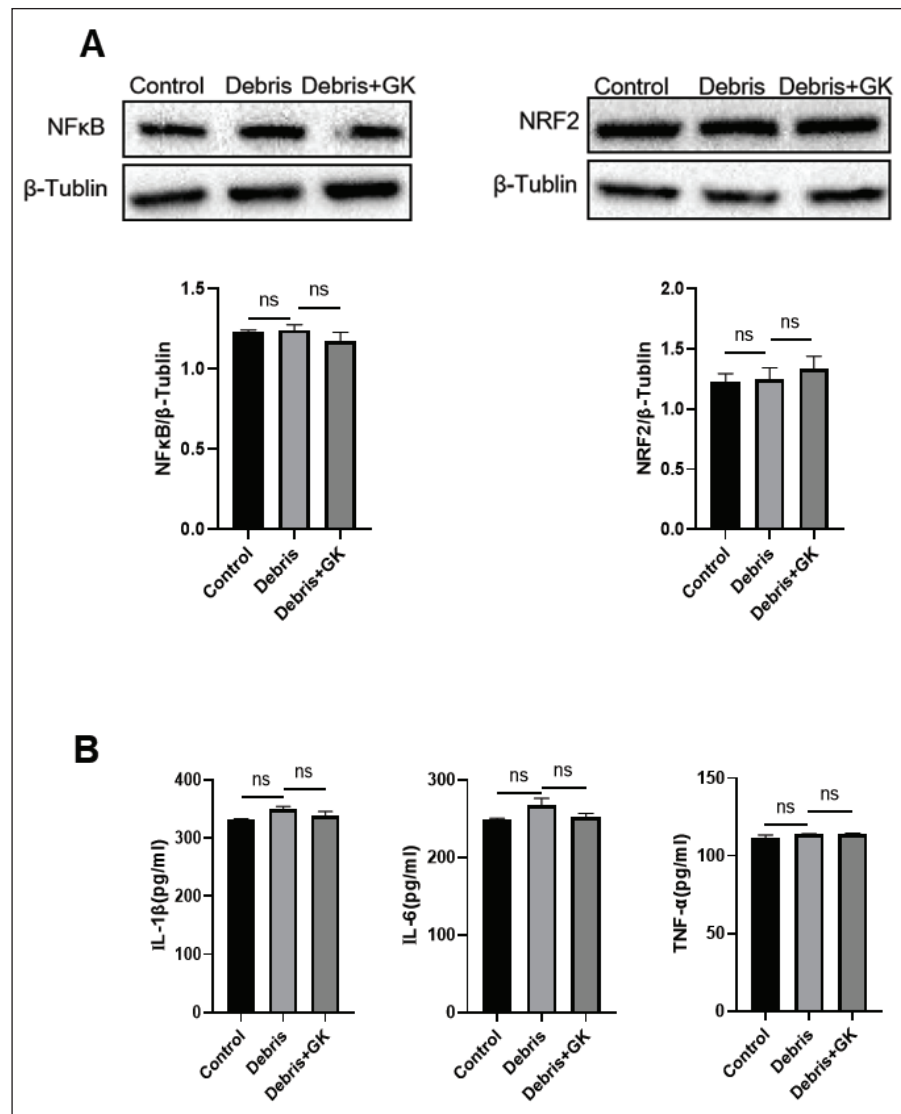


Figure 4. The Phagocytosis of Myelin Debris Is Not Enough to Affect the Inflammatory and Oxidative Responses of Astrocytes *in vitro*. After 3 h of GK Treatment, Primary Astrocytes Were Exposed to Myelin Debris (4 mg/mL) for 24 h. (A) The Expression of NF-κB and Nrf2 Protein in the Supernatant of Cultured Astrocytes. (B) The Level of IL-1β, IL-6, and TNF-α in the Supernatant of Cultured Astrocytes.

Note: The results were obtained from two independent cell experiments with similar results. The results are represented as the mean ± SEM.

