Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2025**; 30(3): 36255
https://doi.org/10.31083/FBL36255

Original Research

Scutellarin Attenuates Pro-Inflammatory Foam Cell Formation and Facilitates M2 Polarization in Microglia during Copper Homeostasis Imbalance via the MAPK Signaling Pathway

Qiting Zhao[1,2,†], Lingyi Chen[1,2,†], Yantuanjin Ma[1,2], Shufen Wang[1,2,*]

[1]Institute of Biomedical Engineering, Kunming Medical University, 650500 Kunming, Yunnan, China
[2]Yunnan Key Laboratory of Breast Cancer Precision Medicine, 650051 Kunming, Yunnan, China
*Correspondence: wangshufen@kmmu.edu.cn (Shufen Wang)
[†]These authors contributed equally.

Academic Editor: Seok-Geun Lee
Submitted: 9 December 2024 Revised: 14 January 2025 Accepted: 22 January 2025 Published: 6 March 2025

Abstract

Background: Clinical and experimental evidence indicates that copper has the ability to promote the progressive development of demyelinating diseases such as multiple sclerosis. Microglia-mediated neuroinflammation is believed to play a crucial role in this process. Scutellarin, a flavonoid compound, has anti-inflammatory, antioxidative, and neuroprotective effects. **Aim**: We investigated the effect of scutellarin on copper-induced inflammatory foam cell formation in microglia. **Methods**: We exposed BV2 murine microglial cells to copper, then collected the conditioned medium and co-cultured it with MO3.13 human glial cells to mimic myelin damage *in vitro*. The Cell Counting kit-8 assay, quantitative (polymerase chain reaction) PCR, enzyme-linked immunosorbent assay, Luxol fast blue staining, and western blotting were used to detect the cell phenotype. To investigate whether exposure of BV2 cells to copper can cause neurotoxicity and indirect damage to myelin cells, we determined whether BV2 cells promote inflammation through foam cell formation by oil red O staining and detection of malondialdehyde (MDA) content. Finally, we treated cells with scutellarin to investigate its therapeutic effects. **Results**: Exposure to copper activated the pro-inflammatory phenotype of microglia, as assessed by measuring the transcription of M1/M2-related biomarkers. In addition, increased copper intake by microglia promoted intracellular lipid accumulation and oxidation, facilitating foam cell formation. Rescue experiments showed that copper chelator ammonium tetrathiomolybdate (ATTM) and the lipid oxidation inhibitor ferrostatin-1 (Fer-1) significantly inhibited copper-induced inflammation, reduced intracellular lipid accumulation and MDA levels, and decreased foam cell formation. Moreover, copper-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) in microglia led to a shift towards the M1 phenotype and foam cell transformation, which were effectively inhibited by ATTM, Fer-1, and the p38 MAPK inhibitor SB203580. Lastly, after treatment with scutellarin, copper-induced foam microglia exhibited inhibited p38 MAPK phosphorylation, increased production of neurotrophic factors, decreased expression of inflammatory mediators, reduced lipid accumulation, and induced polarization towards the M2 phenotype. **Conclusions**: Here, we demonstrated that copper can induce microglia to damage myelinating cells, with the key mechanism involving the phosphorylation of p38 MAPK. Scutellarin partially reversed the positive effects of copper on promoting microglial M1 polarization, lipid deposition, and lipid oxidation by mediating the p38 MAPK signaling pathway. Taken together, these results suggest that scutellarin may be a promising drug for the treatment of demyelinating diseases such as multiple sclerosis.

Keywords: scutellarin; copper; microglia; foam cells; multiple sclerosis

1. Introduction

Multiple sclerosis (MS) is a progressive autoimmune disease that affects the central nervous system (CNS) [1,2]. One of the pathological features of this disease is damage to oligodendrocytes caused by inflammatory mediators produced by microglia, leading to demyelination and ultimately causing neurological decline. Although microglia can produce neurotrophic factors to repair the CNS, microglia can also contribute to neuroinflammation and neurodegeneration in MS by releasing inflammatory mediators. In the early and late stages of MS, activated microglia are present, which secrete a large number of inflammatory and neurotoxic mediators. Microglia activation occurs before the onset of MS; thus, inhibiting this early activation can hinder the development of CNS inflammatory lesions to a certain extent [3–6].

"Foam cells" are a type of cell that have accumulated large amounts of lipids in their cytoplasm, giving them a foamy appearance under a microscope [7]. The formation of foam cells in demyelinating lesions is accompanied by inflammatory responses or repair mechanisms, which can cause damage to the CNS or promote its repair [5,6,8–12]. Studies have shown that microglia form foam cells by phagocytosing myelin debris generated from myelin injury, thereby exacerbating myelin damage and promoting MS progression [5,6]. However, little is known about how pro-inflammatory foam-like microglia participate in the initiation and progression of MS.

Although the pathogenesis of MS remains unclear, studies have shown that toxic substances in the environment can activate the immune system, causing neuronal damage [13–15]. Therefore, it is necessary to study the role of environmental toxins in MS. Humans may inadvertently be exposed to metals in their daily lives, and metals play an important role in neurodegenerative diseases [16,17]. Dysregulation of metal homeostasis may contribute to the onset and progression of MS [18].

Copper is a metal contaminant that has garnered attention due to its widespread use in agriculture, electronics, dyes, cosmetics, food processing, and other industries. Although environmental studies have suggested that copper may increase the risk of MS [19–23], the effects of copper on MS remain unclear. It has been shown that copper accumulation precedes inflammation and myelin lesions in N,N-diethyldithiocarbamate-induced demyelinating diseases. Moreover, its applications in agriculture, medicine, and industry provide opportunities for copper to enter the nervous system and participate in demyelination [24–30]. Currently, there is ample clinical evidence supporting the adverse effects of copper on myelin. Studies have demonstrated that the levels of copper in the cerebrospinal fluid (CSF) and blood of patients with MS are significantly elevated [31–35]. Notably, while the copper content is lower in secondary progressive MS compared to primary progressive MS, levels in both are still higher than those in healthy individuals [32]. Additionally, female patients with MS exhibit increased copper absorption and half-life, resulting in greater copper accumulation compared to male patients and healthy individuals [23]. Proteomic analyses further revealed an imbalance in the expression of copper-related proteins in female patients with relapsing-remitting MS [36]. Moreover, increased copper levels in CSF have been detected in Skogholt disease, a neurological disorder characterized by myelin damage [37,38]. Furthermore, demyelinating lesions have been observed in patients with Wilson's disease, a genetic disorder where the body is unable to metabolize copper [39–44]. Wilson's disease has also been confirmed in animals [45,46]. However, little is known about the potential pathogenic mechanisms of copper in MS. Moreover, there are no reports on the involvement of copper exposure in the formation of foam cells in microglia in the CNS. Therefore, one objective of this study was to investigate whether copper can induce inflammation in the nervous system by promoting the transformation of microglia into foam cells.

Scutellarin, a flavonoid compound, possesses significant neuroprotective effects including anti-inflammatory, antioxidative, and anti-apoptotic properties, and is widely used in the treatment of neurological diseases [47–51]. Scutellarin promotes M2 polarization by inhibiting phosphorylation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Previous studies have demonstrated the therapeutic potential of scutellarin in MS using various cellular and animal models [52,53]. However, the mechanism by which scutellarin treats neuroinflammation induced by microglia with a foam cell phenotype remains unclear.

Generally, excessive metal ions and inflammation significantly contribute to demyelination. The results of this study showed that scutellarin can attenuate copper overdose-induced foam cell formation and inflammation in microglia through the p38 MAPK signaling pathway, and promote myelin sheath protection.

2. Materials and Methods

2.1 Cell Culture and Treatment

BV2 murine microglial cells were kindly provided by Dr. Qiuye Jia (Kunming Medical University, Kunming, China). MO3.13 human glial cells were purchased from Warner Bio (WN-51194; Wuhan, China). All cell lines were validated by short tandem repeat profiling and tested negative for mycoplasma. Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Opcel, Inner Mongolia, China), and incubated at 37 °C in air containing 5% CO_2. A total of 6×10^5 cells in 2 mL DMEM containing 10% FBS were plated per well in 6-well plates. After 1 day, the cells were pretreated overnight with a p38 inhibitor (SB203580, 10 µM; MedChemExpress (MCE), Monmouth Junction, NJ, USA), ferrostatin-1 (Fer-1, 1 µM; MCE), ammonium tetrathiomolybdate (ATTM, 10 µM; MCE), and scutellarin (20 µM; MCE) followed by copper exposure (Merck, Billerica, MA, USA). After 24 h, cell supernatants were collected for co-culture with MO3.13 human glial cells and the enzyme-linked immunosorbent assay (ELISA). Cells were collected for quantitative polymerase chain reaction (qPCR) and western blot analysis. Cell viability (Cell Counting Kit-8 [CCK-8] Assay Kit; Beyotime, Shanghai, China), intracellular lipid level (Oil Red O Staining Kit; Solarbio, Beijing, China), and myelin maturation (Luxol Fast Blue Staining Kit; Servicebio, Wuhan, China) were assessed according to the manufacturers' instructions. Experiments were performed in triplicate and repeated three times.

