Vitis vinifera L. Flavones Alleviate Alzheimer's Disease by Attenuating Microglial Polarization-Mediated Neuroinflammation and Cognitive Decline

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Summary

Alzheimer's disease (AD) is the leading cause of dementia and constitutes a heavy health burden on aging population. Microglia-mediated neuroinflammation is a key pathological contributor for AD pathogenesis. Flavones from *Vitis vinifera* L. (VTF) exert neuroprotective effects against amyloid beta (Aβ)-mimicked AD injury *in vitro*. However, its role in AD remains largely unexplored. In this study, inhibited microglial activation towards M1 phenotype in LPS/Aβ-induced BV2 microglia by decreasing microglial marker IBA-1, iNOS expression and percentage of $CD86^+$ M1 microglial cells. Moreover, VTF also suppressed microglial M1 polarization-driven release of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and inflammatory mediators. Mechanistically, VTF inhibited LPS/Aβ-induced activation of the TLR4/NLRP3 inflammasome axis. Reactivating the TLR4 pathway by transfection with the recombinant TLR4 plasmids reversed the inhibitor effects of VTF on microglial M1 polarization-evoked inflammatory response under LPS/Aβ condition. *In vivo*, administration of VTF improved learning and memory ability in APP/PS1-constructed AD mice and reduced Aβ levels. Furthermore, VTF suppressed M1 microglial polarization in AD mice, concomitant with the decreased levels of pro-inflammatory cytokines. Intriguingly, VTF treatment inhibited activation of the TLR4/NLRP3 inflammasome pathway in AD mice. Moreover, reactivating this pathway by LPS reversed the protective effects of VTF on cognitive impairment, Aβ accumulation and microglial M1 polarization-evoked neuroinflammation in AD model mice. Thus, this study highlights that VTF may alleviate the progression of AD by suppressing microglial M1 polarization-induced neuroinflammation through inhibition of the TLR4/NLRP3 inflammasome axis, supporting VTF as a promising therapeutic intervention for AD.

Key words

Alzheimer's disease • VTF • Neuroinflammation • Microglial polarization • TLR4/NLRP3 inflammasome

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Introduction

Dementia is a common clinical syndrome characterized by progressive deterioration of memory, cognition and daily functioning. As a progressive neurodegenerative disease, Alzheimer's disease (AD) accounts for approximately 70 % of all dementia cases and poses a severe threat to the well-being of aging population [1,2]. Epidemiological data reveal an increasing prevalence of AD in aging population at an alarming rate, affecting approximately 40 % of individuals aged over 80 years [3]. Due to increasing life expectancy and global population aging, AD has affected over 55 million cases and is projected to reach 152 million by 2050 [2,4]. Currently, AD has constituted a dire global health challenge, with an estimated economic burden of $1.1 trillion by 2050 [5]. Although abundant advances, current therapeutic strategies offer only symptomatic relief and fail to effectively halt or cure the progression of AD. Therefore, there is an urgent need for to develop a new therapeutic agent for AD.

Increasing evidence has confirmed that neuroinflammation is one of the cardinal pathological features of neurodegenerative diseases, including

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AD [2,6]. The pathophysiology of AD is characterized by extracellular accumulation of amyloid beta (Aβ), particularly the highly neurotoxic Aβ40 and Aβ42. Microglia are the resident innate immune cells of central nervous system and play the important role in regulating neuroinflammation. In AD, microglia can recognize the deposition of Aβ and undergo phenotypic switching to neurotoxic and pro-inflammatory M1-like phenotype [7]. M1 microglial polarization serves as a reliable indicator of neuroinflammation and is characterized by the abundant release of pro-inflammatory cytokine to drive neuroinflammation and neurotoxicity, such as IL-1β [8,9]. Accumulating evidence has confirmed that microglial polarization-mediated neuroinflammation contributes to the onset, progression, and pathophysiology of AD [2,6,9]. Currently, lowering microglia-driven neuroinflammation represents a promising avenue for the treatment of AD [10,11].