Abbreviations: GK, Ginkgolide K; Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB, noncanonical nuclear factor-kB; IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.

GK Promotes Neurotrophic Factors by Astrocytes Exposed to Myelin Debris

Finally, we tried to understand how GK protects astrocytes from apoptosis induced by exposure to myelin debris. As anticipated, GK significantly increased GFAP⁺ astrocytes expressing CNTF and bFGF in the corpus callosum, as compared with CPZ model mice (Figure 6A; $p < 0.0001$ and $p < 0.01$, respectively).

The expression of CNTF and bFGF protein in brain homogenate of mice treated with GK was also significantly higher than that of CPZ model mice (Figure 6B, both $p < 0.0001$), suggesting that the myelin debris produced by demyelination in CPZ mice could stimulate astrocyte to generate CNTF and bFGF. GK also further promoted the production of CNTF and bFGF *in vitro* cultured astrocytes exposed to myelin debris (Figure 6C; $p < 0.0001$ and $p < 0.001$, respectively).

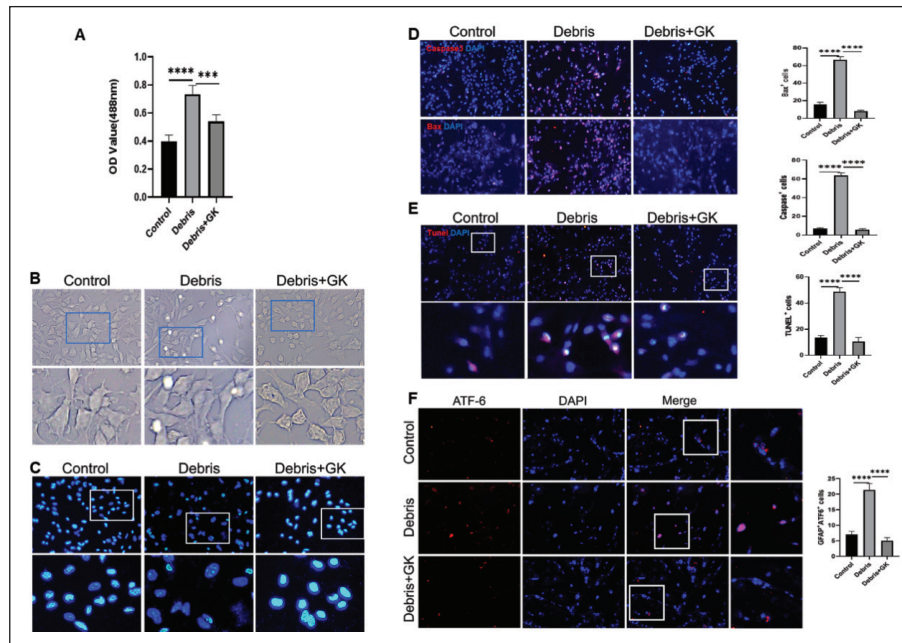


Figure 5. GK Prevented Apoptosis by Astrocytes Exposed to Myelin Debris. After 3 h of GK Treatment, GL261 and Primary Astrocytes Were Exposed to Myelin Debris (4 mg/mL) for 24 h. (A) LDH in the Supernatant of Primary Astrocytes. (B) GL261 astrocytes Were Observed under a Microscope Bright Field. (C) Cell Nucleus of Primary Astrocytes. (D–F) Primary Astrocytes Were Stained with Anti-Caspase-3, Anti-Bax, TUNEL Kit, and Anti-ATF6.

Note: The results were obtained from two independent cell experiments *in vitro*. Micrographs are representative of slices in each sample with similar results. The results are represented as the mean $\pm$ SEM. *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: GK, Ginkgolide K; LDH, lactate dehydrogenase.