2.2 Intracellular Copper Assay

After cells were treated with copper, copper absorption levels were measured using a copper assay kit according to the manufacturer's instructions (Solarbio). Three independent experiments were conducted for statistical analyses.

2.3 Quantitative Polymerase Chain Reaction

Total cellular RNA was extracted with RNAiso Plus (TaKaRa, Dalian, China). For the reverse transcription of total RNA, PrimeScript RT Master Mix (TaKaRa) was used. The primer sequences are listed in **Supplementary Table**

1. After reverse transcription, qPCR analyses of mRNA levels were performed using the SYBR Green Real-Time PCR Master Mix Kit (TaKaRa) under the following conditions: initial pre-incubation at 95 °C for 30 s, followed by 39 cycles at 95 °C for 5 s and 60 °C for 30 s. Gene levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were conducted for statistical analyses.

2.4 ELISA

Tumor necrosis factor alpha (TNF-α) concentrations in the cellular culture medium were determined by an ELISA kit (BOSTER, Wuhan, China) according to the manufacturer's instructions. Three independent experiments were performed for statistical analyses.

2.5 Western Blot Analysis

Cells were lysed in 200 µL Radio Immunoprecipitation Assay Lysis Buffer (RIPA buffer, Beyotime) with 1% Phenylmethanesulfonyl fluoride (PMSF, Beyotime) and 1% phosphoprotein inhibitor, and boiled at 100 °C for 10 min. Protein concentrations were determined with the Bicinchoninic Acid Assay (BCA assay, Beyotime). Proteins (30 mg) were resolved by electrophoresis with 12% polyacrylamide gels (Yamei, Shanghai, China) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Merck). The membrane was then incubated with appropriate primary and secondary antibodies, including JNK Antibody (1:1000, #9252, Cell Signaling Technology (CST), Danvers, MA, USA), p-JNK Antibody (1:1000, #9251, CST), p38 MAPK Antibody (1:1000, #9212, CST), p-p38 MAPK Antibody (1:1000, #9211, CST), ERK1/2 Antibody (1:1000, #9102, CST), p-ERK1/2 Antibody (1:1000, #9101, CST), MBP Antibody (1:1000, #78896, CST), MAG Antibody (1:1000, #9043, CST), and β-actin Antibody (1:10000, #BA2305, BOSTER). Three independent experiments were performed for statistical analyses. All original western blot images are shown in **Supplementary file 1**.

2.6 Statistical Analyses

All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism 8 (GraphPad software Inc., La Jolla, CA, USA). Quantitative results are shown as the mean $\pm$ standard error of the mean. Images were adjusted with Adobe Photoshop and Adobe Illustrator (Adobe Inc., San Jose, CA, USA). $p < 0.05$ was considered statistically significant. * was considered $p < 0.05$ that significant statistically. ** and *** were considered $p < 0.01$ and $p < 0.001$ that highly statistically significant, respectively.

3. Results

3.1 Copper can Induce Indirect Damage to Myelin Cells through Activation of Microglia

The CCK-8 assay was performed to detect the viability of BV2 cells after exposure to different copper concentrations (0, 2.5, 5, 10, 20, 30, 40, 50 µg). The results showed that copper inhibited cell viability in a concentration-dependent manner after 24 h of treatment (Fig. 1A). In addition, the copper content increased with increasing copper concentrations (Fig. 1B); this effect was inhibited by the ATTM copper chelator (Fig. 1C,D). Changes in the gene expression levels of copper transporter 1 and divalent metal transporter 1 copper transporters supported this finding (Fig. 1E,F). To determine whether copper indirectly induced myelin cell damage at doses that activate microglia, we observed the effects of copper treatment on MO3.13 cell viability after 24 h (Fig. 1G). Exposure to 0–20 µg copper for 24 h had no significant effect on MO3.13 cell viability, whereas 40 µg copper for 24 h led to significant MO3.13 cell death. Based on the viability of BV2 and MO3.13 cells under copper exposure, we next treated microglia with 20 µg copper. After 24 h of copper exposure, diluted (50%) microglia cell culture conditioned medium (Cu-BV2 CCM) was used to culture MO3.13 cells. The results showed that MO3.13 cell viability and maturation decreased under Cu-BV2 CCM treatment, and the protein expression levels of MAG and MBP were decreased. These effects were rescued with ATTM treatment (Fig. 1H–J, **Supplementary Fig. 1A,B**). The results demonstrated that copper can induce indirect damage to myelin cells through activation of microglia.

3.2 Copper Promotes Inflammatory Responses by Regulating the Polarization of Microglia towards the M1 Phenotype

Next, we were interested in determining how copper induces microglia-mediated cellular damage. First, BV2 cells were exposed to copper for 24 h, followed by light microscopy to visualize their morphology. We found that microglia were significantly activated after 24 h of copper exposure, as we observed marked changes in the morphology of BV2 cells, transitioning from a resting state with short cell bodies and elongated branches to an activated state with swollen cell bodies, narrow shapes, and shorter branches (Fig. 2A). Subsequently, qPCR analysis revealed significant increases in the gene expression of microglial activation markers cluster of differentiation 68 (*Cd68*) and (*Cd45*) under copper exposure (Fig. 2B,C), confirming the activation of microglia. Given that microglial activation can be categorized into pro-inflammatory M1 and anti-inflammatory M2 phenotypes, we next investigated the phenotypic changes. As shown in Fig. 2D–I, qPCR analysis revealed the significant upregulation of mRNA expression of M1-related markers (*Tnf-*α, prostaglandin-endoperoxide synthase 2 [*Ptgs2*], interleukin 6 [*Il-6*]) and downregulation of M2-related markers (transforming growth factor beta [*Tgf-*β], arginase 1 [*Arg-1*], insulin-like growth factor 1 [*Igf-1*]) after 24 h of copper exposure, indicating a shift towards the M1 phenotype in microglia following copper exposure. Consistently, expression of the pro-inflammatory cytokine (*Tnf-*α) in microglial cultures increased under cop-

