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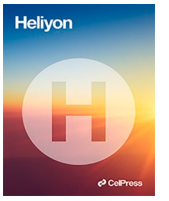

Micheliolide attenuates neuroinflammation to improve cognitive impairment of Alzheimer's disease by inhibiting NF-κB and PI3K/Akt signaling pathways

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ABSTRACT

Inflammatory reaction in the brain activates glial cells, and over-activated glial cells secrete inflammatory mediators, which aggravates the inflammatory response in the brain and accelerates the development of Alzheimer's disease (AD) in turn. Numerous natural compounds from herbs can alleviate inflammation, and it is very promising to find *anti*-neuroinflammatory natural compounds. Micheliolide (MCL) is an asesquiterpene lactone. Studies have proved that MCL showed an obvious anti-inflammatory property. Nevertheless, whether MCL can treat AD has not been determined. In this research, AD model mice were fed with a diet supplemented MCL for 3 months, the cognitive ability and inflammatory state of mice were detected. We found that MCL raised the frequency of touching novel objects, cut down the escape latency, raised the number of crossing platform, inhibited the infiltration of inflammatory cells and the secretion of interleukin-1α (IL-1α), IL-12p40, IL-13, IL-17A, tumor necrosis factor-α (TNF-α), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) in peripheral blood samples, inhibited the hyperplasia of glial cells and the production of IL-1α, IL-4, G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF), MIP-1α and MIP-1β, and reduced the deposition of Aβ peptides in the brain of AD mice. We also concluded that MCL dropped the expression of IL-1β, TNF-α, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and the phosphorylation of IκB, p65 and Akt in BV-2 cells. In conclusion, MCL alleviates the intensity of systemic inflammatory reaction via inhibiting nuclear transcription factor κ gene binding (NF-κB) and phosphoinositide-3-kinase/serine/threonine

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kinase (PI3K/Akt) pathways in glial cells, and improves the cognitive impairment of AD mice. Therefore, MCL could be a therapeutic candidate for AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that brings Serious economic and mental burden for the elderly. It is urgent to develop new drugs to prevent and treat the disease [1]. Inflammation usually is essential for the repair process. However, if inflammation is prolonged, it can cause detrimental effects on brain functions due to persistent release of cytotoxic pro-inflammatory mediators. Lots of evidence has indicated that neuro-inflammation acts a significant role in the pathophysiology of AD, and may promote the development of AD [2]. Glial cells, cytokines, chemokines, oxygen-free radicals and complements are involved in the neuro-inflammation. Microglia is the main phagocyte with the function of eliminating dangerous signals in the central nervous system. During presymptomatic stage of AD, microglia is activated by pro-inflammatory mediators. Over-activated microglia significantly promotes neuro-inflammation by secreting various pro-inflammatory mediators including tumor necrosis factor-α (TNF-α) [3], interleukin-1β (IL-1β) [4], IL-6 [5], IL-10 [6], nitric oxide (NO), and cyclooxygenase-2 (COX-2) [7], *etc,* and leads to synaptic dysfunction and neuronal death. Eventually, the interaction among the inflammatory mediators, Aβ plaques and neuronal debris established a low-grade, chronic inflammation state. Multiple inflammatory signals are involved in the regulation of microglia activity. Nuclear transcription factor κ gene binding (NF-κB) and phosphoinositide-3-kinase/serine/threonine kinase (PI3K/Akt) are two core neuroinflammatory signaling pathways [8]. Akt can promote the phosphorylation of IKKα, accelerate the degradation of IκB and the phosphorylation of p65, The translocation of p-p65 to nucleus induces the transcription and expression of inflammatory mediators [9]. The NF-κB signaling pathway regulates the transcription of genes such as cytokines, chemokines and adhesion molecules, which closely associates with the development of AD [10–12]. The PI3K/Akt is identified as a signaling pathway linking with the production of amyloid-β and neurofibrillary tangles [13]. Thus, inhibition the activation of NF-κB and PI3K/Akt signaling pathways and alleviation the sustained activation of microglia may have therapeutic effects for AD [14].

There are only a few drugs applied to the treatment of AD currently, and show some disadvantages. For example, cholinesterase inhibitors have severe liver toxicity. Memantine, commonly only used for the treatment of moderate to severe AD. For the reason that the destructive effect of inflammation in the pathogenesis of neurodegenerative disorders, drugs with anti-inflammatory or anti-oxidative properties have been investigated [15]. Various anti-inflammatory agents have been taken to modify AD progression over the past two decades, ranging from non-steroidal anti-inflammatory drugs (NSAIDs) to TNF-α inhibitors. Researches indicated that peroxisome proliferator-activated receptor c (PPARc) agonists inhibited the production of inflammatory mediators in microglia and the differentiation of monocytes to macrophages [16]. Over the past 5 years, several placebo-controlled, randomized trials have investigated the efficacy of NSAIDs, the epidemiology and clinical trial results have proved that NSAIDs can change the degradation of Aβ precursor protein (APP). However, it seems to work only in the early stage of AD. Disappointingly, there is no evidence to fully prove that NSAIDs have obvious effects in moderate to severe AD patients, and long-term use of NSAIDs will cause side effects [17]. Nimesulide, naproxen, celecoxib and rofecoxib have no long-term beneficial effects in the treatment of AD. There are quite a few herbs with anti-inflammatory properties without significant side effects [18]. At present, traditional Chinese herbs used for the treatment of inflammatory diseases have gradually become a research hotspot [19]. In recent years, some traditional Chinese herbs such as *Ginseng*, *Polygala*, *Acorus calamus*, *Michelia*, *Epimedium*, *Tripterygium wilfordii*, *Magnolia officinalis*, *Cinnamon*, *Andrographis paniculata*, *Portulaca oleracea*, *Cornus officinalis*, *Scutellaria baicalensis* and *Ginkgo biloba* have been found to improve the cognitive impairment in AD model mice [20–23]. The potential active ingredients of these herbs were extracted, purified and analyzed, and it was found that many polyterpenoids, carbohydrates, flavonoids and alkaloids have powerful anti-inflammatory activity and are promising drugs for AD treatment [24]. Studies have illustrated that sesquiterpene lactones are easy to penetrate the brain, and have *anti*-neuroinflammatory properties [25], such as micheliolide (MCL). MCL distributes in the root bark of *Michelia champacalinn* and *Michelia compressa*, both of which are magnoliaceae plants. MCL monomers extracted from plants are colorless crystals, stable in blood [26], and can be synthesized from parthenolide. It has been discovered that MCL can inhibit the activation of inflammatory signaling pathways and the expression of inflammatory mediators in macrophages [27]. However, the therapeutic roles of MCL on AD has not been reported. Therefore, we investigated the protective effects and the underlying mechanisms of MCL on AD in 5 × FAD mice.

2. Materials and methods

2.1. Reagents

MCL was isolated from *Michelia compressa* (*Magnoliaceae*), and HPLC purity was ≥99%. Its molecular weight is 248.32 KDa, and the molecular formula is $C_{15}H_{20}O_4$ (Fig. S1). Primary Iba1 antibody was ordered from Wako (Japan). *Anti*-GFAP antibody was ordered from Abcam (Japan). Biotin-rabbit anti-mouse IgG and SYBR master mix were ordered from Life Technologies (USA). The second antibody and TRIzol were ordered from Invitrogen (USA). 3,3′-diaminobenzidine (DAB), thioflavin-s, LPS and BCA protein assay reagents were ordered from Sigma (Germany). Genomic DNA isolation solutions were ordered from Promega (USA). Agarose was ordered from Biowest (France). Bio-Plex Pro mouse chemokine panel 23-plex was ordered from Bio-Rad (USA). Reverse transcriptase was ordered from Takara (Japan). RIPA was ordered from Beyotime (China). A phosphatase inhibitor was ordered from Roche (Switzerland). AF488-labeled goat anti-rabbit antibody was ordered from Molecular Probes (USA).