Vitis Vinifera L. is one of the most widely cultivated fruit crops worldwide and has been utilized in in traditional Chinese medicine to treat various diseases based on its broad-spectrum biological activities, particularly in the management of inflammatory-related and neurodegenerative diseases [12,13]. Epidemiological and clinical studies confirm that consumption of flavonoid-rich fruits has been shown to exert the beneficial effects on inflammatory disease and age-related cognitive decline [14,15]. *Vitis vinifera* L. flavones (VTF) is a common active phenolic isolated from *Vitis Vinifera* L. Moreover, the active compounds isolated from *Vitis vinifera* L. have been proved to exert the anti-oxidant, anti-inflammation and anti-nociceptive properties, including VTF [16,17]. VTF has been reported to possess the immunoregulatory properties in mice with lipopolysaccharide(LPS)-induced immunological liver injury [18]. Notably, our previous study revealed that VTF confers significant neuroprotection in an Aβ-simulated model of AD *in vitro* [19]. However, the functions of VTF in neuroinflammation and progression of AD remain unclear. In this study, we sought to explore the roles of VTF in microglia-mediated neuroinflammation *in vitro* and APP/PS1 mice models of AD. Moreover, the effects of VTF on cognitive decline and the underlying mechanism were also investigated.

Materials and Methods

Cell culture and treatment

The immortalized murine microglial cell line BV2 was purchased from the Procell system (Wuhan, China). For culture, BV2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (HyClone Inc., Logan, UT, USA) and 1 % antibiotics of penicillin/streptomycin. BV2 cells were exposed to various doses of VTF (2-100 μg/ml) for 1 h prior to treatment with Aβ1-42 (10 μM) and LPS (1 μg/ml) (Sigma-Aldrich; St. Louis, MO, USA) for 24 h. VTF (purity >92 %) was prepared by our institute as previously described [19]. All cells were maintained at 37 °C with 5 % CO_2.

Cell cytotoxicity assay

To explore the cytotoxicity of VTF on BV2 cells, cells were stimulated with VTFat concentrations ranging from 2 to 100 μg/ml. Twenty-four hours later, a commercial Cell Counting Kit (CCK)-8 kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to evaluate cytotoxicity according to the manufacturer's instructions. Briefly, cells were then incubated with medium contain 10 μl of CCK-8 for 3 h. Then, the absorbance at 450 nm was determined to assess cytotoxicity.

Construction and transfection of recombinant TLR4 expression vectors

Before construction of the recombinant TLR4 (rTLR4) expression plasmids, total RNA was extracted from BV2 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, the first-strand cDNA was prepared using the isolated RNA as a template according to the instruction of a commercial SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Then, the TLR4 cDNA was synthesized by PCR amplification, followed by the digestion and inserting into a pDNA3.1(+) vector to generate rTLR4 vector. Afterwards, BV2 cells were transfected with the pcDNA-TLR4 (rTLR4) and empty plasmids. Forty-eight hours later, the transfected efficacy on the expression of TLR4 was assessed by Western assay.

Experimental animals and ethics statement

Male C57BL/6 mice (weighing 18-22 g) and APP/PS1 transgenic mice on a C57BL/6 background were purchased from the Promoter Biotechnology Co., Ltd. (Beijing, China). All mice were acclimatized for one week prior to the subsequent experiments and housed in a room with a 12-h light/dark cycle, a constant temperature of 23±2 °C and 55-60 % humidity. All

animals had free access to standard food and water. All animal procedures were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of Xinjiang Medical University (No. #IACUC-20210507-07). During the above process, all reasonable efforts were made to minimize animal suffering.