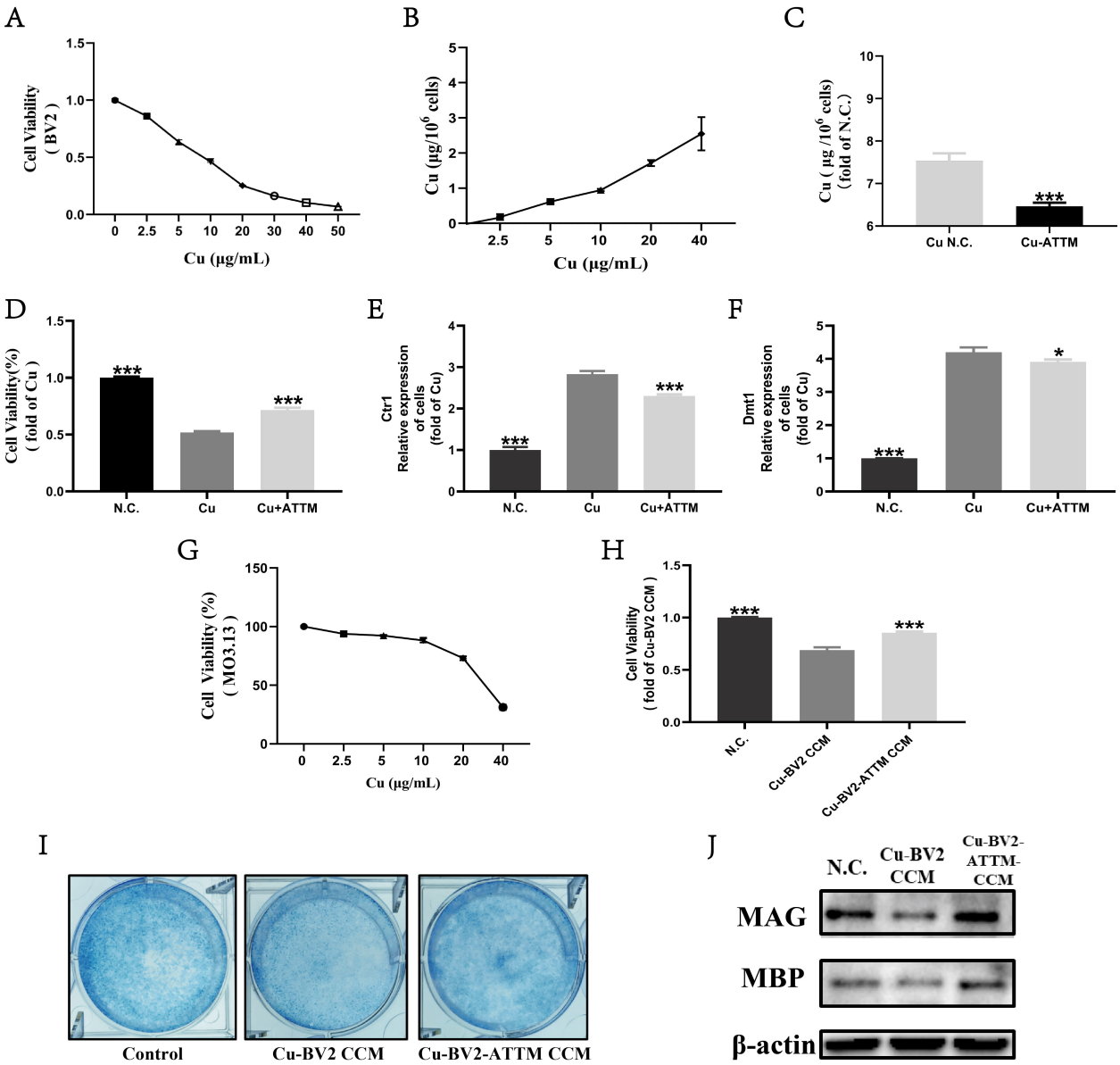

Fig. 1. Imbalance in copper homeostasis of microglia can cause neurotoxicity, resulting in indirect damage to myelin sheath cells. (A) Detection of BV2 cell viability treated with different concentrations of copper using the CCK-8 method. (B) Cu content in BV2 cells after 24-hour copper exposure. (C,D) Changes in BV2 cell viability and Cu content in cells under copper exposure after overnight ATTM pretreatment. (E,F) qPCR analysis of the mRNA levels of *Ctr1* and *Dmt1* in copper-treated BV2 cells after overnight ATTM pretreatment. (G) Detection of MO3.13 cell viability treated with different concentrations of copper using the CCK-8 method. (H–J) Changes in myelin sheath cell damage after CCM treatment following overnight ATTM pretreatment. Diameter = 40 mm. All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism. Quantitative results were shown as mean ± SEM. $*p < 0.05$, $***p < 0.001$. CCK-8, Cell Counting Kit-8; ATTM, ammonium tetrathiomolybdate; qPCR, quantitative polymerase chain reaction; CCM, culture conditioned medium; SEM, standard error of the mean; N.C., negative control; MBP, myelin basic protein; MAG, myelin-associated glycoprotein.

per stimulation (Fig. 2K), with Tnf-α being a key inflammatory factor contributing to myelin damage in MS [54]. Importantly, ATTM treatment reversed these phenomena, promoting the transition of microglia from the M1 to M2 phenotype. These results demonstrate that copper promotes inflammatory responses by regulating the polarization of microglia towards the M1 phenotype.

3.3 Imbalances in Intracellular Copper Homeostasis Trigger Foam Cell Formation in Microglia, Contributing to Myelin Damage

Previous studies have shown that microglia in MS phagocytose myelin debris, leading to the formation of foam cells [5,6]. However, our study found that the transition of microglia into foam cells could be induced solely by

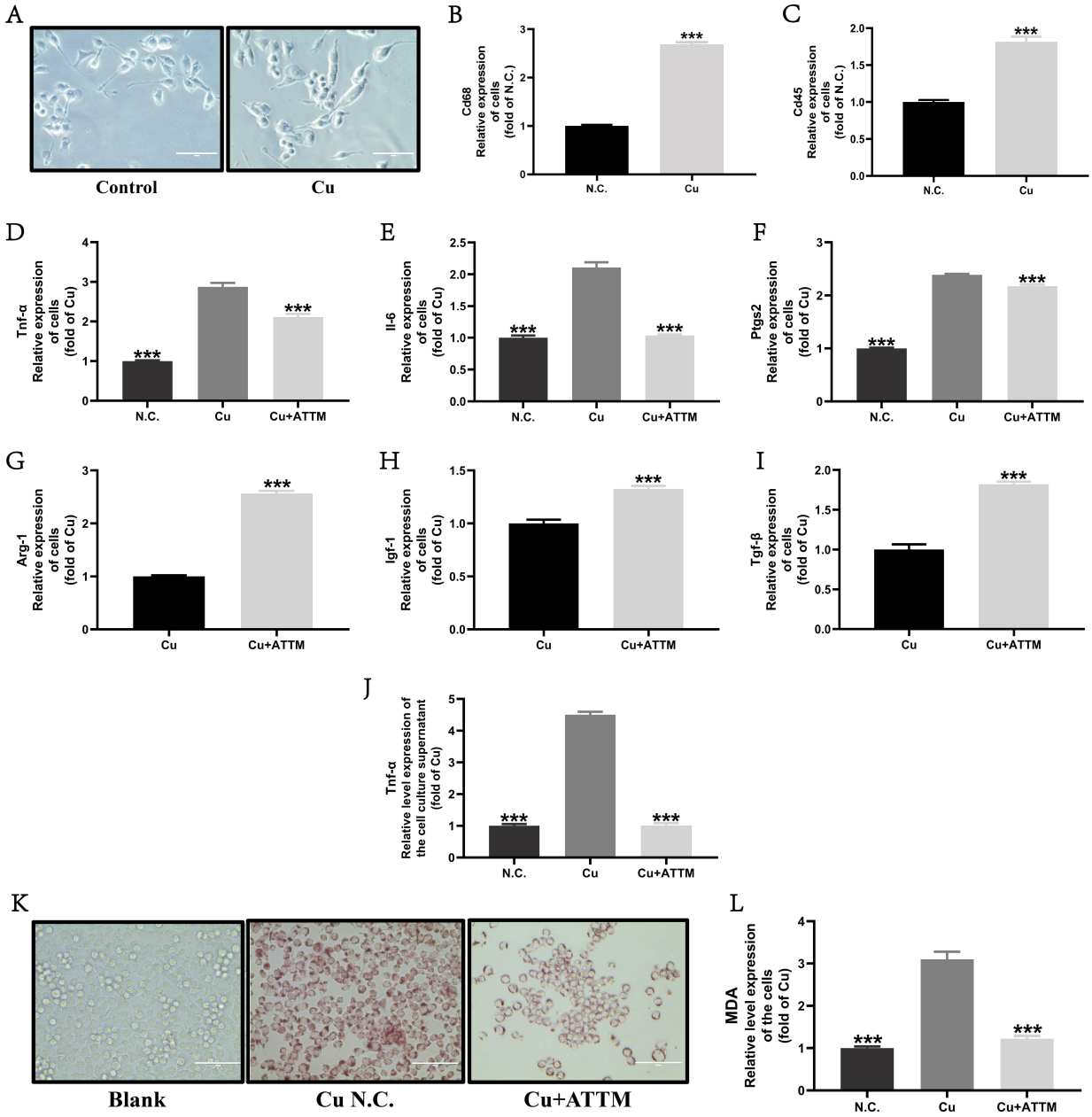

Fig. 2. Microglia promote inflammatory expression through foam cell formation under copper stimulation. (A) Morphological observation of BV2 cells after 24 h of copper treatment. Scale bar = 100 µm. (B,C) qPCR analysis of BV2 cell activation after copper treatment (changes in mRNA levels of Cd68 and Cd45). (D–I) Changes in mRNA expression of M1 (*Tnf-α*, *Il-6*, *Ptgs2*) and M2 (*Arg-1*, *Igf-1*, *Tgf-β*) related markers in BV2 cells exposed to copper after overnight ATTM pretreatment. (J) Elisa analysis of pro-inflammatory cytokine (Tnf-α) secretion by BV2 cells after copper treatment with overnight ATTM pretreatment. (K) Oil Red O staining reflects that ATTM pretreatment can reduce copper-induced lipid droplet deposition in BV2 cells. Scale bar = 100 µm. (L) Detection of MDA content in BV2 cells after 24 h of copper and ATTM treatment. All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism. Quantitative results were shown as mean ± SEM. ***$p < 0.001$. Tnf-α, tumor necrosis factor alpha; Il-6, interleukin 6; Ptgs2, prostaglandin-endoperoxide synthase 2; Arg-1, arginase 1; Igf-1, insulin-like growth factor 1; Tgf-β, transforming growth factor beta; MDA, malondialdehyde.

copper exposure. We hypothesize that imbalances in intracellular copper homeostasis may trigger inflammatory foam cell formation in microglia, contributing to myelin damage. To verify this assumption, we first treated cells with ATTM, which significantly inhibited foam cell formation and reduced lipid oxidation levels (Fig. 2J–L). These results demonstrate that under copper stimulation, microglia promote inflammatory expression through foam cell formation. Next, to confirm the regulatory role of lipid oxidation, we conducted reverse validation using the lipid oxidation

inhibitor Fer-1. We found that Fer-1 treatment significantly reduced lipid oxidation levels in microglia and effectively controlled foam cell formation (Fig. 3A,B). Additionally, Fer-1 promoted M2 polarization in microglia (*Tnf-α*, *Ptgs2*, *Il-6* mRNA expression was downregulated, and *Tgf-β*, *Arg-1*, *Igf-1* mRNA expression was upregulated) (Fig. 3C–I) and effectively mitigated the immune damage to MO3.13 cells under copper exposure (Fig. 3J,K). In addition, the decreased protein expression levels of MAG and MBP were increased (Fig. 3L, **Supplementary Fig. 1C,D**). These results showed that the decreased cell viability and maturation of MO3.13 cells were restored. In summary, copper promotes foam cell formation in microglia, activating M1 polarization, which indirectly causes myelin cell damage.