2.2. Animal and drug administration

5 × FAD mice (overexpress human APP695, with five familial Alzheimer's disease (FAD) gene mutations: K670 N/M671L (Swedish)/I716V (Florida)/V717I (London) of amyloid-beta (Aβ) precursor protein (APP) and M146L/L286V of presenilin 1 (PS1)) were ordered from Nanjing Institute of Biomedicine (China) (qualification certificate number: 201400975). The Newborn mice with mutated *APP* and *PS1* genes identified by PCR and electrophoresis of DNA (See supplementary materials and methods, Fig. S2) were taken as 5 × FAD mice. All mice were kept in specific pathogen-free (SPF) conditions. Four groups of mice: wild-type C57/BL6J mice raised with food without MCL (WT group), 5 × FAD model mice raised with food without MCL (AD group), 5 × FAD model mice raised with food supplemented low dose of MCL (10 mg/kg/day) (MCLL group), 5 × FAD model mice raised with food supplemented high dose of MCL (20 mg/kg/day) (MCLH group). We mixed a certain amount of MCL powder with diet, and fed the treated-group mice which could eat diet freely. The diet mixed evenly with MCL was processed by Zhicheng (Jiangsu, China). Generally speaking, the mouse eats 5 g diet every day. At the beginning of administration, the mouse weighed about 25 g. The mouse in the MCLL group ate 5 g of diet plus 0.25 mg MCL per day, and the mouse in the MCLH group ate 5 g of diet plus 0.5 mg MCL per day. The consumed gram of feed was calculated by (the feed weight of the previous day - the remaining feed weight on the day)/the number of mice in the cage. There were 10 male mice in each group and raised food mixed MCL from 2 months old to 5. The behavioral experiments were carried out when the mice were 5 months old. All the animal experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (the animal ethics certificate number: PZSHUTCM210108002). Animal pain and discomfort were minimized with effort.

2.3. Novel object recognition task

As reference described and slightly modified [28], in the course of the training phase, two objects of the same color and shape (X and Y) were placed in a 50 × 50 × 40 cm square opaque white box and 18 cm away from the box wall. The mice moved freely for 5 min in the box. After 1 h and 24 h of the training phase, object X was replaced with object Z, different color and shape but similar size to object X, the mice were tested again in the box, respectively. Mouse is searching for objects when mouse nose contacts with objects or mouse nose tip is not more than 2 cm away from the objects. The preference index is the ratio of time to search for object X over the total time to search for object X and Y (training phase), or the ratio of time to search for object Z over the total time to search for object Z and Y (1 h and 24 h after the training phase).

2.4. Morris water maze

The Morris water maze was performed as cited in Ref. [29]. The water pool is 100 cm in a diameter, and surrounded by a black curtain, evenly pasted with four cards with different colors and shapes, marked four equidistant points on the pool wall as the starting point of the test. A platform with a diameter of 7 cm was fixed in the central location of the fourth quadrant and submerged 1 cm underwater. There were three trials per mouse per day to look for the hidden platform in 60 s, and keep staying on the platform for 10 s. The platform was taken away on the 6th day, and the mice swam freely for 60 s again in the pool beginning from the center of the second quadrant. The escape latency, swimming track, time staying at the target quadrant, and frequency crossing the platform region were recorded. The data were analyzed by Noldus software (China).

2.5. Immunohistochemistry staining

Mice were anesthetized by isoflurane and perfused with 4% paraformaldehyde (PFA). Mouse brain frozen sections were cut into 40 μm in thickness through the coronal plane. Every fifth section was collected and stained. The histosections were rinsed in PBS, immersed in a penetrating membrane agent containing formaldehyde and 3% H_2O_2 for 30 min, and immersed in a penetrating membrane agent containing 0.1% Triton X-100 for 10 min, blocked by 5% BSA in TBST. The histosections were immersed in diluted specific primary antibody overnight at 4 °C. After rinsing with PBS, the histosections were immersed in a diluted secondary antibody. After rinsing with PBS, the histosections were subsequently immersed in streptavidin horseradish peroxidase. Sections were thereafter visualized by DAB staining. After gradient alcohol dehydration and xylene degreasing, sections were read with a Zeiss microscope (Germany). The $Iba1^+$ and $GFAP^+$ cells in brain sections were calculated through Image J.

2.6. Thioflavin-S fluorescent staining assay

According to the protocol described in cited reference [30], the mice's brain histosections were adhered to slides and immersed in 0.05% Thio-S dye agent for 8 min, washed twice with 80% alcohol, and washed 4 times with ddH_2O. Brain sections were immersed in high PB solution, and washed 4 times with ddH_2O. The histosections were read under a Zeiss microscope. The burden of Aβ plaques in the cortex and hippocampus was analyzed.

2.7. Liquid suspension chip for the detection of inflammatory mediators

The blood of mice was drained by PBS perfusion, and the brain tissue was rapidly isolated and put in a tube with 0.5 mL lysis agent (RIPA lysate:phosphatase inhibitor:protease inhibitor = 100:1:0.1). Brain tissues were homogenated by an ultrasonic oscillator on ice.

A total of 90 μL of serum or brain homogenate supernatant per mouse was taken. The concentration of inflammatory mediators was detected with Bio-Plex Pro mouse chemokine panel 23-plex of Bio-Rad. Briefly, the bottom of the well is coated with cytokine antibodies attached to microbeads. 70 μL of the sample was added into the well, then a secondary antibody labeled with biotin was added, and finally, streptavidin labeled with PE fluorescein was added. The data were read with Luminex 100/200™.

2.8. Quantitative real-time PCR analysis

2×10^6 BV-2 cells were treated with 0, 6, and 12 μM of MCL under the stimulation of 100 ng/mL LPS for 6 h. Total RNA was extracted with TRIzol, and was reverse transcribed into cDNA. The fluorescent quantitative PCR-specific primers were as the following: iNOS (FP: *CTGCAGCACTTGGATCAGGAACCTG*, RP: *GGGAGTAGCCTGTGTGCACCTGGAA*); COX-2 FP: *TGAAGACCAGGAGTACAG*.

C, RP:GGTACAGTTCCATGACATCG); TNF-α (FP: GTCAGCCGATTTGCTATCT

RP: *CGGACTCCGCAAAGTCTAAG*); IL-1β (FP: *GCCCATCCTCTGTGACTCA*, RP: *AGGCCACAGGTATTTTGTCG*); GAPDH (FP: *ATGTGTCCGTCGTGGATCTGA*, RP: *ATGCCTGCTTCACCACCTTCT*). The data were analyzed based on the $2^{-\Delta\Delta CT}$. PCR was performed under the condition: 95 °C, 30 s, 95 °C, 5 s, 60 °C, 34 s, 95 °C, 15 s, 60 °C, 1 min, 40 cycles in the Prism®7500 PCR system (ABI, USA).

2.9. Western-blot analysis

BV-2 cells were incubated in 12 μM of MCL under the stimulation of LPS for 15, 30 or 60 min. 2×10^6 cells were harvested and