Animal grouping and administration

The APP/PS1 transgenic mice were widely applied to construct model of AD [20]. The C57BL/6 mice served as the control group (n=10). The APP/PS1 mice were randomly divided into the following groups (n=10 in each group): model group, model+VTF group, and model+VTF+LPS group. In the control and model group (APP/PS1), mice were treated with 0.5 % sodium carboxymethyl cellulose (CMC-Na). In the model and VTF groups, APP/PS1 mice were intragastrically administrated with various doses of VTF (5 mg/kg, 10 mg/kg and 20 mg/kg, 50 mg/kg) once a day for eight weeks. In the model+VTF+LPS (TLR4 activator) group, APP/PS1 mice were intraperitoneally injected with LPS (5 mg/kg, S1732; Beyotime, Shanghai, China) every other day for eight weeks.

Morris Water Maze test

Spatial learning and reference memory ability of mice were assessed using the Morris water maze test as previously described [20]. Briefly, mice under various groups were conducted in a water maze pool (120 cm in diameter, 50 cm in height) filled with water. Furthermore, a hidden platform was immersed 1 cm beneath the surface of water throughout the training phase. Before the training phase, mice were allowed to swim freely for 1 min in the pool without platform to familiarize them with apparatus. During the training days for five consecutive days, mice were placed into water and subject to 60 s of spatial acquisition for four sessions per day, with a 15 min intertrial interval. The test automatically terminated when mice found and climbed onto platform. Mice that failed to find platform were manually guided to the platform, with a 15 s stay on the platform. On day six, to evaluate the spatial memory consolidation of animals, the platform was removed. Then, mice were allowed to freely swim for 1 min. The escape latency to find the hidden platform, percentage of platform quadrant movement distance, percentage of time spent in target quadrant and average swim speed were recorded and analyzed using a tracking device (MWT-100; Taimeng Tech. Co. Ltd., Chengdu, China).

qRT-PCR

Mice were anesthetized and sacrificed by cervical dislocation to collect brain tissues. Then, the prepared first-strand cDNA was used to perform real-time PCR assay to determine the mRNA levels of IBA-1 and iNOS in BV2 cells and tissues. All experimental procedures were carried out according to manufacturer's instructions of the SYBR Premix Kit (TaKaRa, Dalian, China). For amplification, the specific primer sequences were synthesized by Sangon Biotech (Shanghai, China) and used as follows: IBA-1 (sense, 5′-GTGTGC-AGACCTAGGGAAATAG-3′; anti-sense, 5′-CATCTT-GAACCTCCACGTACTC-3′) and iNOS (sense, 5′-GGA-ATCTTGGAGCGAGTTGT-3′; anti-sense, 5′-CCTCTT-GTCTTTGACCCAGTAG-3′). All reaction was performed on an ABI PRISM 7000 system (Applied Biosystems; Foster City, CA, USA). All data was evaluated using the $2^{-\Delta\Delta Ct}$ equation and an internal reference of β-actin.

Determination of Nitric Oxide (NO) levels

BV2 cells under various groups were collected, followed by centrifuging to prepare the supernatants. Then, the contents of NO were detected using an NO Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the Griess reaction. All protocols were conducted in accordance with the manufacturer's instructions.

ELISA assay

To quantify the concentrations of inflammation-related mediators, the commercially available ELISA kits (Beyotime, Shanghai, China) were applied to determine the levels of IL-1β, IL-6, TNF-α and IL-10 in cells and brain tissues. The contents of soluble Aβ40 and Aβ42 were measured using the commercial Mouse Aβ1-40 and Aβ1-42 ELISA kits (CUSABIO, Wuhan, China). During this process, the experimental protocols were carried out according to the instructions of ELISA kits.

Western assay

BV2 cells and tissue samples were lysed to extract total protein. Then, a commercial BCA Protein Assay Kit (Beyotime) was used to quantify protein concentration. Subsequently, equal amounts of protein samples (approximately 25 μg) were separated by 10 %

SDS-PAGE, followed by electrically transferring to PVDF membranes. After interdicting with 5 % non-fat milk, the primary antibodies against IBA-1, TLR4, NLRP3, and ASC (all from Abcam, Cambridge, MA, USA) were added for further incubation at 4 °C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, the membranes were exposed to an ECL reagent (Beyotime) to visualize the binding signals. An Image J software was applied to quantify the binding protein bands. β-actin was used as the internal control, and all experiments were independent replicated (n=3) for each quantified blot.