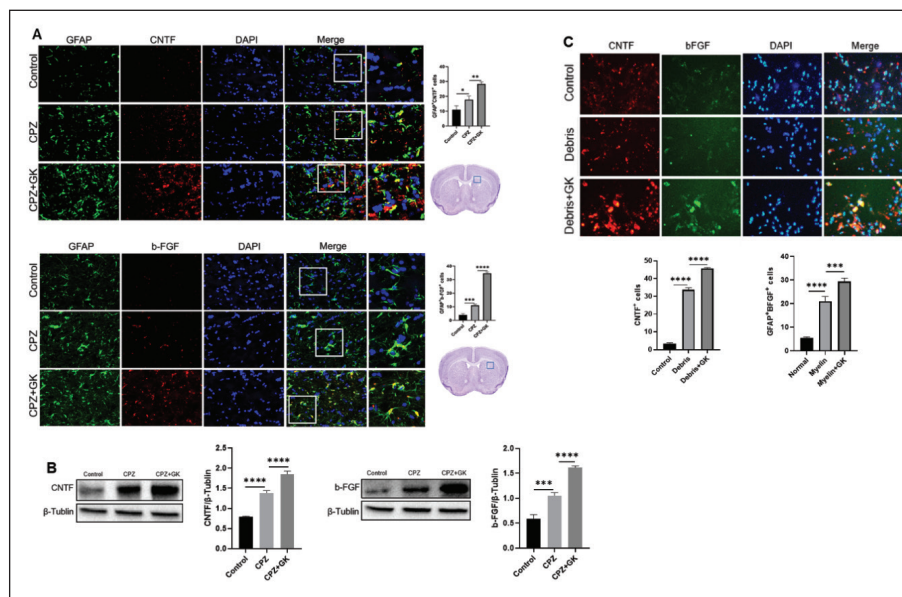


Figure 6. GK Promoted CNTF and bFGF Production by Astrocytes Exposed to Myelin Debris *in vitro* and *in vivo*. (A) CNTF and bFGF Expression in Brain Slices, as well as GFAP + CNTF or GFAP + bFGF + Cells in the Corpus Callosum. (B) The Expression of CNTF and bFGF in Brain Homogenate. (C) The Expression of CNTF and bFGF in Astrocytes.

Note: The results were obtained from two independent cell experiments *in vitro* and four mice/each group *in vivo*. Micrographs are representative of slices in each sample with similar results. The results are represented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: GK, Ginkgolide K; CNTF, ciliary neurotrophic factor; bFGF, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein.

Discussion

We previously found that *GK* treatment effectively alleviated behavioral abnormalities and improved demyelination in CPZ mice (Li et al., 2019). In this study, we further focused on the role of *GK* in targeting astrocytes to phagocytize myelin debris. The results showed that *GK* promoted astrocytes to phagocytize myelin debris, inhibited inflammatory signals, and induced antioxidant molecules. Astrocytes that have phagocytized myelin debris exhibited nuclear pyknosis, up-regulated the expression of caspase-3 and Bax, and increased TUNEL⁺ cell apoptosis, suggesting that astrocytes phagocytized myelin debris may lead to self-apoptosis. Meanwhile, *GK* promoted the formation of CNTF and bFGF, which should be related to anti-apoptosis and remyelination.

Efficient clearance of myelin debris by phagocytosis is critical to eliminate inhibitory signals interfering with oligodendrocyte progenitor cells (OPCs) activation, recruitment to the site of demyelination, and/or differentiation into myelinating mature oligodendrocytes (Franklin & Ffrench-Constant, 2017; Lampron et al., 2015). Traditionally, microglia are considered the primary phagocytes. Recent studies have reported that astrocytes also have phagocytic functions (Konishi et al., 2020; Lee & Chung, 2021; Lee et al., 2021; Ponath et al., 2017; Sanchez-Mico et al., 2021; Tao et al., 2017), providing a new idea for the therapy of demyelination. ABCA1 participates in astrocytic phagocytosis to remove damaged debris (Chen et al., 2021). Consistent with this observation, our results also revealed that *GK* promoted astrocytes to phagocytize myelin debris, possibly through the up-regulation of ABCA1 expression. How *GK* induces ABCA1 to promote astrocytic phagocytosis of myelin debris remains unclear. Limited information demonstrated that SIRT1 activator E1231 protects from experimental atherosclerosis and lowers plasma cholesterol and triglycerides by enhancing ABCA1 expression (Feng et al., 2018). Loss of SIRT1 *in vivo* reduces expression of a variety of liver X receptor targets involved in lipid metabolism, including ABCA1 (Li et al., 2007). Our latest results showed that *GK* induced the expression of SIRT1 in H₂O₂-induced PC12 neurons and reduced cellular senescence (unpublished data). These results suggest that *GK* induced AST upregulates the expression of ABCA1 and promotes the phagocytosis of myelin debris, which might be related to SIRT1. The detailed cellular and molecular mechanisms remain to be further clarified.