3.4 Copper Promotes Pro-inflammatory Microglia and Foamy Cell Formation via the MAPK Signaling Pathway

To investigate the underlying mechanisms in the aforementioned studies, based on previous work [52], we examined the MAPK signaling pathway. Western blot analysis revealed that copper upregulated the total protein levels of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 and promoted their phosphorylation (Fig. 4A). The results showed that the MAPK signaling pathway was activated under copper stimulation. However, under ATTM and Fer-1 treatment, only the total protein levels and phosphorylation levels of p38 were consistently and effectively inhibited compared to copper exposure alone, whereas the expression trends of ERK1, ERK2, JNK1, and JNK2 were inconsistent and unstable in three independent experiments (Fig. 4B,C). Additionally, this finding is in line with our previous research [52]. Therefore, we selected p38 for further research. Subsequently, we repeated the experiments using the p38 inhibitor SB203580. We found that similar to ATTM and Fer-1 treatment, SB203580 also reversed MO3.13 cell damage (increased cell viability and restored cell maturation of MO3.13 cells) by promoting microglia polarization from M1 to M2 (Fig. 4D–L). In addition, the decreased protein expression levels of MAG and MBP were reversed (Fig. 4M, **Supplementary Fig. 1E,F**). Furthermore, the results of Oil Red O staining and MDA detection showed that SB203580 pretreatment mitigated copper-induced lipid accumulation and oxidation (Fig. 4N,O). Together, these results suggest that copper may interfere with the p38 MAPK signaling pathway, characterized by p38 phosphorylation, participating in microglia-mediated foam cell formation and subsequent inflammatory responses.

3.5 Scutellarin Attenuates Pro-inflammatory Foam Cell Formation and Facilitates M2 Polarization via Suppression of the MAPK Signaling Pathway

Based on previous experimental evidence of scutellarin regulating MS, oxidative stress, and MAPK signaling pathways [52,53], we further investigated the effects of scutellarin on MAPK signaling pathway-mediated inflammatory foam cell transformation in microglia under copper stimulation. Cytotoxicity was evaluated by the CCK-8 assay, which showed that scutellarin had no significant effect on the viability of BV2 cells. In addition, cell viability was significantly increased at a scutellarin concentration of 20 μM/mL (**Supplementary Fig. 2**). Indeed, consistent with the aforementioned results, under copper exposure, scutellarin reduced the phosphorylation level of intracellular p38 in microglia (Fig. 5A). However, the expression trend of ERK and JNK in three independent experiments showed inconsistent and unstable results. After scutellarin treatment, the phosphorylation level of ERK was mostly increased, whereas the phosphorylation level of JNK tended to be unchanged. Additionally, as indicated by Oil Red O staining and MDA detection, scutellarin eliminated abnormal foam cell transformation and lipid oxidation, thereby significantly reducing the inhibition of BV2 cell viability by copper (Fig. 5B,C) and the indirect damage to MO3.13 cells (increased cell viability and restored cell maturation of MO3.13 cells) (Fig. 5K,L). In addition, the decreased protein expression levels of MAG and MBP were increased (Fig. 5M, **Supplementary Fig. 1G,H**). In particular, our data analysis showed that scutellarin significantly reversed the M1 polarization in microglia, promoted the expression of M2-related biomarkers (*Tgf-β*, *Arg-1*, *Igf-1*), and inhibited secretion of the inflammatory factor TNF-α (Fig. 5D–J). These findings are consistent with our previous research results in MS animal models [52].

4. Discussion

Neurological diseases have significantly impacted human health and social development. MS is an autoimmune disease that affects the CNS. The main pathological feature of this disease is the production of demyelination [1,2]. MS has many possible causes, one of which is damage to oligodendrocytes caused by inflammatory mediators produced by microglia [3–6].

Humans may unintentionally come into contact with metals in their daily lives, and an imbalance in metal homeostasis may lead to the occurrence and development of MS [18]. Copper is a widely used metal contaminant in various industries that can easily pollute the environment. Environmental investigations have shown that copper is one of the risk factors for the development of MS [19–23]; however, it remains unclear how copper exposure causes MS, and it is even less clear how copper directly affects microglia in MS. Therefore, we investigated the effects of copper on microglia in MS as our research direction, and found that neuroinflammation triggered by imbalanced copper levels can cause demyelination.

Previous studies on copper in MS have mainly focused on environmental investigations, clinical samples, and detection of copper content in animal models. However, there is no clear evidence confirming whether copper can activate microglia and damage myelin cells. In this study, we

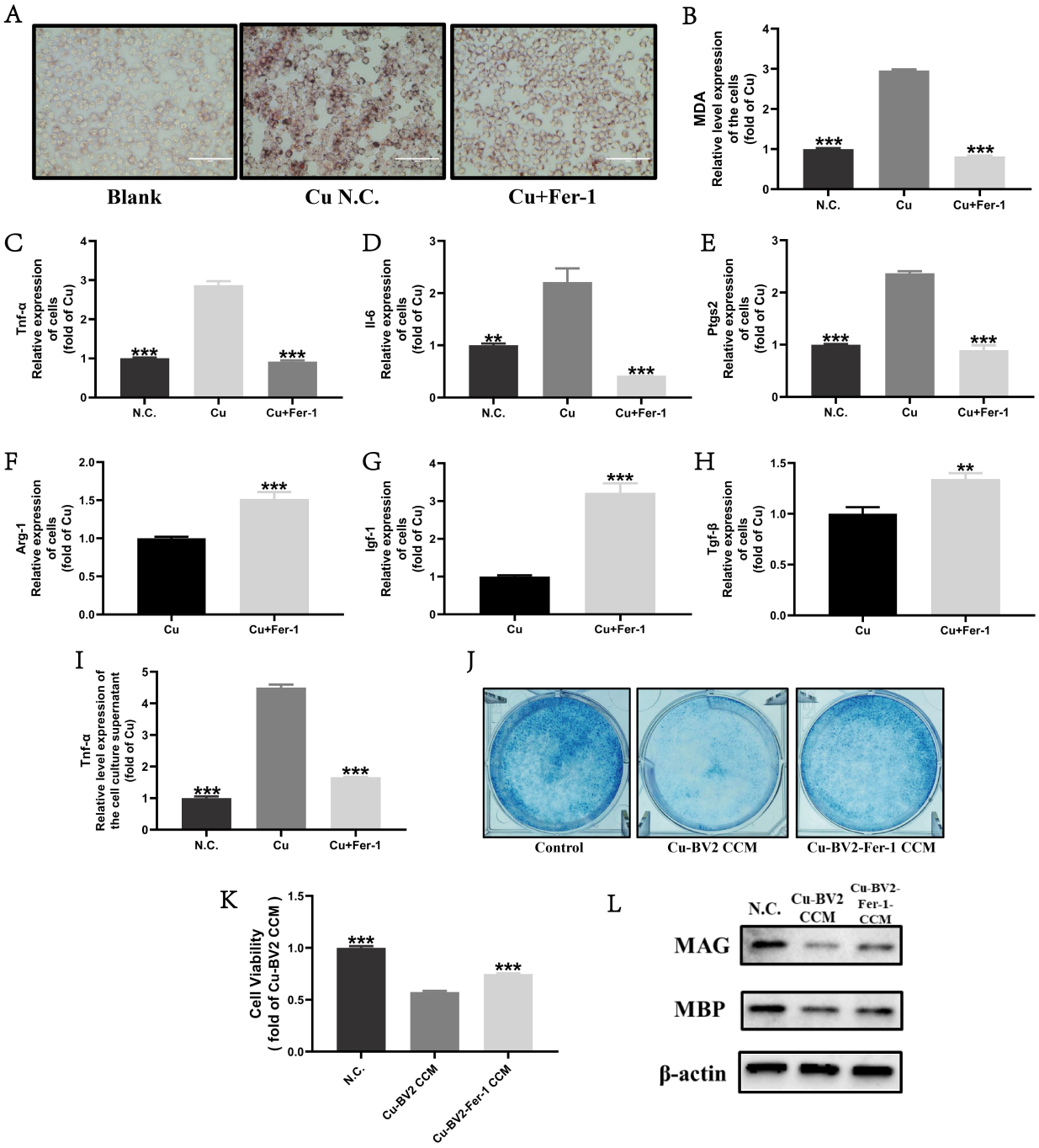

Fig. 3. Lipid oxidation inhibitors have a protective effect on copper-induced neuroinflammation. (A) Oil Red O staining analysis of copper-induced lipid droplet deposition in BV2 cells under Fer-1 pretreatment. Scale bar = 100 μm. (B) Detection of MDA content in BV2 cells after 24 hours of copper and Fer-1 treatment. (C–H) Changes in mRNA expression of M1 (*Tnf-α*, *Il-6*, *Ptgs2*) and M2 (*Arg-1*, *Igf-1*, *Tgf-β*) related markers in BV2 cells exposed to copper after overnight Fer-1 pretreatment. (I) Elisa analysis of copper-induced proinflammatory cytokine (Tnf-α) secretion by BV2 cells after overnight Fer-1 pretreatment. (J–L) Fer-1 pretreatment can reduce myelin cell damage induced by CCM. Diameter = 40 mm. All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism. Quantitative results were shown as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. Fer-1, ferrostatin-1.

employed a conditioned media co-culture method to investigate the cytotoxicity of microglia towards myelin cells. Under the premise of excluding the direct effects of copper on myelin cells, we found that the supernatant collected from microglia exposed to copper could damage the cell viability and maturity of oligodendrocytes (Fig. 3G,H).