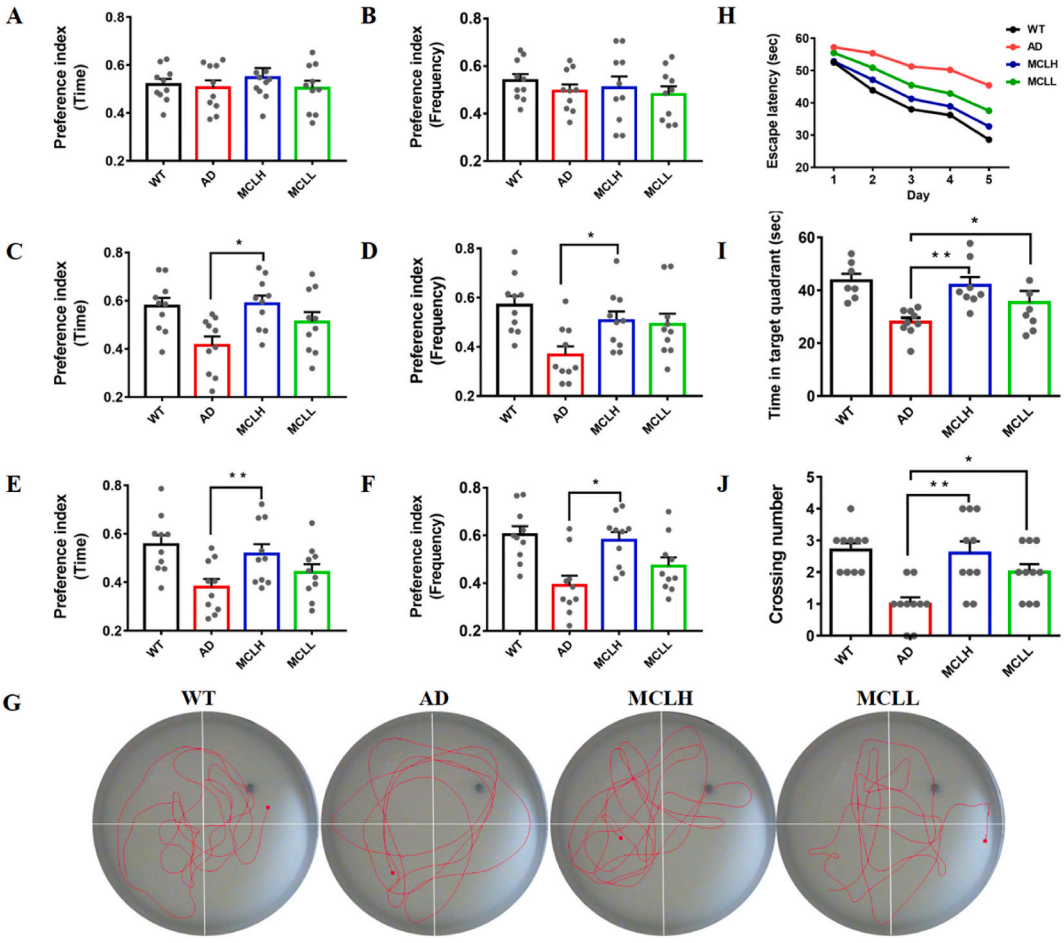

Fig. 1. MCL improves cognitive impairment in 5 × FAD mice. A. Percentage of time to recognize object X vs. object X and Y at 0 h. B. Percentage of frequency to recognize object X vs. object X and Y at 0 h. C. Percentage of time to recognize object Z vs. object Z and Y at 1 h (*$P < 0.05$, MCLH group vs. AD group). D. Percentage of frequency to recognize object Z vs. object Z and Y at 1 h (*$P < 0.05$, MCLH group vs. AD group). E. Percentage of time to recognize object Z vs. object Z and Y at 24 h (**$P < 0.01$, MCLH group vs. AD group). F. Percentage of frequency to recognize object Z vs. object Z and Y at 24 h (*$P < 0.05$, MCLH group vs. AD group). G. Representative swimming trajectory of each group of mice during the spatial exploration. H. Escape latency in the water pool during the stage of positioning navigation. I. Time spending in the platform quadrant during the stage of spatial exploration (**$P < 0.01$, MCLH group vs. AD group). J. Number of crossing platform region during the stage of spatial exploration (**$P < 0.01$, MCLH group vs. AD group). Statistical results were presented as the mean ± SD, 10 mice per group.

lysed. Protein quantification was made by BCA kit. Protein bands were separated by SDS-polyacrylamide gel electrophoresis and transferred to the NC membrane. NC membrane was blocked in TBST solution with 5% BSA, and immersed in diluted primary antibodies overnight at 4 °C. Followed by washing in TBST, immersed in diluted secondary antibody. The protein band was detected by an enhanced chemiluminescent solution according to the instruction. GAPDH was used as the internal control.

2.10. Cell immunofluorescence analysis

BV-2 cells 2×10^4/well were cultured on sterile coverslips and treated with 12 μM of MCL for 1 h. Cells were fixed with 4% PFA for 20 min and rinsed with PBS 3 times. The fixed cells were then immersed in 1% Triton X-100 for 10 min, blocked with 5% BSA in TBST for 1 h, and then incubated in diluted primary antibody overnight at 4 °C. The cells were rinsed and incubated in diluted second antibody for 1 h, rinsed with PBS, incubated in DAPI for 10 min, and then rinsed. Finally, the coverslips with cells were dried in the air and sealed with nail polish. Representative pictures were read with Zeiss confocal microscope (Germany).

2.11. Statistical analysis

The analysis was performed by Graphpad software, and the results were analyzed in means ± standard deviation (SD) or means ± standard error (SE). Differences between the two groups were obtained by Student's *t*-test analysis. The differences between multiple groups were obtained by one-way ANOVA. Statistical difference was considered as **, $P < 0.05$ or *, $P < 0.01$. The DOI of raw data is DOI: 10.6084/m9.figshare.22219165.

3. Results

3.1. MCL improves cognitive impairment in 5 × FAD mice

AD patients have difficulty in movement, and their gaits are abnormal compared to those of healthy people in the late stage of disease [31]. Therefore, this study analyzed the difference in gaits among WT, AD and MCL-treated AD mice (See supplementary materials and methods). The results suggested that there were no statistical differences in gaits among the four groups of mice at the age of 5 months ($P > 0.05$). Therefore, MCL does not affect the movement of mice (Fig. S3). AD patients also accompanied by emotional anxiety and depression, which will affect learning and memory abilities [32]. This study also analyzed the difference in emotion among WT, AD and MCL-treated AD mice through open field tests (See supplementary materials and methods). The results suggested that there were no statistical differences in emotion among the four groups of mice at the age of 5 months ($P > 0.05$). Therefore, MCL does not result in anxiety and depression in mice (Fig. S4). In a word, MCL does not affect movement and emotion in mice and is safe at the therapeutic dose to mice.

AD patients suffer from short-and long-period memory damage. To evaluate the protective effect of MCL on memory in AD mice, we conducted a novel object recognition task based on the nature that mice have exploring preferences for novel objects. At the training phase (0 h), the percentage of time and frequency looking for object X vs. object X and Y were no significant differences among the four groups of mice ($P > 0.05$). So the mice among the four groups show the same nature of looking for novel objects (Fig. 1A and B). 1 h later of the training phase, the percentage of time and frequency looking for object Z vs. object Z and Y decreased significantly in AD model mice compared to those in WT mice, while increased in MCL-treated mice compared to those in AD model mice ($P < 0.05$). The data revealed that the short-period memory of AD mice was damaged, MCL improved the short-term memory damage of AD mice (Fig. 1C and D). 24 h later of the training phase, the percentage of time and frequency looking for object Z vs. object Z and Y decreased significantly in AD model mice compared to those in WT mice, while increased in MCL-treated mice compared to those in AD model mice (MCLH group vs. AD group, $P < 0.01$). The data showed that the long-period memory of AD mice was also damaged, and MCL improved the long-period memory damage of AD mice (Fig. 1E and F).

Significant spatial orientation damage is also a major clinical symptom of AD patients [33]. In this study, a Morris water maze was conducted to evaluate the effect of MCL on spatial orientation in AD mice. In the positioning navigation stage, the latency of AD model mice escaping from the water was longer than that of WT, MCLL and MCLH groups on the first day. From the second day to the 5th day, the escape latency was shortened slowly in AD model mice compared to that in WT mice, while was shortened obviously in MCL-treated mice compared to that in AD model mice ($P < 0.01$) (Fig. 1H). In the stage of spatial exploration, the time staying in the target quadrant and the frequency crossing the platform region decreased significantly in AD model mice compared to those in WT mice, while increased significantly in MCLH ($P < 0.01$) and MCLL ($P < 0.05$) treated mice compared to those in AD model mice (Fig. 1I and J). Representative swimming trajectories of four groups were shown in Fig. 1G. These data indicated that the spatial orientation abilities of AD mice were damaged, and MCL improved their spatial orientation damage.

3.2. MCL reduces the formation and accumulation of Aβ plaques in 5 × FAD mice

The mass production and accumulation of Aβ plaques in the brain of patients is a major pathological characteristic of AD [34], and is a vital dangerous signal to stimulate inflammation. Inhibition of plaque formation and accumulation will be beneficial to delay the development of AD. The burden of Aβ plaques in AD mice brains after MCL administration was determined by immunofluorescent staining of brain sections. Representative scanned images of each group were shown in Fig. 2A. The data indicated that the burden of Aβ plaques in the cortex and hippocampus DG, CA1 region increased significantly in AD model mice vs. those in WT mice ($P < 0.01$).