Besides, ABCA1 is known to have anti-inflammatory and anti-oxidative effects in macrophages (Tang et al., 2009). Interestingly, overexpression of ABCA1 decreased TNF- α and IL-6 production in endothelial cells (Stamatikos et al., 2019), suggesting that ABCA1 could form a negative feedback regulatory axis with inflammatory responses. Therefore, the mechanism by which ABCA1 participates in phagocytosis and anti-inflammation still needs to be further

explored. The last puzzling question is why astrocytes that phagocytize myelin debris will undergo self-apoptosis while *GK* further promotes phagocytosis but reduces apoptosis. Consistent with our results, telocytes enhanced M1 macrophage differentiation and phagocytosis while inhibiting apoptosis (Huang et al., 2021). When a particle is recognized by phagocytic receptors, various signaling pathways are activated to initiate phagocytosis. Generally speaking, cell responses associated with phagocytosis can be controlled by parallel signaling pathways. There are several studies to clarify the signal cascade of astrocyte apoptosis, including cytosolic Ca²⁺ elevation, oxidative stress, NF- κ B activation, mitochondrial dysfunction, ER stress, and protease activation. Therefore, we speculate that myelin debris stimulates astrocytes to enter a reactive state, including activation, inflammatory response, and oxidative stress, which may promote cell apoptosis, while *GK* may reduce apoptosis by inhibiting inflammatory and oxidative stress.

Microglia are classically considered phagocytes. However, astrocytes have been highlighted as phagocytes in the last few years. Microglia and astrocytes participate in developmental myelination by engulfing excessive, unneeded myelin. They are also involved in the clearance of degenerated myelin debris for accelerating remyelination or engulfing healthy myelin sheaths for inhibiting remyelination (Xu et al., 2023). Astrocytes are capable of phagocytosis and can provide a compensatory mechanism if microglia are unable to execute phagocytosis (Konishi et al., 2020). It was reported that astrocytes phagocytosed adult hippocampal synapses and eliminated unnecessary excitatory synaptic connections for circuit homeostasis, thereby supporting cognitive function (Lee et al., 2021). However, LCN2-expressing astrocytes acquire a phagocytic phenotype and are able to uptake myelin, leading to progressive demyelination in *Lcn2*^{-/-} mice (Wan et al., 2022). These studies raised the following questions: (1) what is the essential difference between astrocyte and microglia phagocytosis; (2) how microglia and astrocytes cooperate to complete phagocytic function; and (3) whether different signal molecules and pathways closely regulate astrocytic phagocytosis, demyelination, and remyelination. Based on these limited results, we speculate that *GK* protection against myelin debris-mediated apoptosis in astrocytes should be related to the regulation between ATF6, NF- κ B, and Nrf2 and the increase of CNTF and bFGF production. Of course, how *GK* affects these molecules and thus affects cell apoptosis still needs careful stripping and verification.

Conclusion

GK promoted astrocytes to phagocytize myelin debris, accompanied by the upregulation of ABCA1, inhibition of NF- κ B, and increase of Nrf2, CNTF, and bFGF, ultimately reducing the apoptosis of astrocytes stimulated with myelin

debris. Future research is anticipated to generate novel strategies for treating CNS demyelination.