The aforementioned findings raise new questions such as does an imbalance in copper homeostasis cause dam-

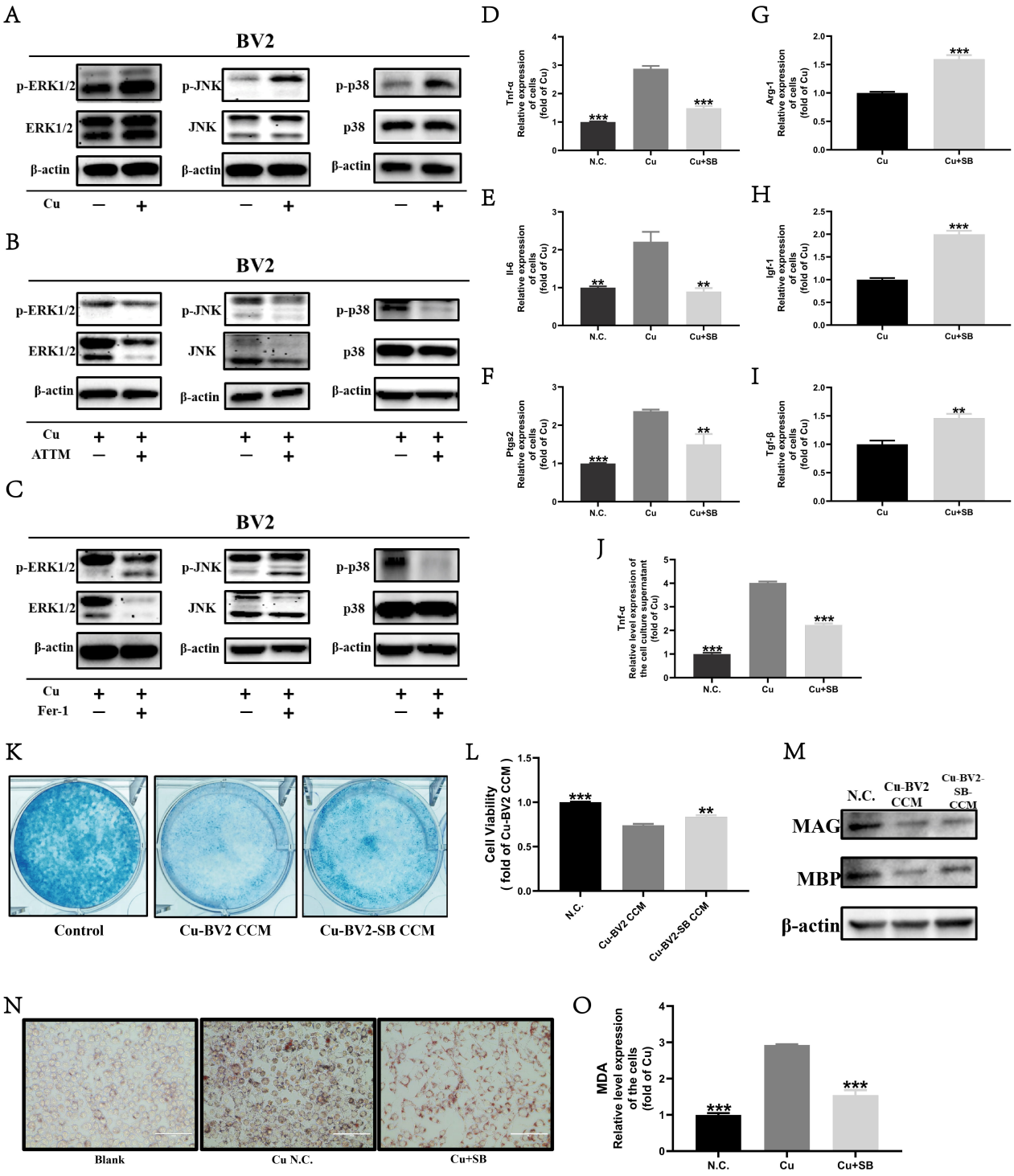

Fig. 4. Copper promotes M1 polarization and lipid accumulation in microglia mainly through activating p38 MAPK phosphorylation. (A–C) After pretreatment with ATTM and Fer-1 respectively, the total protein expression and protein phosphorylation of MAPKs were analyzed by Western Blot. (D–I) After pretreatment with the p38 MAPK inhibitor SB203580 overnight, changes in mRNA expression of M1 (*Tnf-α*, *Il-6*, *Ptgs2*) and M2 (*Arg-1*, *Igf-1*, *Tgf-β*) related markers were observed in BV2 cells exposed to copper. (J) Elisa analysis was used to assess the secretion of proinflammatory cytokine (Tnf-α) in BV2 cells induced by copper after overnight pretreatment with SB203580. (K–M) Treatment with SB203580 can reduce CCM-induced myelin cell damage. Diameter = 40 mm. (N,O) Oil Red O staining and MDA content analysis can reflect that SB203580 can reduce copper-induced lipid droplet deposition and lipid oxidation levels in BV2 cells. Scale bar = 100 μm. All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism. Quantitative results were shown as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. MAPKs, mitogen-activated protein kinases.

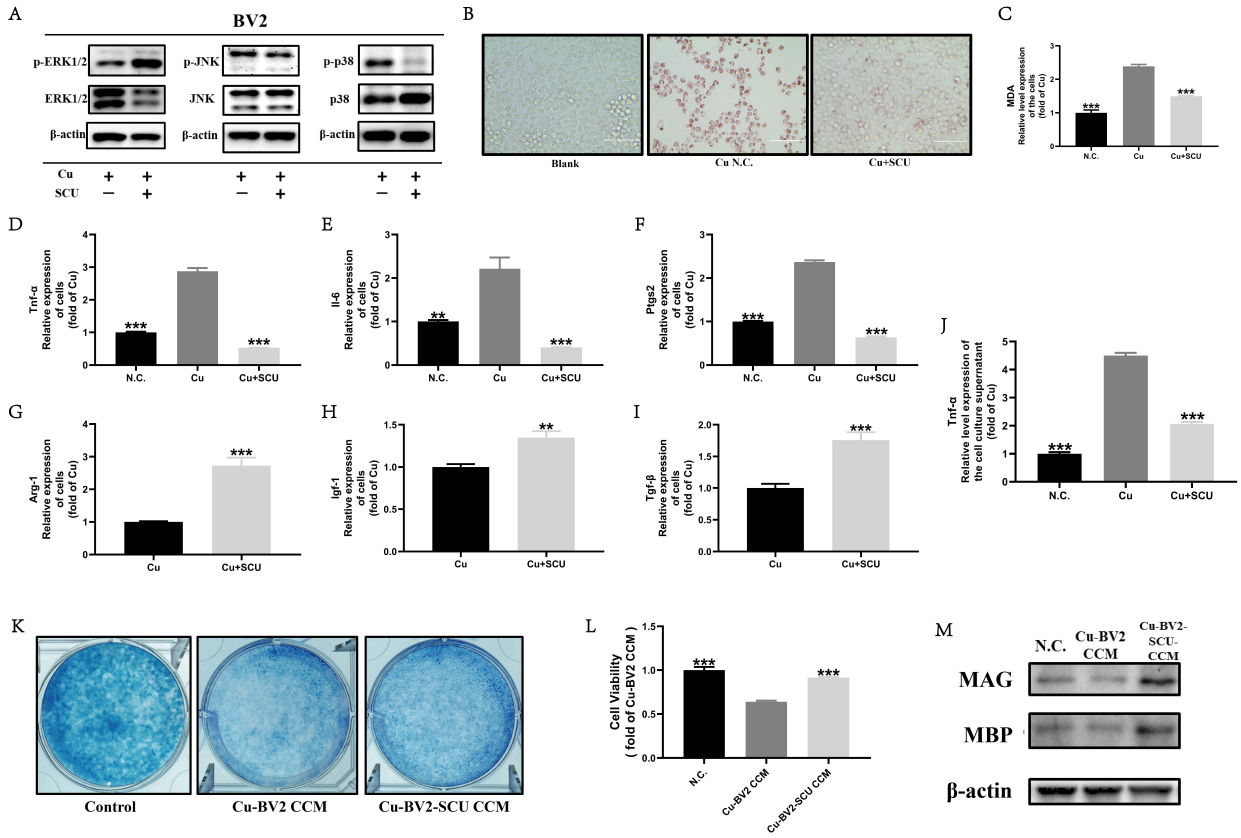

Fig. 5. Scutellarin partially reverses the positive promotion of copper on microglial M1 polarization, lipid deposition, and lipid oxidation by mediating the p38 MAPK signaling pathway. (A) Total protein expression and protein phosphorylation of MAPKs were analyzed by Western Blot after pretreatment with Scutellarin. (B,C) Lipid droplet deposition and lipid oxidation in copper-induced BV2 cells were analyzed by Oil Red O staining and MDA content analysis. Scale bar = 100 μm. (D–I) Changes in mRNA expression of M1 (*Tnf-α*, *Il-6*, *Ptgs2*) and M2 (*Arg-1*, *Igf-1*, *Tgf-β*) related markers in BV2 cells under copper exposure after overnight pretreatment with Scutellarin were observed. (J) Elisa analysis of the secretion of pro-inflammatory cytokine (Tnf-α) in BV2 cells induced by copper after overnight pretreatment with Scutellarin. (K–M) Scutellarin treatment can alleviate CCM-induced myelin sheath cell damage. Diameter = 40 mm. All experiments were independently repeated at least three times. Pairwise *t*-test analysis of data was performed using GraphPad Prism. Quantitative results were shown as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. SCU, Scutellarin.

age to myelin cells and then trigger an inflammatory response in microglia, thereby promoting the development of MS? Similar to previous research hypotheses [55], in the early stages of copper homeostasis disorder, copper may first directly cause oligodendrocyte death, leading to demyelination. Then in such circumstances where oligodendrocytes are damaged, microglia may be activated through the release of activating factors, mediating an immune response that exacerbates the occurrence and development of demyelination. Additionally, copper may also induce innate immune responses. When excessive copper is ingested, innate immune cells such as microglia are activated, which then induces damage to oligodendrocytes. Importantly, these mechanisms are likely to occur simultaneously. Therefore, future research is necessary.