Immunofluorescence staining

The harvested brain tissues were fixed, dehydrated and cut into serial sections. For cell-based assays, BV2 cells were fixed in 4 % paraformaldehyde for 30 min. Subsequently, cells and tissue sections were permeabilized with 0.3 % Triton X-100 for 15 min at room temperature. After incubation with the blocking solution, cells were treated with anti-mouse CD86 antibody (M1 microglial marker) and tissue sections were incubated with primary antibodies against CD86 and IBA-1 (Abcam, Cambridge, MA, USA) at 4 °C overnight. After rinsing with PBS, samples were incubated with secondary antibodies. One hour later, the specimens were counterstained with DAPI to stain cellular nuclear for 5 min in darkness and analyzed using an Olympus fluorescence microscope.

Statistical analysis

All data from at least three-independent experiments were analyzed using a SPSS24.0 software and are presented as mean ± standard deviation (mean ± SD). A significant difference among multiple groups were analyzed by ANOVA with Turkey's *post hoc* test. Student *t*-test was used for comparisons between two groups. $P<0.05$ means statistically significant.

Results

VTF inhibits microglial activation towards M1 phenotype in LPS/Aβ-stimulated BV2 microglia

We first investigated the effects of VTF on microglia under LPS/Aβ-mimicked AD environment *in vitro*, and revealed that VTF ranging from 2 to 100 μg/ml exhibited little cytotoxicity on BV2 microglia; however, cell viability decreased upon exposure to 200 μg/ml VTF (Fig. 1A). Intriguingly, LPS/Aβ stimulation obviously increased the mRNA levels of microglial marker IBA-1, which were dose-dependently attenuated by VTF (2-100 μg/ml) (Fig. 1B). Therefore, dose of 100 μg/ml VTF was chosen for the next experiments. Concomitantly, VTF suppressed LPS/Aβ-induced protein expression of IBA-1 (Fig. 1C, D) and transcriptional levels of M1 microglial marker iNOS (Fig. 1E). Further immunofluorescence analysis also confirmed the increased percentage of $CD86^+$ M1 microglial phenotype, which was reversed by VTF administration (Fig. 1F, G).

VTF suppresses microglial M1 polarization-mediated pro-inflammatory response in LPS/Aβ-treated BV2 cells

Further analysis corroborated that LPS/Aβ stimulation dramatically elevated inflammatory mediator NO levels in BV2 microglia, which was abrogated by VTF. Concomitantly, M1 microglia-meditated releases of pro-inflammatory cytokine IL-1β (Fig. 2B), IL-6 (Fig. 2C) and TNF-α (Fig. 2D) in LPS/Aβ-treated microglia were reduced after VTF treatment. Thus, these findings indicate that VTF may inhibit M1 microglia-evoked inflammatory response under LPS/Aβ conditions.

The activation of the TLR4/NLRP3 inflammasome axis is abrogated after VTF treatment in microglia under LPS/Aβ exposure

A large body of research indicates a pivotal role of the TLR4/NLRP3 inflammasome axis in neuroinflammatory processes underlying the pathological progression of AD [21]. Therefore, we investigated the relationship between VTF and TLR4/NLRP3 inflammasome axis. As expected, the increased expression of TLR4 protein was validated in microglia under LPS/Aβ exposure (Fig. 2E). Simultaneously, LPS/Aβ stimulation elevated protein levels of NLRP3 and down-stream ASC (Fig. 2E-G). However, these up-regulation was abrogated following VTF administration. These data reveal the inhibitory effects of VTF on LPS/Aβ-activated TLR4/NLRP3 inflammasome axis in microglia.