Summary

When myelin debris was exposed to astrocytes, *GK* intervention promoted astrocytes to engulf myelin debris, possibly through the upregulation of ABCA1 expression, accompanied by the inhibition of inflammatory molecules and the formation of neurotrophic factors, leading to the improvement of the microenvironment for myelin sheath protection or regeneration.

Abbreviations

MS: Multiple sclerosis; CNS: central nervous system; *GK*: *Ginkgolide K*; EAE: experimental autoimmune encephalomyelitis; CPZ: cuprizone; CFSE: carboxyfluorescein succinimide ester; RT: room temperature; DMEM: Dulbecco's modified eagle medium; PBS: phosphate buffered saline; DMSO: dimethyl sulfoxide; BCA: bicinchoninic acid; ABCA1: ATP binding cassette transporter A1; GFAP: glial fibrillary acidic protein; Iba1: ionized calcium binding adapter molecule 1; DMBP: degraded myelin basic protein; CNTF: ciliary neurotrophic factor; bFGF: basic fibroblast growth factor; Nrf2: nuclear factor erythroid 2-related factor 2; NF- κ B: noncanonical nuclear factor-kappa B; Bax, BCL2-associated X; ATF6: activating transcription factor 6; HRP: horseradish peroxidase; PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; LDH: lactate dehydrogenase; IL-1 β : interleukin 1 β ; IL-6: interleukin 6; TNF- α : tumor necrosis factor- α ; ROS: reactive oxidative species.

Authors' Contributions

BX and CM designed the experiments and checked all experimental data. XL, KL, and ZD involved in the establishment of CPZ model and intervention of *GK*. QW performed Western blot. XL, KL, and LS performed astrocyte culture and treatment, ELISA, RT-PCR, immunocytochemistry, and immunohistochemistry. JY participated in the data analysis. DM, BX, and CM proofread and finalized the article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

All experimental protocols were approved by the Ethics Committee of Shanxi University of Chinese Medicine (AWE202209006, Approval date: October 7, 2022).

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References

- Attfield, K. E., Jensen, L. T., Kaufmann, M., Friese, M. A., & Fugger, L. (2022). The immunology of multiple sclerosis. *Nature Reviews Immunology*, 22(12), 734–750. <https://doi.org/10.1038/s41577-022-00718-z>
- Chen, W., Li, L., Wang, J., Zhang, R., Zhang, T., Wu, Y., Wang, S., & Xing, D. (2021). The ABCA1-efferocytosis axis: A new strategy to protect against atherosclerosis. *Clinica Chimica Acta*, 518, 1–8. <https://doi.org/10.1016/j.cca.2021.02.025>
- Feng, T., Liu, P., Wang, X., Luo, J., Zuo, X., Jiang, X., Liu, C., Li, Y., Li, N., Chen, M., Zhu, N., Han, X., Liu, C., Xu, Y., & Si, S. (2018). SIRT1 activator E1231 protects from experimental atherosclerosis and lowers plasma cholesterol and triglycerides by enhancing ABCA1 expression. *Atherosclerosis*, 274, 172–181. <https://doi.org/10.1016/j.atherosclerosis.2018.04.039>
- Franklin, R. J. M., & Ffrench-Constant, C. (2017). Regenerating CNS myelin: From mechanisms to experimental medicines. *Nature Reviews Neuroscience*, 18(12), 753–769. <https://doi.org/10.1038/nrn.2017.136>
- Huang, Y. L., Zhang, F. L., Tang, X. L., & Yang, X. J. (2021). Telocytes enhances M1 differentiation and phagocytosis while inhibits mitochondria-mediated apoptosis via activation of NF- κ B in macrophages. *Cell Transplantation*, 30, 9636897211002762. <https://doi.org/10.1177/09636897211002762>
- Konishi, H., Okamoto, T., Hara, Y., Komine, O., Tamada, H., Maeda, M., Osako, F., Kobayashi, M., Nishiyama, A., Kataoka, Y., Takai, T., Udagawa, N., Jung, S., Ozato, K., Tamura, T., Tsuda, M., Yamanaka, K., Ogi, T., Sato, K., & Kiyama, H. (2020). Astrocytic phagocytosis is a compensatory mechanism for microglial dysfunction. *EMBO Journal*, 39(22), e104464. <https://doi.org/10.15252/embj.2020104464>
- Lampron, A., Larochelle, A., Laflamme, N., Préfontaine, P., Plante, M. M., Sánchez, M. G., Yong, V. W., Stys, P. K., Tremblay, M. È., & Rivest, S. (2015). Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *Journal of Experimental Medicine*, 212(4), 481–495. <https://doi.org/10.1084/jem.20141656>
- Lassmann, H., van Horssen, J., & Mahad, D. (2012). Progressive multiple sclerosis: Pathology and pathogenesis. *Nature Reviews Neurology*, 8(11), 647–656. <https://doi.org/10.1038/nrneurol.2012.168>
- Lee, J. H., Kim, J. Y., Noh, S., Lee, H., Lee, S. Y., Mun, J. Y., Park, H., & Chung, W. S. (2021). Astrocytes phagocytose adult hippocampal synapses for circuit homeostasis. *Nature*, 590(7847), 612–617. <https://doi.org/10.1038/s41586-020-03060-3>
- Lee, S. Y., & Chung, W. S. (2021). The roles of astrocytic phagocytosis in maintaining homeostasis of brains. *Journal of Pharmacological Sciences*, 145(3), 223–227. <https://doi.org/10.1016/j.jphs.2020.12.007>
- Li, Q. Y., Miao, Q., Sui, R. X., Cao, L., Ma, C. G., Xiao, B. G., Xiao, W., & Yu, W. B. (2019). Ginkgolide K supports remyelination