Copper indirectly damages myelin cells by promoting the formation of foam cells in microglia and activating M1 polarization. The involvement of foam cells in the occurrence and development of MS has been reported. Although foam cells mainly function to repair myelin damage, inflammatory phenotype activation and neurological injury of foam cells have also been observed in MS [3,8–12]. Previous studies have shown that microglia form foam cells by phagocytosing myelin debris, which exacerbates the progression of MS [5,6]. However, our experimental results indicate that the imbalance in intracellular copper homeostasis can directly cause the formation of inflammatory foam cells in microglia, leading to myelin damage. Furthermore, there have been no reports on the involvement of copper exposure in the formation of foam cells in microglia of the CNS. Accordingly, we propose a scientific hypothesis, namely, that high levels of copper exposure in the cellular microenvironment cause an imbalance in copper homeostasis in microglia, leading to the formation of inflammatory foam cells and resulting in myelin damage *in vitro*.

Differences in research results may be due to variations in cell models, cell culture environments, media, and

other factors. In this study, we determined that copper can directly mediate the transformation of proinflammatory foam cells in the BV2 cell model (Fig. 2). The inhibition of copper chelator ATTM and lipid oxidation inhibitor Fer-1 confirms the relevance of this discovery (Figs. 2,3). Additionally, Fer-1 promotes M2 polarization of microglia and effectively alleviates the immune damage caused by copper exposure to MO3.13 cells (Fig. 3). Therefore, copper is a poor prognostic marker for MS, which is complementary to our observations in environmental, clinical, and animal models.

The formation of copper-induced inflammatory foam cells is achieved by targeting the MAPK signaling pathway. Since the hypothesis that demyelination can be altered by the interaction between copper and microglia to form foam cells has not been studied, as mechanistic evidence, we chose and identified an effective mechanism to support this hypothesis. Based on previous experimental results and literature review, we selected the MAPK signaling pathway and conducted mechanistic validation. Although the promoting function of the MAPK signaling pathway in MS has been reported, and treatment with copper chelators has been shown to alleviate demyelination in the experimental autoimmune encephalomyelitis mouse model [56,57], before this study, targeting MAPKs by copper in microglia had not been reported from a positive perspective. The results indicate that copper stimulation can activate phosphorylation of the MAPK signaling pathway. However, only the total protein and phosphorylation levels of p38 were stably and effectively inhibited under the treatment of ATTM and Fer-1 (Fig. 4A–C). Therefore, we chose p38 as the main research direction. The p38 inhibitor SB203580 promoted the conversion of microglia to an anti-inflammatory phenotype (Fig. 4D–J), thereby alleviating damage to myelin cells (Fig. 4K,L) and reducing fat accumulation and lipid oxidation (Fig. 4M,N). Overall, we demonstrate that copper mediates the transformation of foam cells and immune responses in microglia by targeting the p38 MAPK signaling pathway. The comprehensive mechanism of the copper-p38 MAPK-foam cell-lipid oxidation-immune response axis deserves further investigation, but it is beyond the scope of the current study.

It has been reported that cuprizone (CPZ)-induced demyelination in the mouse brain may not be dependent on copper chelation, but rather is caused by CPZ+copper complexes [54]. In BV2 cells treated with CPZ+copper complexes, cell viability was significantly decreased, while the marker of copper-induced cell death, Fdx1, increased. This phenomenon can be alleviated by copper chelators ATTM and BCS (data not shown). Future research is needed to investigate the role of CPZ in regulating copper-induced cell death in MS.

Scutellarin is a flavonoid activity extracted from the traditional Chinese medicine herb Erigeron breviscapus Hand-Mazz from Yunnan Province, China. In the last century, scutellarin has been widely used in clinical practice, mainly to treat cardiovascular and cerebrovascular diseases and degenerative diseases of the nervous system [47–51]. After entering the brain, scutellarin is widely distributed, thus playing a protective role. Scutellarin can reduce vascular resistance, increase blood–brain barrier (BBB) permeability, and improve the microenvironment of brain tissue [58]. The possible protective effects of scutellarin against cerebral infarction and Alzheimer's disease have been studied. Scutellarin was shown to penetrate the BBB, reduce nitric oxide release in brain tissue, and reduce cerebellar infarct size in cerebral infarction [50]. In addition, scutellarin can inhibit the production and deposition of amyloid beta (Aβ) and protect neurons from Aβ toxicity and oxidative stress, thereby alleviating the neurological dysfunction in Alzheimer's disease [59]. Because scutellarin can pass the BBB and has therapeutic effects on CNS diseases, we chose scutellarin as our research direction. Previous studies by our group [52] and Ying *et al*. [53] have demonstrated the therapeutic effects of scutellarin on different animal models of MS.

Furthermore, studies have shown that scutellarin promotes M2 polarization by inhibiting phosphorylation of the p38 signaling pathway. Treatment with a p38 MAPK inhibitor suppresses the expression level of phosphorylated p38 protein and decreases the expression of proinflammatory mediators in BV2 microglia, while increasing the expression of anti-inflammatory mediators. However, treatment with p38 MAPK activators increases the expression of proinflammatory mediators, and reduces the expression of anti-inflammatory mediators. This trend is consistent with the expression of microglia after scutellarin treatment [47–49]. There is no clear report on how this drug treats neuroinflammation induced by the foam cell phenotype of microglia. In this study, we demonstrate for the first time that scutellarin can inhibit myelin damage caused by abnormal activation of microglia by reducing the formation of inflammatory foam cells. This is crucial for the treatment and repair of neurological damage in MS.

Finally, since our study mainly focused on *in vitro* experiments, the next step of our research is to conduct *in vivo* experiments based on the *in vitro* data. Although scutellarin is soluble in saline, its structure is hydrophobic. Thus, because of its physical properties, scutellarin has very low absorption, low bioavailability, and does not easily cross the BBB. The same is true for the physical properties of its analogue baicalin. However, they do have significant therapeutic effects on neurological disorders. Therefore, how to better solve the problems of extremely low absorption, low bioavailability, and difficult passage of scutellarin through the BBB will be our next concern.

In summary, the results of this study showed that copper exposure induces an increase in the lipid uptake capacity of microglia, followed by activation towards the M1 pro-inflammatory phenotype, which promote the pro-

gression of MS, all of which may be mediated by p38 MAPK phosphorylation. This phenomenon could be reversed by treatment with scutellarin. Scutellarin targets microglia and is expected to be an effective treatment for demyelinating diseases. Therefore, inhibiting abnormal activation of microglia through scutellarin is crucial for understanding the pathogenesis and repair therapy of MS. Importantly, this study indicates the possibility of targeting pro-inflammatory foam cells as a therapeutic target for microglia-mediated neuroinflammation. However, there is a lack of *in vivo* evidence in this study. In addition, the specific connections among copper, foam cells, and neuroinflammation underlying the mechanism of action of scutellarin need to be further studied and explored. Therefore, *in vivo* experiments and further elucidation of the mechanism of action of scutellarin will provide insights into future clinical applications of scutellarin.

5. Conclusion

Our study found that copper can induce the activation of copper homeostasis imbalance in microglia, thereby secreting TNF-α and causing indirect damage to myelin cells. In addition, the activation of microglia may be related to copper-induced foam cell formation and further activation of the p38 MAPK pathway. Finally, with the treatment of scutellarin, the active promotion of copper on M1 polarization, lipid deposition and lipid oxidation of microglia were inhibited via the p38 MAPK signaling pathway, thus alleviating the sustained release of inflammation. However, the therapeutic effects of scutellarin *in vivo* need to be further studied.

Availability of Data and Materials

The data used to support the findings of this study are available from every author upon reasonable request.

Author Contributions

QZ and LC performed the experiments and analyzed data. QZ and LC contributed to the writing of this manuscript. YM participated in the analyzed data. SW designed this study, oversaw the execution of this study, contributed to the writing and revision of this manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research was supported by a grant of the National Natural Science Foundation of China (No. 81960240), by Yunnan Applied Basic Research—Yunnan Provincial Science and Technology Department—Kunming Medical University joint projects (202001AY070001-001) and by Scientific Research Fund of Education Department of Yunnan Province (Grant No. 2023Y0782).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL36255.