However, the burden of Aβ plaques in the cortex ($P < 0.05$) and hippocampus DG ($P < 0.01$), CA1 ($P < 0.01$) decreased significantly in MCLH-treated mice vs. those in AD model mice (Fig. 2B). These results indicated that MCL reduced the burden of Aβ plaques significantly in the brain of AD mice.

3.3. MCL inhibits the activation of glial cells in 5 × FAD mice

Microglia and astrocyte are the main phagocytes in the brain. The hyperplasia of glial cells is obvious in AD patients [35]. To verify the anti-inflammatory effect of MCL, Iba1$^+$ microglia and GFAP$^+$ astrocyte in the brain sections were analyzed by immunohistochemical staining. Representative scanned images of four groups were shown in Figs. 3A and 4A. The amount of Iba1$^+$ microglia in the mouse brain increased significantly, and lots of Iba1$^+$ cells gathered into clusters in AD model mice vs. those in WT mice. In contrast, the numbers of Iba1$^+$ cells dropped significantly ($P < 0.01$), and the aggregation of microglia were less obviously in MCL-treated mice than those in AD model mice (Fig. 3B). Similarly, the number of GFAP$^+$ astrocyte in the cortex and hippocampus of mice decreased significantly ($P < 0.05$), and the aggregated astrocytes were less obviously in MCL-treated mice than those in AD model mice (Fig. 4B). Those results indicated that glial cells in the brain of AD mice were activated obviously, and MCL inhibited the activation and proliferation of glial cells in the brain of AD mice.

3.4. MCL reduces the production of inflammatory mediators in the brain of 5 × FAD mice

Inflammatory reaction in AD patients is intense, and various inflammatory mediators participate in the inflammatory reaction [36]. We further analyzed the production of 23 inflammatory mediators in the brain of mice with chemokine chips. The level of 23 inflammatory mediators in the brain of mice showed in Fig. 5A. The concentrations of most inflammatory mediators were significantly higher in AD model mice than those in WT mice. In contrast, cysteine-X-cysteine motif (CXCL)1 ($P < 0.05$), granulocyte colony stimulating factor (G-CSF) ($P < 0.05$), granulocyte-macrophage colony stimulating factor (GM-CSF) ($P < 0.05$), IL-1α ($P < 0.05$), IL-4 ($P < 0.05$), macrophage inflammatory protein-1α (MIP-1α) ($P < 0.01$) and MIP-1β ($P < 0.05$) were down-regulated in MCLH-treated mice vs. those in AD model mice (Fig. 5B–H). These results suggested that the level of inflammatory mediators in AD mice increased, MCL reduced the level of inflammatory mediators in the AD mice brain. Thus, MCL can inhibit the inflammatory response in the brain of AD mice.

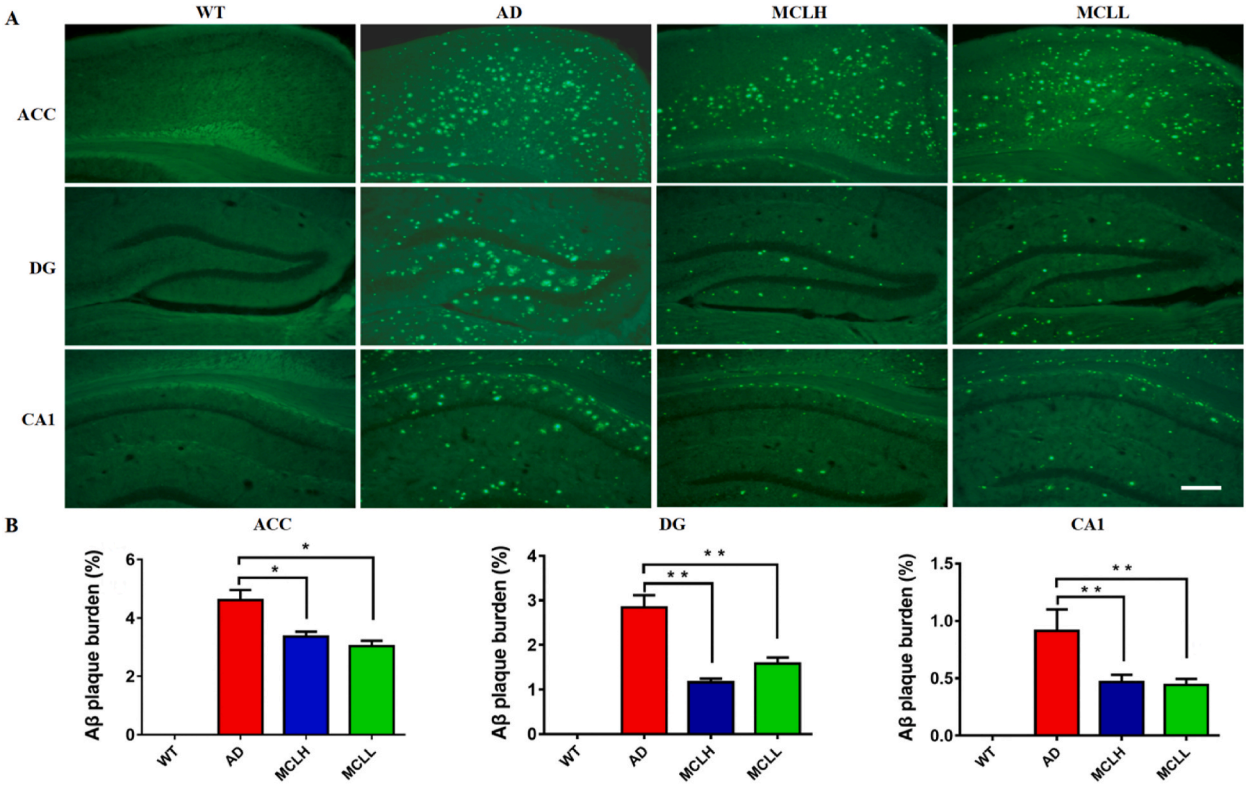

Fig. 2. MCL reduces the formation and accumulation of A β plaques in the brain of 5 × FAD mice. A. Representative images of the burden of Aβ plaques in the cortex, hippocampus DG and CA1 in the group of WT, AD, MCLH and MCLL. B. Quantitative histogram of the percentage of Aβ plaques area in the cortex (*$P < 0.05$, MCLH group vs. AD group), hippocampus DG (**$P < 0.01$, MCLH group vs. AD group) and CA1 (**$P < 0.01$, MCLH group vs. AD group) region/the whole brain slice area. Statistical results were shown as the mean ± SE, 5 mice per group. Scale: 200 μm.