- via induction of astrocytic IGF/PI3K/Nrf2 axis. *International Immunopharmacology*, 75, 105819. <https://doi.org/10.1016/j.intimp.2019.105819>
- Li, X., Zhang, S., Blander, G., Tse, J. G., Krieger, M., & Guarente, L. (2007). SIRT1 deacetylates and positively regulates the nuclear receptor LXR. *Molecular Cell*, 28(1), 91–106. <https://doi.org/10.1016/j.molcel.2007.07.032>
- Miao, Q., Chai, Z., Song, L. J., Wang, Q., Song, G. B., Wang, J., Yu, J. Z., Xiao, B. G., & Ma, C. G. (2022). The neuroprotective effects and transdifferentiation of astrocytes into dopaminergic neurons of ginkgolide K on Parkinson's disease mice. *Journal of Neuroimmunology*, 364, 577806. <https://doi.org/10.1016/j.jneuroim.2022.577806>
- Morizawa, Y. M., Hirayama, Y., Ohno, N., Shibata, S., Shigetomi, E., Sui, Y., Nabekura, J., Sato, K., Okajima, F., Takebayashi, H., Okano, H., & Koizumi, S. (2017). Reactive astrocytes function as phagocytes after brain ischemia via ABCA1-mediated pathway. *Nature Communications*, 8(1), 28. <https://doi.org/10.1038/s41467-017-00037-1>
- Motavaf, M., Sadeghizadeh, M., & Javan, M. (2017). Attempts to overcome remyelination failure: Toward opening new therapeutic avenues for multiple sclerosis. *Cellular and Molecular Neurobiology*, 37(8), 1335–1348. <https://doi.org/10.1007/s10571-017-0472-6>
- Piehl, F. (2021). Current and emerging disease-modulatory therapies and treatment targets for multiple sclerosis. *Journal of Internal Medicine*, 289(6), 771–791. <https://doi.org/10.1111/joim.13215>
- Plemel, J. R., Liu, W.-Q., & Yong, V. W. (2017). Remyelination therapies: A new direction and challenge in multiple sclerosis. *Nature Reviews Drug Discovery*, 16(9), 617–634. <https://doi.org/10.1038/nrd.2017.115>
- Ponath, G., Ramanan, S., Mubarak, M., Housley, W., Lee, S., Sahinkaya, F. R., Vortmeyer, A., Raine, C. S., & Pitt, D. (2017). Myelin phagocytosis by astrocytes after myelin damage promotes lesion pathology. *Brain*, 140(2), 399–413. <https://doi.org/10.1093/brain/aww298>
- Sanchez-Mico, M. V., Jimenez, S., Gomez-Arboledas, A., Muñoz-Castro, C., Romero-Molina, C., Navarro, V., Sanchez-Mejias, E., Nuñez-Díaz, C., Sanchez-Varo, R., Galea, E., Davila, J. C., Vizuite, M., Gutierrez, A., & Vitorica, J. (2021). Amyloid- β impairs the phagocytosis of dystrophic synapses by astrocytes in Alzheimer's disease. *Glia*, 69(4), 997–1011. <https://doi.org/10.1002/glia.23943>