References

[1] Koch-Henriksen N, Sørensen PS. The changing demographic pattern of multiple sclerosis epidemiology. The Lancet. Neurology. 2010; 9: 520–532. https://doi.org/10.1016/S1474-4422(10)70064-8.

[2] Weiner HL. The challenge of multiple sclerosis: how do we cure a chronic heterogeneous disease? Annals of Neurology. 2009; 65: 239–248. https://doi.org/10.1002/ana.21640.

[3] Bogie JFJ, Stinissen P, Hendriks JJA. Macrophage subsets and microglia in multiple sclerosis. Acta Neuropathologica. 2014; 128: 191–213. https://doi.org/10.1007/s00401-014-1310-2.

[4] Prineas JW, Parratt JDE. Multiple Sclerosis: Microglia, Monocytes, and Macrophage-Mediated Demyelination. Journal of Neuropathology and Experimental Neurology. 2021; 80: 975–996. https://doi.org/10.1093/jnen/nlab083.

[5] Garcia Corrales AV, Verberk SGS, Haidar M, Grajchen E, Dehairs J, Vanherle S, *et al*. Fatty acid elongation by ELOVL6 hampers remyelination by promoting inflammatory foam cell formation during demyelination. Proceedings of the National Academy of Sciences of the United States of America. 2023; 120: e2301030120. https://doi.org/10.1073/pnas.2301030120.

[6] Bogie JFJ, Grajchen E, Wouters E, Corrales AG, Dierckx T, Vanherle S, *et al*. Stearoyl-CoA desaturase-1 impairs the reparative properties of macrophages and microglia in the brain. The Journal of Experimental Medicine. 2020; 217: e20191660. https://doi.org/10.1084/jem.20191660.

[7] Orekhov AN. LDL and foam cell formation as the basis of atherogenesis. Current Opinion in Lipidology. 2018; 29: 279–284. https://doi.org/10.1097/MOL.0000000000000525.

[8] Wang C, Lu J, Sha X, Qiu Y, Chen H, Yu Z. TRPV1 regulates ApoE4-disrupted intracellular lipid homeostasis and decreases synaptic phagocytosis by microglia. Experimental & Molecular Medicine. 2023; 55: 347–363. https://doi.org/10.1038/s12276-023-00935-z.

[9] Loppi SH, Tavera-Garcia MA, Scholpa NE, Maiyo BK, Becktel DA, Morrison HW, *et al*. Boosting Mitochondrial Biogenesis Diminishes Foam Cell Formation in the Post-Stroke Brain. International Journal of Molecular Sciences. 2023; 24: 16632. https://doi.org/10.3390/ijms242316632.

[10] Bogie JFJ, Jorissen W, Mailleux J, Nijland PG, Zelcer N, Vanmierlo T, *et al*. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. Acta Neuropathologica Communications. 2013; 1: 43. https://doi.org/10.1186/2051-5960-1-43.

[11] Bogie JFJ, Timmermans S, Huynh-Thu VA, Irrthum A, Smeets

HJM, Gustafsson JÅ, *et al*. Myelin-derived lipids modulate macrophage activity by liver X receptor activation. PloS One. 2012; 7: e44998. https://doi.org/10.1371/journal.pone.0044998.

[12] Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, *et al*. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain: a Journal of Neurology. 2006; 129: 517–526. https://doi.org/10.1093/brain/awh707.

[13] Hametner S, Wimmer I, Haider L, Pfeifenbring S, Brück W, Lassmann H. Iron and neurodegeneration in the multiple sclerosis brain. Annals of Neurology. 2013; 74: 848–861. https://doi.org/10.1002/ana.23974.

[14] Ghoreishi A, Mohseni M, Amraei R, Alizadeh AM, Mazloomzadeh S. Investigation the Amount of Copper, Lead, Zinc and Cadmium Levels in Serum of Iranian Multiple Sclerosis Patients. Journal of Chemical and Pharmaceutical Sciences. 2015; 8: 40–45.

[15] Aliomrani M, Sahraian MA, Shirkhanloo H, Sharifzadeh M, Khoshayand MR, Ghahremani MH. Blood Concentrations of Cadmium and Lead in Multiple Sclerosis Patients from Iran. Iranian Journal of Pharmaceutical Research: IJPR. 2016; 15: 825–833.

[16] Que EL, Domaille DW, Chang CJ. Metals in neurobiology: probing their chemistry and biology with molecular imaging. Chemical Reviews. 2008; 108: 1517–1549. https://doi.org/10.1021/cr078203u.

[17] Crichton RR, Dexter DT, Ward RJ. Metal based neurodegenerative diseases - From molecular mechanisms to therapeutic strategies. Coordination Chemistry Reviews. 2008; 252: 1189–1199. https://doi.org/10.1016/j.ccr.2007.10.019.

[18] Jopowicz A, Wiśniowska J, Tarnacka B. Cognitive and Physical Intervention in Metals' Dysfunction and Neurodegeneration. Brain Sciences. 2022; 12: 345. https://doi.org/10.3390/brainsci12030345.

[19] Layton W, Sutherland JM. Geochemistry and multiple sclerosis: a hypothesis. The Medical Journal of Australia. 1975; 1: 73–77. https://doi.org/10.5694/j.1326-5377.1975.tb111236.x.

[20] Åström ME, Roos PM. Geochemistry of multiple sclerosis in Finland. The Science of the Total Environment. 2022; 841: 156672. https://doi.org/10.1016/j.scitotenv.2022.156672.

[21] Monti MC, Guido D, Montomoli C, Sardu C, Sanna A, Pretti S, *et al*. Is Geo-Environmental Exposure a Risk Factor for Multiple Sclerosis? A Population-Based Cross-Sectional Study in South-Western Sardinia. PloS One. 2016; 11: e0163313. https://doi.org/10.1371/journal.pone.0163313.

[22] Etemadifar M, Mehrabi B, Kiani-Peykani R, Abtahi SH, Nekouie-Isfahani K, Ramagopalan SV, *et al*. Soil heavy metals are associated with the distribution of multiple sclerosis in Isfahan, Iran. Acta Neurologica Scandinavica. 2016; 134: 292–299. https://doi.org/10.1111/ane.12543.

[23] Johnson S. The possible role of gradual accumulation of copper, cadmium, lead and iron and gradual depletion of zinc, magnesium, selenium, vitamins B2, B6, D, and E and essential fatty acids in multiple sclerosis. Medical Hypotheses. 2000; 55: 239–241. https://doi.org/10.1054/mehy.2000.1051.

[24] Viquez OM, Valentine HL, Amarnath K, Milatovic D, Valentine WM. Copper accumulation and lipid oxidation precede inflammation and myelin lesions in N,N-diethyldithiocarbamate peripheral myelinopathy. Toxicology and Applied Pharmacology. 2008; 229: 77–85. https://doi.org/10.1016/j.taap.2008.01.005.

[25] Valentine HL, Amarnath K, Amarnath V, Valentine WM. Dietary copper enhances the peripheral myelinopathy produced by oral pyrrolidine dithiocarbamate. Toxicological Sciences: an Official Journal of the Society of Toxicology. 2006; 89: 485–494. https://doi.org/10.1093/toxsci/kfj047.

[26] Valentine HL, Does MD, Marshall V, Tonkin EG, Valentine WM. Multicomponent T2 analysis of dithiocarbamate-mediated peripheral nerve demyelination. Neurotoxicology. 2007; 28: 645–654. https://doi.org/10.1016/j.neuro.2007.01.009.

[27] Tonkin EG, Valentine HL, Milatovic DM, Valentine WM. N,N-diethyldithiocarbamate produces copper accumulation, lipid peroxidation, and myelin injury in rat peripheral nerve. Toxicological Sciences: an Official Journal of the Society of Toxicology. 2004; 81: 160–171. https://doi.org/10.1093/toxsci/kfh190.

[28] Viquez OM, Lai B, Ahn JH, Does MD, Valentine HL, Valentine WM. N,N-diethyldithiocarbamate promotes oxidative stress prior to myelin structural changes and increases myelin copper content. Toxicology and Applied Pharmacology. 2009; 239: 71–79. https://doi.org/10.1016/j.taap.2009.05.017.

[29] Valentine HL, Viquez OM, Amarnath K, Amarnath V, Zyskowski J, Kassa EN, *et al*. Nitrogen substituent polarity influences dithiocarbamate-mediated lipid oxidation, nerve copper accumulation, and myelin injury. Chemical Research in Toxicology. 2009; 22: 218–226. https://doi.org/10.1021/tx8003714.

[30] Valentine HL, Viquez OM, Valentine WM. Peripheral nerve and brain differ in their capacity to resolve N,N-diethyldithiocarbamate-mediated elevations in copper and oxidative injury. Toxicology. 2010; 274: 10–17. https://doi.org/10.1016/j.tox.2010.04.018.

[31] Smith DK, Feldman EB, Feldman DS. Trace element status in multiple sclerosis. The American Journal of Clinical Nutrition. 1989; 50: 136–140. https://doi.org/10.1093/ajcn/50.1.136.