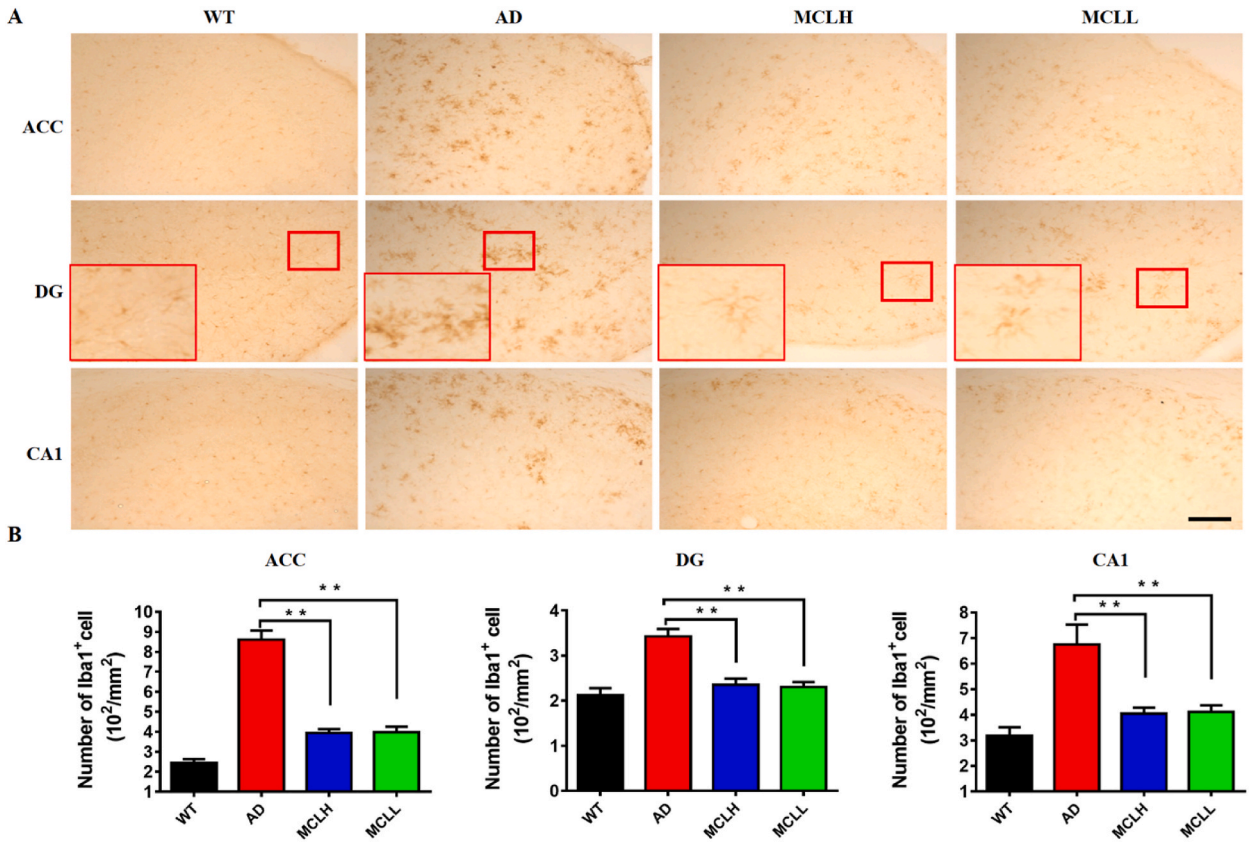

Fig. 3. MCL inhibits the activation of microglia in the brain of 5 × FAD mice. A. Staining images of $Iba1^+$ cells in brain sections in the group of WT, AD, MCLH and MCLL. B. Statistical histogram of the amount of $Iba1^+$ microglia in the cortex (**$P < 0.01$, MCLH group vs. AD group), hippocampus DG (**$P < 0.01$, MCLH group vs. AD group) and hippocampus CA1 (**$P < 0.01$, MCLH group vs. AD group) region. Statistical results were shown as the mean ± SE, 5 mice per group. Scale: 100 μm.

3.5. MCL reduces peripheral inflammatory cells infiltration in 5 × FAD mice

The disorder of brain inflammatory regulation is a local manifestation of the disorder of systemic inflammatory regulation in AD patients [37]. Inhibiting the intensity of systemic inflammation can delay the progress of AD. Therefore, we further analyzed the regulatory effect of MCL on peripheral inflammation in AD mice. The number of leukocytes involved in the inflammatory response in peripheral blood increased significantly in AD model mice vs. that in WT mice, while leukocytes ($P < 0.01$), lymphocytes ($P < 0.05$), neutrophils ($P < 0.05$) and monocytes ($P < 0.05$) decreased in MCLH-treated mice vs. those in AD mice (Fig. 6A–D). These results indicated that inflammatory cells increased significantly in the peripheral blood of mice in the process of AD development, and MCL reduced the number of inflammatory cells in the peripheral blood of AD mice.

3.6. MCL reduces the level of inflammatory mediators in peripheral blood of 5 × FAD mice

The effect of MCL on the level of inflammatory mediators in the peripheral blood of AD mice was also further analyzed. The level of 23 inflammatory mediators in the brain of mice showed in Fig. 7A. The concentration of inflammatory mediators increased significantly, and anti-inflammatory mediators decreased significantly in the peripheral blood in AD model mice vs. those in WT mice. While the concentrations of IL-1α ($P < 0.05$), IL-12p40 ($P < 0.05$), IL-13 ($P < 0.01$), IL-17A ($P < 0.01$), monocyte chemotactic protein-1 (MCP-1) ($P < 0.01$), MIP-1α ($P < 0.05$), G-CSF ($P < 0.05$) and TNF-α ($P < 0.05$) decreased significantly (Fig. 7B–I), and IL-10 ($P < 0.01$), IL-12p70 ($P < 0.05$), CCL5 ($P < 0.05$), CCL11 ($P < 0.05$) and CXCL1 ($P < 0.01$) increased significantly in MCLH-treated mice vs. those in AD model mice (Fig. 7J-N). These results showed that MCL reduced the level of inflammatory mediators significantly and increased the level of anti-inflammatory mediators in AD mice peripheral blood. Thus, MCL can inhibit the inflammatory response in AD mice's peripheral blood.

3.7. MCL inhibits the activation of BV-2 cells stimulated by LPS by inhibiting NF-κB and PI3K/Akt signaling pathways

To analyze the anti-inflammatory mechanisms of MCL, BV-2 cells were stimulated by LPS, and the morphology of BV-2 cells was

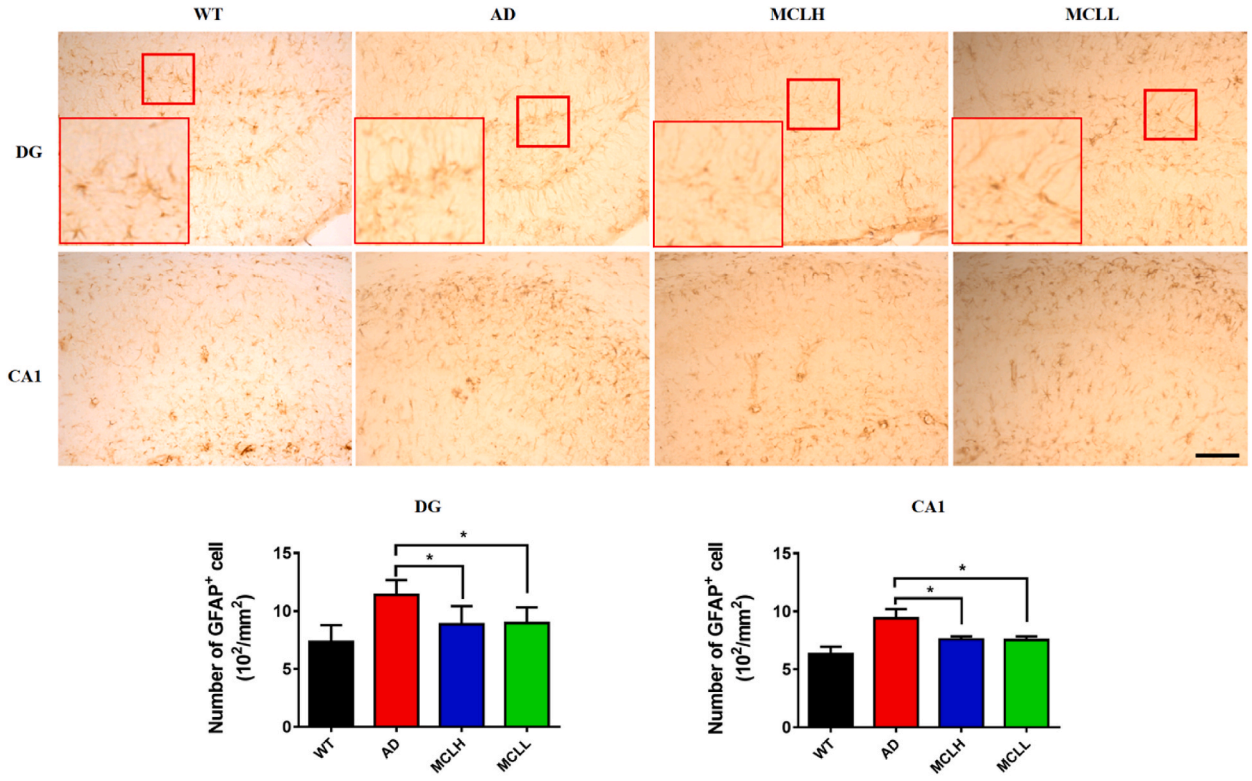

Fig. 4. MCL inhibits the activation of astrocyte in the brain of 5 × FAD mice. A. Staining images of $GFAP^+$ astrocyte in DG and CA1 region of hippocampus in the group of WT, AD, MCLH and MCLL. B. Statistical histogram of the amount of $GFAP^+$ astrocyte in DG (*$P < 0.05$, MCLH group vs. AD group) and CA1 (*$P < 0.05$, MCLH group vs. AD group) region of the hippocampus. Statistical results were shown as the mean ± SE, 5 mice per group. Scale: 100 μm.