- Sen, M. K., Mahns, D. A., Coorsen, J. R., & Shortland, P. J. (2022). The roles of microglia and astrocytes in phagocytosis and myelination: Insights from the cuprizone model of multiple sclerosis. *Glia*, 70(7), 1215–1250. <https://doi.org/10.1002/glia.24148>
- Stamatikos, A., Dronadula, N., Ng, P., Palmer, D., Knight, E., Wacker, B. K., Tang, C., Kim, F., & Dichek, D. A. (2019). ABCA1 overexpression in endothelial cells in vitro enhances apoAI-mediated cholesterol efflux and decreases inflammation. *Human Gene Therapy*, 30(2), 236–248. <https://doi.org/10.1089/hum.2018.120>
- Tang, C., Liu, Y., Kessler, P. S., Vaughan, A. M., & Oram, J. F. (2009). The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *Journal of Biological Chemistry*, 284(47), 32336–32343. <https://doi.org/10.1074/jbc.M109.047472>
- Tao, X. Q., Cao, Z. Y., Cao, L., & Xiao, W. (2017). Effects of ginkgolide K on platelet aggregation activity and neuroprotection. *Zhongguo Zhong Yao Za Zhi*, 42(24), 4727–4732. <https://doi.org/10.19540/j.cnki.cjcm.2017.0208>
- Wan, T., Zhu, W., Zhao, Y., Zhang X., Ye, R., Zuo, M., Xu, P., Huang Z., Zhang, C., Xie, Y., & Liu, X. (2022). Astrocytic phagocytosis contributes to demyelination after focal cortical ischemia in mice. *Nature Communications*, 13(1), 1134. <https://doi.org/10.1038/s41467-022-28777-9>
- Xu, T., Liu, C., Deng, S., Gan, L., Zhang, Z., Yang, G. Y., Tian, H., & Tang, Y. (2023). The roles of microglia and astrocytes in myelin phagocytosis in the central nervous system. *Journal of Cerebral Blood Flow and Metabolism*, 43(3), 325–340. <https://doi.org/10.1177/0271678X221137762>
- Yu, W. B., Wang, Q., Chen, S., Cao, L., Tang, J., Ma, C. G., Xiao, W., & Xiao, B. G. (2019). The therapeutic potential of ginkgolide K in experimental autoimmune encephalomyelitis via peripheral immunomodulation. *International Immunopharmacology*, 70, 284–294. <https://doi.org/10.1016/j.intimp.2019.02.035>
- Zhou, H., Hu, L., Li, J., Ruan, W., Cao, Y., Zhuang, J., Xu, H., Peng, Y., Zhang, Z., Xu, C., Yu, Q., Li, Y., Dou, Z., Hu, J., Wu, X., Yu, X., Gu, C., Cao, S., Yan, F., & Chen, G. (2021). AXL kinase-mediated astrocytic phagocytosis modulates outcomes of traumatic brain injury. *Journal of Neuroinflammation*, 18(1), 154. <https://doi.org/10.1186/s12974-021-02201-3>