[32] Pomary PK, Eichau S, Amigó N, Barrios L, Matesanz F, García-Valdecasas M, *et al*. Multifaceted Analysis of Cerebrospinal Fluid and Serum from Progressive Multiple Sclerosis Patients: Potential Role of Vitamin C and Metal Ion Imbalance in the Divergence of Primary Progressive Multiple Sclerosis and Secondary Progressive Multiple Sclerosis. Journal of Proteome Research. 2023; 22: 743–757. https://doi.org/10.1021/acs.jproteome.2c00460.

[33] Melø TM, Larsen C, White LR, Aasly J, Sjøbakk TE, Flaten TP, *et al*. Manganese, copper, and zinc in cerebrospinal fluid from patients with multiple sclerosis. Biological Trace Element Research. 2003; 93: 1–8. https://doi.org/10.1385/BTER:93:1-3:1.

[34] Sarmadi M, Bidel Z, Najafi F, Ramakrishnan R, Teymoori F, Zarmehri HA, *et al*. Copper concentration in multiple sclerosis: a systematic review and meta-analysis. Multiple Sclerosis and Related Disorders. 2020; 45: 102426. https://doi.org/10.1016/j.msard.2020.102426.

[35] De Riccardis L, Buccolieri A, Muci M, Pitotti E, De Robertis F, Trianni G, *et al*. Copper and ceruloplasmin dyshomeostasis in serum and cerebrospinal fluid of multiple sclerosis subjects. Biochimica et Biophysica Acta. Molecular Basis of Disease. 2018; 1864: 1828–1838. https://doi.org/10.1016/j.bbadis.2018.03.007.

[36] Jankovska E, Lipcseyova D, Svrdlikova M, Pavelcova M, Kubala Havrdova E, Holada K, *et al*. Quantitative proteomic analysis of cerebrospinal fluid of women newly diagnosed with multiple sclerosis. The International Journal of Neuroscience. 2022; 132: 724–734. https://doi.org/10.1080/00207454.2020.1837801.

[37] Aspli KT, Flaten TP, Roos PM, Holmøy T, Skogholt JH, Aaseth J. Iron and copper in progressive demyelination–New lessons from Skogholt's disease. Journal of Trace Elements in Medicine and Biology: Organ of the Society for Minerals and Trace Elements (GMS). 2015; 31: 183–187. https://doi.org/10.1016/j.jtemb.2014.12.002.

[38] Aspli KT, Holmøy T, Flaten TP, Whist JE, Aaseth JO. Skogholt's disease-A tauopathy precipitated by iron and copper? Journal of Trace Elements in Medicine and Biology: Organ of the Society for Minerals and Trace Elements (GMS). 2022; 70: 126915. https://doi.org/10.1016/j.jtemb.2021.126915.

[39] Miyakawa T, Murayama E. An autopsy case of the "demyeli-

nating type" of Wilson's disease. Acta Neuropathologica. 1976; 35: 235–241.

[40] Dezortova M, Lescinskij A, Dusek P, Herynek V, Acosta-Cabronero J, Bruha R, *et al*. Multiparametric Quantitative Brain MRI in Neurological and Hepatic Forms of Wilson's Disease. Journal of Magnetic Resonance Imaging: JMRI. 2020; 51: 1829–1835. https://doi.org/10.1002/jmri.26984.

[41] Dzieżyc K, Litwin T, Członkowska A. Multiple sclerosis in two patients with coexisting Wilson's disease. Multiple Sclerosis and Related Disorders. 2014; 3: 387–390. https://doi.org/10.1016/j.msard.2013.09.002.

[42] Dusek P, Litwin T, Członkowska A. Neurologic impairment in Wilson disease. Annals of Translational Medicine. 2019; 7: S64. https://doi.org/10.21037/atm.2019.02.43.

[43] Despotov K, Klivényi P, Nagy I, Pálvölgyi A, Vécsei L, Rajda C. Rare co-occurrence of multiple sclerosis and Wilson's disease - case report. BMC Neurology. 2022; 22: 178. https://doi.org/10.1186/s12883-022-02691-5.

[44] Meenakshi-Sundaram S, Mahadevan A, Taly AB, Arunodaya GR, Swamy HS, Shankar SK. Wilson's disease: a clinico-neuropathological autopsy study. Journal of Clinical Neuroscience: Official Journal of the Neurosurgical Society of Australasia. 2008; 15: 409–417. https://doi.org/10.1016/j.jocn.2006.07.017.

[45] VOGEL FS, EVANS JW. Morphologic alterations produced by copper in neural tissues with consideration of the role of the metal in the pathogenesis of Wilson's disease. The Journal of Experimental Medicine. 1961; 113: 997–1004. https://doi.org/10.1084/jem.113.6.997.

[46] Dong J, Wang X, Xu C, Gao M, Wang S, Zhang J, *et al*. Inhibiting NLRP3 inflammasome activation prevents copper-induced neuropathology in a murine model of Wilson's disease. Cell Death & Disease. 2021; 12: 87. https://doi.org/10.1038/s41419-021-03397-1.

[47] You P, Fu S, Yu K, Xia Y, Wu H, Yang Y, *et al*. Scutellarin suppresses neuroinflammation via the inhibition of the AKT/NF-κB and p38/JNK pathway in LPS-induced BV-2 microglial cells. Naunyn-Schmiedeberg's Archives of Pharmacology. 2018; 391: 743–751. https://doi.org/10.1007/s00210-018-1503-7.

[48] Chen HL, Jia WJ, Li HE, Han H, Li F, Zhang XLN, *et al*. Scutellarin Exerts Anti-Inflammatory Effects in Activated Microglia/Brain Macrophage in Cerebral Ischemia and in Activated BV-2 Microglia Through Regulation of MAPKs Signaling Pathway. Neuromolecular Medicine. 2020; 22: 264–277. https://doi.org/10.1007/s12017-019-08582-2.

[49] Chen HL, Yang L, Zhang XLN, Jia QY, Duan ZD, Li JJ, *et al*. Scutellarin Acts via MAPKs Pathway to Promote M2 Polarization of Microglial Cells. Molecular Neurobiology. 2023; 60: 4304–4323. https://doi.org/10.1007/s12035-023-03338-3.

[50] Guo H, Hu LM, Wang SX, Wang YL, Shi F, Li H, *et al*. Neuroprotective effects of scutellarin against hypoxic-ischemic-induced cerebral injury via augmentation of antioxidant defense capacity. The Chinese Journal of Physiology. 2011; 54: 399–405. https://doi.org/10.4077/CJP.2011.AMM059.

[51] Zhang HF, Hu XM, Wang LX, Xu SQ, Zeng FD. Protective effects of scutellarin against cerebral ischemia in rats: evidence for inhibition of the apoptosis-inducing factor pathway. Planta Medica. 2009; 75: 121–126. https://doi.org/10.1055/s-0028-1088368.

[52] Wang WW, Lu L, Bao TH, Zhang HM, Yuan J, Miao W, *et al*. Scutellarin Alleviates Behavioral Deficits in a Mouse Model of Multiple Sclerosis, Possibly Through Protecting Neural Stem Cells. Journal of Molecular Neuroscience: MN. 2016; 58: 210–220. https://doi.org/10.1007/s12031-015-0660-0.

[53] Ying S, Yang H, Gu Q, Wu Z, Zou N, Wang CZ, *et al*. The Small-Molecule compound baicalein alleviates experimental autoimmune encephalomyelitis by suppressing pathogenetic $CXCR6^{+}$ CD4 cells. International Immunopharmacology. 2023; 114: 109562. https://doi.org/10.1016/j.intimp.2022.109562.

[54] Morgan ML, Teo W, Hernandez Y, Brideau C, Cummins K, Kuipers HF, *et al*. Cuprizone-induced Demyelination in Mouse Brain is not due to Depletion of Copper. ASN Neuro. 2022; 14: 17590914221126367. https://doi.org/10.1177/17590914221126367.

[55] Zirngibl M, Assinck P, Sizov A, Caprariello AV, Plemel JR. Oligodendrocyte death and myelin loss in the cuprizone model: an updated overview of the intrinsic and extrinsic causes of cuprizone demyelination. Molecular Neurodegeneration. 2022; 17: 34. https://doi.org/10.1186/s13024-022-00538-8.

[56] Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, *et al*. A low molecular weight copper chelator crosses the blood-brain barrier and attenuates experimental autoimmune encephalomyelitis. Journal of Neurochemistry. 2004; 89: 1241–1251. https://doi.org/10.1111/j.1471-4159.2004.02428.x.

[57] Hou G, Abrams GD, Dick R, Brewer GJ. Efficacy of tetrathiomolybdate in a mouse model of multiple sclerosis. Translational Research: the Journal of Laboratory and Clinical Medicine. 2008; 152: 239–244. https://doi.org/10.1016/j.trsl.2008.09.003.

[58] Liu M, Li H, Luo G, Liu Q, Wang Y. Pharmacokinetics and biodistribution of surface modification polymeric nanoparticles. Archives of Pharmacal Research. 2008; 31: 547–554. https://doi.org/10.1007/s12272-001-1191-8.

[59] Guo LL, Guan ZZ, Huang Y, Wang YL, Shi JS. The neurotoxicity of β-amyloid peptide toward rat brain is associated with enhanced oxidative stress, inflammation and apoptosis, all of which can be attenuated by scutellarin. Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft Fur Toxikologische Pathologie. 2013; 65: 579–584. https://doi.org/10.1016/j.etp.2012.05.003.