analyzed. Resting BV-2 cells are polygonal and protuberant. LPS-stimulated BV-2 cells became round, and the protuberances disappeared after 12 h of incubation *in vitro*. MCL-treated cells began to grow antennae after 12 h of incubation *in vitro*. Most MCL-treated cells became triangular after 24 h of incubation *in vitro,* and grow antennal branches obviously. Representative morphology images of BV-2 cells of each group were shown in . The results showed that MCL inhibited the activation of BV-2 cells. To analyze the inhibitory effect of MCL on the inflammatory response, BV-2 cells were stimulated by LPS, the RNA expression levels of IL-1β, TNF-α, COX-2 and iNOS were detected by qRT-PCR. We found that the expression levels of four inflammatory mediators increased significantly after LPS stimulation. While the increased expressions of IL-1β ($P < 0.01$), TNF-α ($P < 0.01$), COX-2 ($P < 0.01$) and iNOS ($P < 0.01$) were down-regulated significantly by MCL (Fig. 8G–J). Thus MCL can inhibit LPS-stimulated inflammatory response in BV-2 cells.

NF-κB and PI3K/Akt are the main signaling pathways involved in inflammation. To clarify the underlying mechanisms of the anti-inflammatory role of MCL, the phosphorylation levels of IκB, p65 and Akt were detected by Western-blot. The phosphorylation levels of IκB, p65 and Akt increased significantly after LPS stimulation. While the increased phosphorylation level of IκB ($P < 0.05$), p65 ($P < 0.01$) and Akt ($P < 0.01$) were inhibited significantly in MCL-treated cells (Fig. 8A, C and 8E). The statistical results were shown in Fig. 8B. 8D and 8F. The nuclear localization of phosphorylated p65 (p-p65) was detected by cell immunofluorescent staining. The fluorescence intensity of p-p65 in the nucleus increased significantly in BV-2 cells stimulated by LPS, while decreased significantly after MCL treatment. Representative images of p-p65 localization were shown in Fig. 8K. The results suggested that MCL inhibited the phosphorylation of p65 and its subsequent translocation into nuclei stimulated by LPS. In short, MCL can alleviate inflammatory response in glial cells by inhibiting the activation of NF-κB and PI3K/Akt inflammatory signaling pathways.

4. Discussion

AD is the major type of dementia. How to prevent AD is a knotty problem in the face of the whole world at present. It is a great challenge for researchers to develop safe and reliable drugs to prevent and treat AD effectively. There is no fully effective cure for AD yet. Several AD clinical trials have been demonstrated failure. Although there have been many failures in AD drug development, there is still a glimmer of hope to find potential drugs to prevent and treat the disease. Since AD is a multifactorial disease, AD therapy has been shifted from single target approach to developing drugs targeted at multiple disease aspects. Traditional Chinese herbs are an important source of drug candidates because of their multi-target characteristics. Pseudopurslane saponin, andrographolide and other traditional Chinese herbs can inhibit the activity of microglia and reduce the formation of Aβ plaques in the brain of AD mice [38–40].

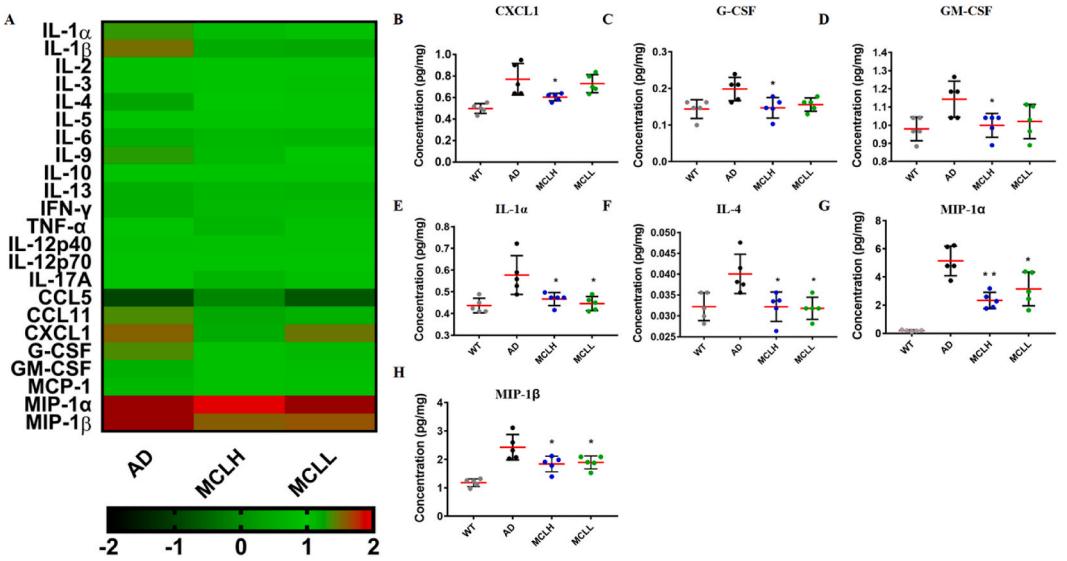

Fig. 5. MCL reduces the level of inflammatory mediators in the brain of 5 × FAD mice. A. The hotmap of level of 23 inflammatory mediators in AD, MCLH and MCLL groups. Color bar of the heat map: red, up-regulated; green, down-regulated. The signal density of inflammatory mediators was turned into Log2, and subtracted the density value-wise mean from the values of inflammatory mediators, so that the mean value per group was 0. Multiply all values in each group of data by scale factor S, so that the sum of the squares of the values in each row is 1.0. B. Quantitative histogram of CXCL1 (*$P < 0.05$, MCLH group vs. AD group). C. Quantitative histogram of G-CSF (*$P < 0.05$, MCLH group vs. AD group). D. Quantitative histogram of GM-CSF (*$P < 0.05$, MCLH group vs. AD group). E. Quantitative histogram of IL-1α (*$P < 0.05$, MCLH group vs. AD group). F. Quantitative histogram of IL-4 (*$P < 0.05$, MCLH group vs. AD group). G. Quantitative histogram of MIP-1α (*$P < 0.01$, MCLH group vs. AD group). H. Quantitative histogram of MIP-1β (*$P < 0.05$, MCLH group vs. AD group). 6 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

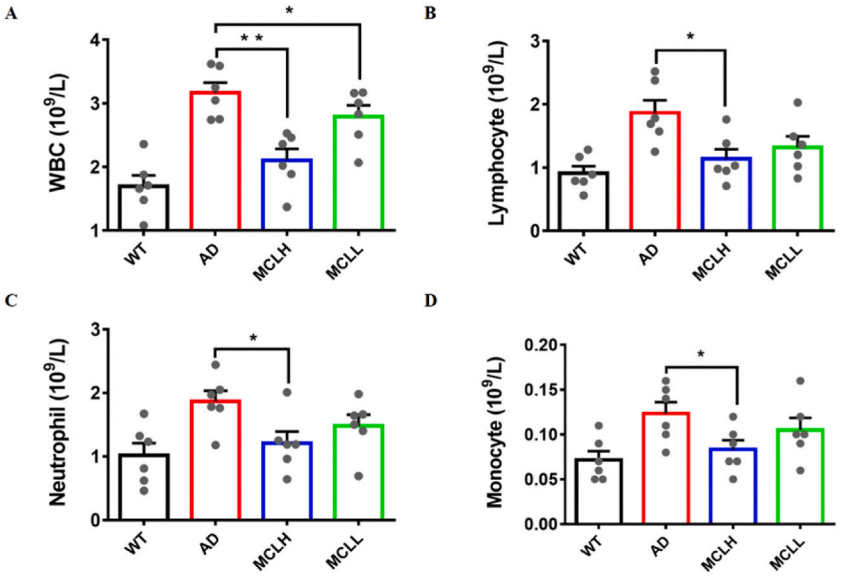

Fig. 6. MCL decreases the inflammatory cells in the peripheral blood of 5 × FAD mice. A. MCL reduced the number of white blood cells in the peripheral blood of 5 × FAD mice (**$P < 0.01$, MCLH group vs. AD group). B. MCL reduced the number of lymphocytes in the peripheral blood of 5 × FAD mice (*$P < 0.05$, MCLH group vs. AD group). C. MCL reduced the number of neutrophils in the peripheral blood of 5 × FAD mice (*$P < 0.05$, MCLH group vs. AD group). D. MCL reduced the number of monocytes in the peripheral blood of 5 × FAD mice (*$P < 0.05$, MCLH group vs. AD group). Statistical results were shown as the mean ± S, 6 mice per group.