Reactivation of the TLR4-NLRP3 signaling reverses VTF-mediated protection against microglial M1 polarization-evoked neuroinflammation

To further elaborate the role of TLR4-NLRP3 signaling in VTF-mediated protection against microglia-evoked neuroinflammation, we re-activated this pathway by transfection with recombinant TLR4 vector (Fig. 3A, B). Further assay revealed that LPS/Aβ stimulation-increased upregulation of M1 microglial marker IBA-1 (Fig. 3C) and iNOS (Fig. 3D) were suppressed by VTF, which was

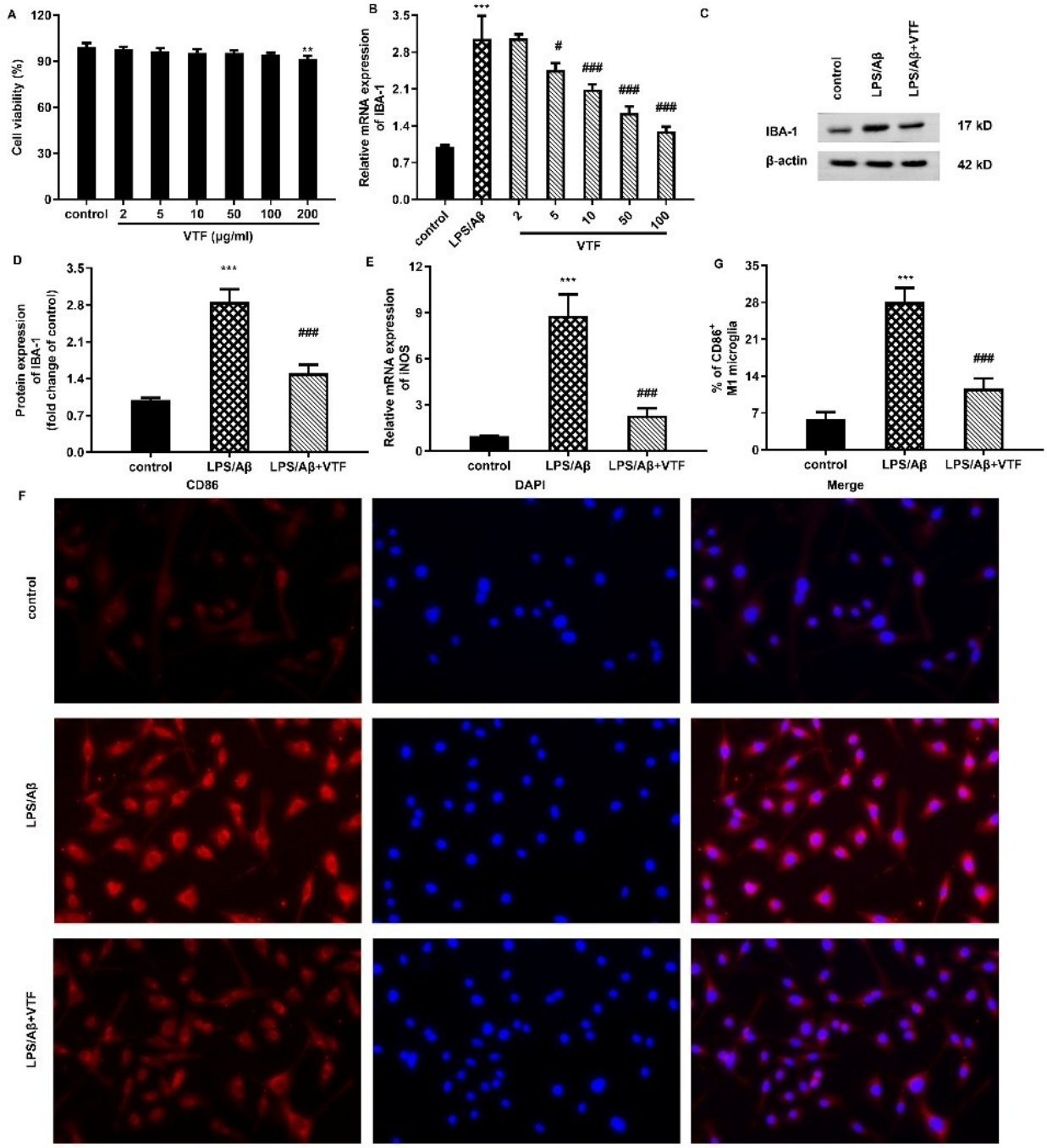


Fig. 1. VTF inhibits microglial activation to M1 phenotype under LPS/Aβ condition. (**A**) After treatment with various does of VTF, the effects on cytotoxicity of BV2 microglial cells were analyzed by CCK-8 assay. (**B**) Microglial cells were stimulated with the indicated doses of VTF, then, the mRNA (**B**) and protein (**C**) expression of IBA-1 were analyzed. (**D**) The quantification of IBA-1 protein was evaluated by ImageJ software. (**E**) The mRNA levels of M1 microglial marker iNOS were detected by qRT-PCR assay. (**F, G**) Immunofluorescence assay was performed to detect the percentage of $CD86^+$ M1 microglial cells. ** $P<0.01$, *** $P<0.001$ vs. control group, # $P<0.05$, ### $P<0.001$ vs. LPS/Aβ group.

reversed after TLR4 plasmid transfection. Moreover, treatment with VTF reduced percentage of $CD86^+$ M1 microglia, but this effect was attenuated upon reactivation of TLR4 signaling (Fig. 3E). Additionally, the suppressive effects of VTF on LPS/Aβ-induced releases of NO (Fig. 3F), IL-1β (Fig. 3G), IL-6 (Fig. 3H) and TNF-α (Fig. 3I) were abolished by TLR4 overexpression.

Treatment with VTF ameliorates cognitive impairment and Aβ levels in AD model

To evaluate the effects of VTF on spatial learning and memory, the Morris Water Maze Test was performed. Compared with the control C57BL/6 mice, the APP/PS1 mice were used to construct AD model, and exhibited a higher escape latency (Fig. 4A) and lower