Thus, natural plant extracts will be feasible candidates for AD drug development in the future. However, the gap between natural plant extracts and diseases is the blood-brain barrier, and many natural plant extracts can not enter the central nervous system due to their structural characteristics, which limits their effectiveness in neurodegenerative diseases. Studies have shown that sesquiterpene

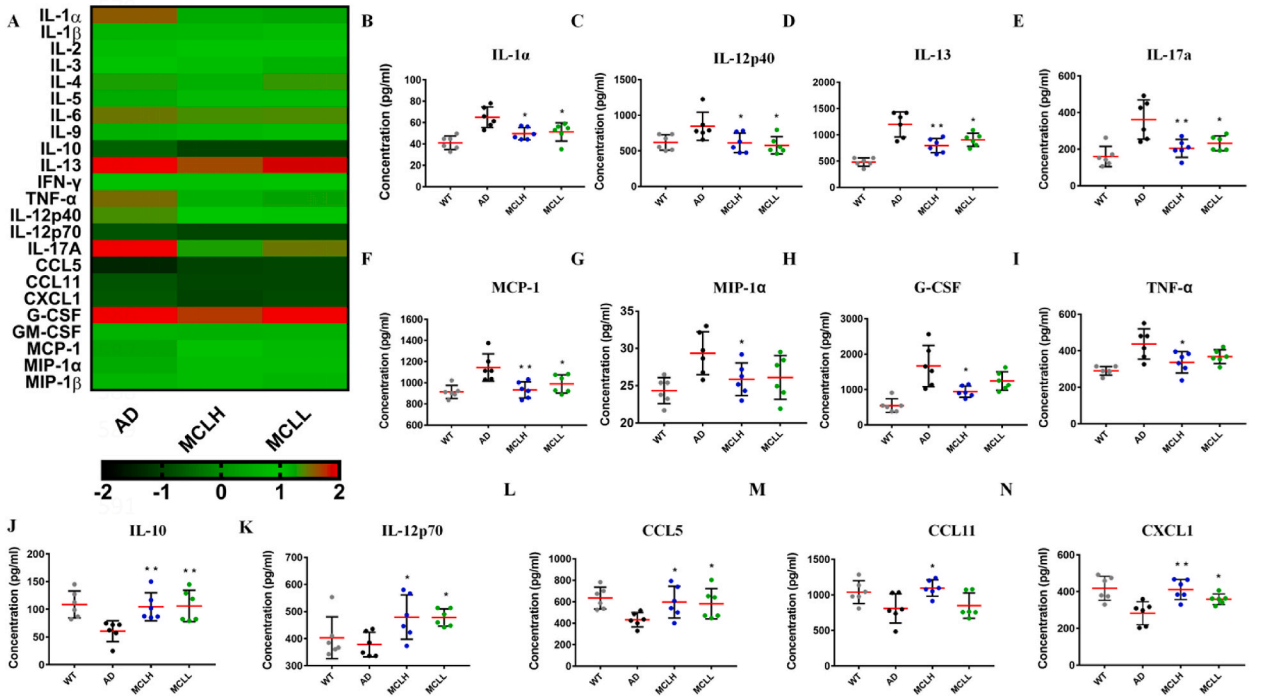

Fig. 7. MCL inhibits inflammation in the peripheral blood of 5 × FAD mice. A. The hot map of level of 23 inflammatory mediators in AD, MCLH and MCLL groups. Color bar of the heat map: red, up-regulated; green, down-regulated. The signal density of inflammatory mediators was turned into Log2, and subtracted the density value-wise mean from the values of inflammatory mediators, so that the mean value per group was 0. Multiply all values in each group of data by scale factor S, so that the sum of the squares of the values in each row is 1.0. B. Quantitative histogram of IL-1α (*$P <$ 0.05, MCLH group vs. AD group). C. Quantitative histogram of IL-12p40 (*$P < 0.05$, MCLH group vs. AD group). D. Quantitative histogram of IL-13 (*$P < 0.01$, MCLH group vs. AD group). E. Quantitative histogram of IL-17A (*$P < 0.01$, MCLH group vs. AD group). F. Quantitative histogram of MCP-1 (*$P < 0.05$, MCLH group vs. AD group). G. Quantitative histogram of MIP-1α (*$P < 0.05$, MCLH group vs. AD group). H. Quantitative histogram of G-CSF (*$P < 0.05$, MCLH group vs. AD group). I. Quantitative histogram of TNF-α (*$P < 0.05$, MCLH group vs. AD group). J. Quantitative histogram of IL-10 (*$P < 0.01$, MCLH group vs. AD group). K. Quantitative histogram of IL-12p70 (*$P < 0.05$, MCLH group vs. AD group). L. Quantitative histogram of CCL5 (*$P < 0.05$, MCLH group vs. AD group). M. Quantitative histogram of CCL11 (*$P < 0.05$, MCLH group vs. AD group). N. Quantitative histogram of CXCL1 (*$P < 0.01$, MCLH group vs. AD group). 6 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lactones are easy to penetrate the blood-brain barrier and have *anti*-neuroinflammatory activity [41]. MCL is a guaianolide sesquiterpene lactone and can permeate the blood-brain barrier, preferentially accumulating in the brain [42]. Studies have shown that MCL has a therapeutic effect on acute myelogenous leukemia [43], malignant gliomas, breast cancer [44] and ovarian cancer [45]. Despite the therapeutic potential of MCL shown in these studies, there are no reports on MCL to treat AD. Therefore, this study evaluated the efficacy of MCL in the treatment of AD. We found that MCL could shorten the escape latency, and increase the number of crossing platform in the target quadrant of 5 × FAD mice. Therefore MCL can improve the learning and memory impairment of 5 × FAD transgenic mice at 5 months old. Chronic inflammation in the central nervous system plays an important role in the occurrence and progression of AD. There is evidence showing the involvement of inflammation in AD, including activated microglia within and surrounding senile plaques. Epidemiological studies suggest the use of anti-inflammatory drugs to reduce incidence of AD. However, clinical trials with anti-inflammatory drugs have not been successful. MCL was reported to treat inflammatory enteritis, colitis-associated cancer [46] and rheumatic arthritis [47]. MCL suppressed inflammatory response stimulated by LPS and protected mice from inflammatory injury through inhibition of NF-κB and PI3K/Akt activities [27]. MCL protected mice against the infection of *Staphylococcus aureus* and MRSA by down-regulating the inflammatory response [48]. MCL played an anti-inflammatory role in *Mycobacterium tuberculosis*-induced immune response by inhibiting NF-κB and NLRP3 inflammasome activation [49]. Although MCL has been proven to have anti-inflammatory properties in many diseases, it is not clear whether MCL treats AD by inhibiting neuroinflammation. We here further clarified the mechanism of MCL in the treatment of AD. Unlike other risk factors and genetic causes of AD, neuroinflammation is not typically the cause, but rather a result of AD pathologies or risk factors associated with AD and increases the severity of the disease by exacerbating Aβ and tau pathologies. Glial cells are the main immune cells involved in the inflammatory response in the brain. The proliferation of glial cells and accumulation of Aβ plaques induced by long-term chronic inflammation are important reasons for the occurrence and development of AD [50]. Microglia and astrocytes are the major source of cytokines in AD. Cytokines contribute to the development of neuroinflammation. Restoring the physiological function of microglia and astrocytes might be a new treatment modality for AD therapy. Different strategies are developed to modulate immune cell function in

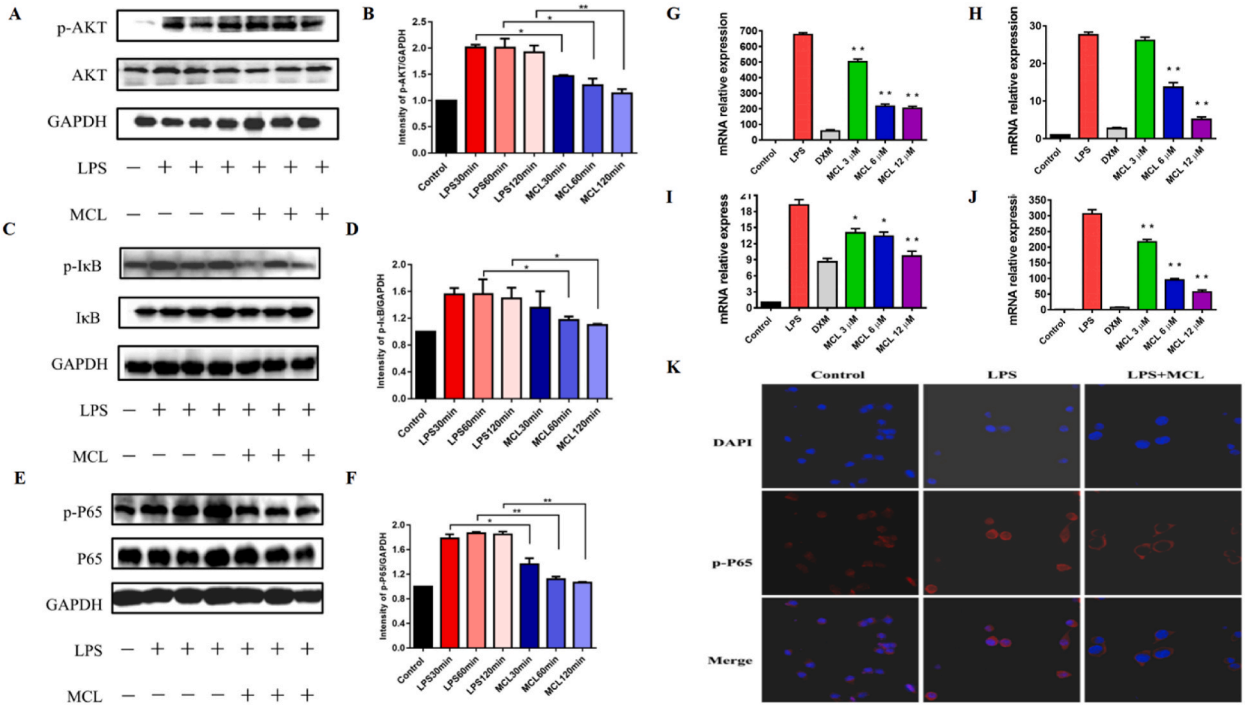

Fig. 8. MCL inhibits the activation of BV-2 cells stimulated by LPS by inhibiting NF-κB and PI3K/Akt signaling pathways. A. Inhibition of MCL on Akt phosphorylation level in BV-2 cells in Western-blot. B. Quantitative histogram of inhibition of MCL on Akt phosphorylation level in BV-2 cells (**$P < 0.01$, 12 μM MCL 120 min vs. LPS 120 min). C. Inhibition of MCL on IκB phosphorylation level in BV-2 cells in Western-blot. D. Quantitative histogram of inhibition of MCL on IκB phosphorylation level in BV-2 cells (*$P < 0.05$, 12 μM MCL 120 min vs. LPS 120 min). E. Inhibition of MCL on p65 phosphorylation level in BV-2 cells in Western-blot. F. Quantitative histogram of inhibition of MCL on p65 phosphorylation level in BV-2 cells (**$P < 0.01$, 12 μM MCL 120 min vs. LPS 120 min). Data from Western-blot were shown as mean ± SD in three independent tests. G. Inhibition of MCL on the expression of IL-1β (**$P < 0.01$, 12 μM MCL vs. LPS). H. Inhibition of MCL on the expression of COX-2 (**$P < 0.01$, 12 μM MCL vs. LPS). I. Inhibition of MCL on the expression of TNF-α (**$P < 0.01$, 12 μM MCL vs. LPS). J. Inhibition of MCL on the expression of iNOS (**$P < 0.01$, 12 μM MCL vs. LPS). Statistical results of qRT-PCR were shown as mean ± SD of 3 independent tests. K. Representative immunofluorescent staining images of the inhibitory effect of MCL on p-p65 nuclear localization. Scale: 50 μm.

neuroinflammation such as reducing expression of cytokines, inhibiting cytokine release and preventing cytokines to bind to their receptors. Reducing oxidative injury via inhibition of COX-2 and iNOS has been shown to have neuroprotective effects *in vitro* and *in vivo* animal studies. Recruitment of microglia around amyloid plaques and promoting microglial encapsulation of amyloid plaques, formation of microglial barrier around amyloid plaques to reduce plaque load have been tried in experimental animal models with promising results [51]. In this study, it was found that MCL could significantly inhibit the activation and proliferation of microglia and astrocyte, reduce the aggregation of Aβ plaques, and reduce the secretion of IL-1α, IL-4, G-CSF, GM-CSF, MIP-1α and MIP-β in the brain. MCL could also reduce the number of white blood cells, and decrease the level of IL-1α, IL-12p40, IL-13, IL-17A, G-CSF, MCP-1, MIP-1α and TNF-α in peripheral blood of AD mice. Furthermore, it was also proven that MCL could inhibit the activation of NF-κB and PI3K/Akt inflammatory pathways *in vitro*. Thus, MCL inhibits neuroinflammatory response. Intervention with immune mechanisms is likely to lead to future preventive or therapeutic strategies for AD.

This study aims to clarify the effects and possible mechanisms of MCL on AD *in vivo* and *in vitro*. But the exact target of MCL in the treatment of AD has not been deciphered yet. Further studies need to be focused on the interaction of MCL and important proteins in NF-κB and PI3K/Akt pathways and its verification using multiple strategies.

Although some AD cases are genetically linked, there are many diseases and lifestyle factors that can lead to an increased risk of developing AD, including traumatic brain injury, diabetes, hypertension, obesity, and other metabolic syndromes. Patients with these diseases are usually accompanied by symptoms of systemic chronic inflammation, so inflammation is an important pathogenesis of AD. Anti-inflammatory strategy may play a positive role in the treatment of these previous AD patients. For other AD patients without inflammation, unique anti-inflammatory strategy may not benefit them. MCL is a purified and stable molecule derived from natural herb and can permeate the blood-brain barrier to accumulate in the brain. Its significant anti-inflammatory effects are verified in our current and previous studies. In our current AD mouse model, MCL can play an impressive protective role by the addition to food. It is a promising drug candidate for AD and worthy for clinical trials. But we can't anticipate the result of clinical trials. If possible, we will refer to reference [52] to design a clinical trial to further validate the therapeutic effect of MCL on delaying the onset of AD in future.

5. Conclusion

We can conclude that MCL inhibits the phosphorylation and activation of NF-κB and PI3K/Akt inflammatory signaling pathways, reduces the secretion of inflammatory mediators, inhibits the activation of glial cells, reduces the accumulation of Aβ plaques, alleviates systemic inflammation, and thus improves cognitive impairment in AD mice. In a word, MCL is a very promising drug candidate for AD treatment. The underlying mechanisms of MCL improving AD cognitive impairment were shown in the graphical abstract.

Conflicts of Interest The authors declare no commercial or financial conflicts.

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Author contribution statement

Guizhen Yang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. You Hu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Xiangyang Qin: Contributed reagents, materials, analysis tools or data; Wrote the paper. Jinxia Sun; Zhulei Miao; Lixin Wang: Contributed reagents, materials, analysis tools or data. Zunji Ke; Yuejuan Zheng: Conceived and designed the experiments.

6. Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e17848.

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