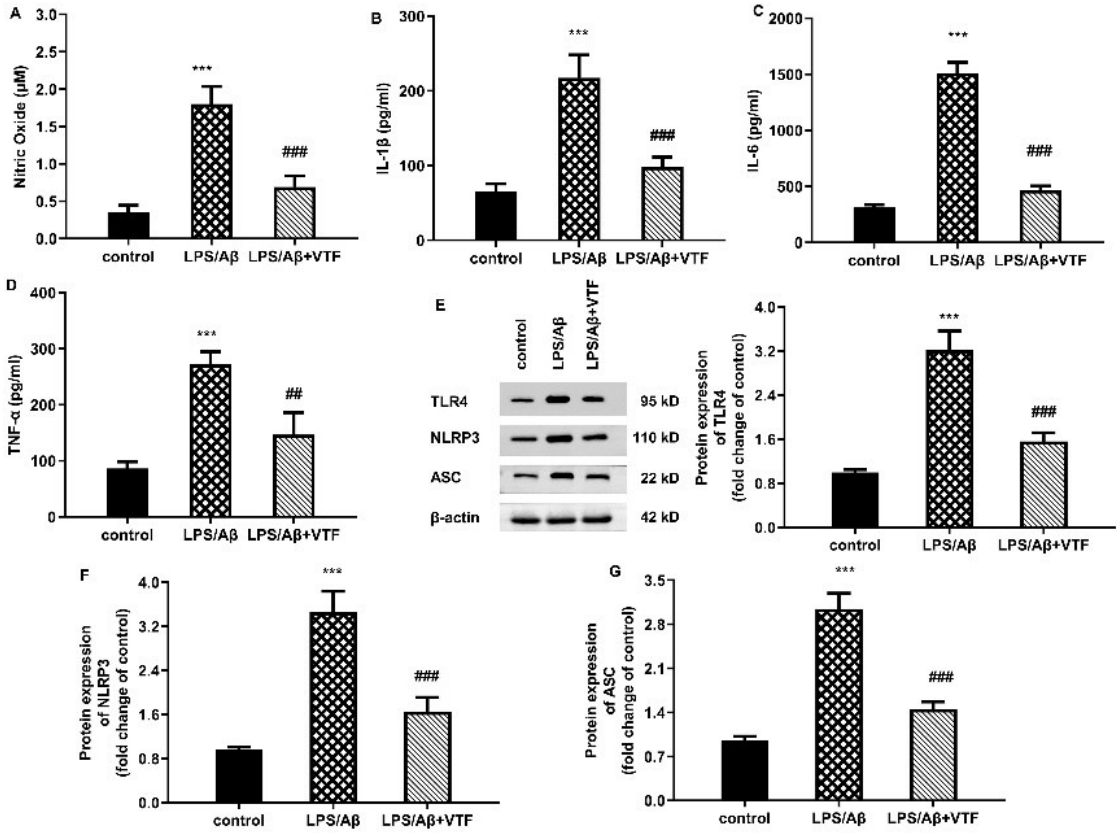


Fig. 2. VTF inhibits M1 microglial polarization-mediated pro-inflammatory properties and activation of the TLR4/NLRP3 inflammasome axis. BV2 microglial cells were stimulated with 100 μg/ml VTF under LPS/Aβ exposure. Then, the levels of NO (**A**), IL-1β (**B**), IL-6 (**C**) and TNF-α (**D**) were analyzed by the commercial ELISA Kits. (**E-G**) The protein levels of TLR4, NLRP3 and ASC in BV2 cells were determined. The corresponding protein bands were quantitatively analyzed using an ImageJ software. *** P<0.001 vs. control group, ### P<0.001 vs. LPS/Aβ group.

percentage of time spent in target quadrant (Fig. 4B) in the model groups. Intriguingly, the preliminary results observed that the higher escape latency and lower percentage of time spent in target quadrant in model groups were dose-dependently reversed after treatment with 10, 20 and 50 mg/kg of VTF, suggesting the protective efficacy of VTF in ameliorating cognitive impairment in AD mice. Nevertheless, no obvious difference was found between the 20 and 50 mg/kg of VTF groups (Suppl. Fig. 1). Thus, the dose of 20 mg/kg VTF was selected for subsequent experiments. Moreover, the obvious decreases in swim speed (Fig. 4C) and percentage of platform quadrant movement distance (Fig. 4D) were validated in AD model groups relative to the control groups; in contrast, administration of VTF significantly improved swim speed and percentage of platform quadrant movement distance of model mice. Additionally, VTF treatment also attenuated the levels of Aβ1-40 (Fig. 4E) and Aβ1-42 (Fig. 4F) in brain tissues of APP/PS1 model mice. Therefore, these findings suggest that administration of VTF may alleviate cognitive impairment in AD mice.

VTF protects against AD symptoms in model mice by inhibiting activation of the TLR4-NLRP3 axis

Further mechanistic investigations revealed that activation of the TLR4-NLRP3 axis in model groups was inhibited by VTF, which were reactivated by the activator of this pathway, LPS (Fig. 4G-I). Notably, the suppressive effects of VTF on escape latency in model mice were reversed by LPS (Fig. 4A). Moreover, reactivating the TLR4 pathway by LPS further offset VTF-increased % of time spent in target quadrant (Fig. 4B), swim speed (Fig. 4C) and % of platform quadrant movement distance (Fig. 4D) in the model groups. Additionally, the elevated levels of Aβ1-40 (Fig. 4E) and Aβ1-42 (Fig. 4F) in APP/PS1 model mice were alleviated after VTF administration , which were reversed by LPS.

VTF attenuates M1 microglial polarization-induced neuroinflammation via inhibition of the TLR4-NLRP3 axis in AD model

We next explored the function of VTF in neuroinflammation during AD, and confirmed the

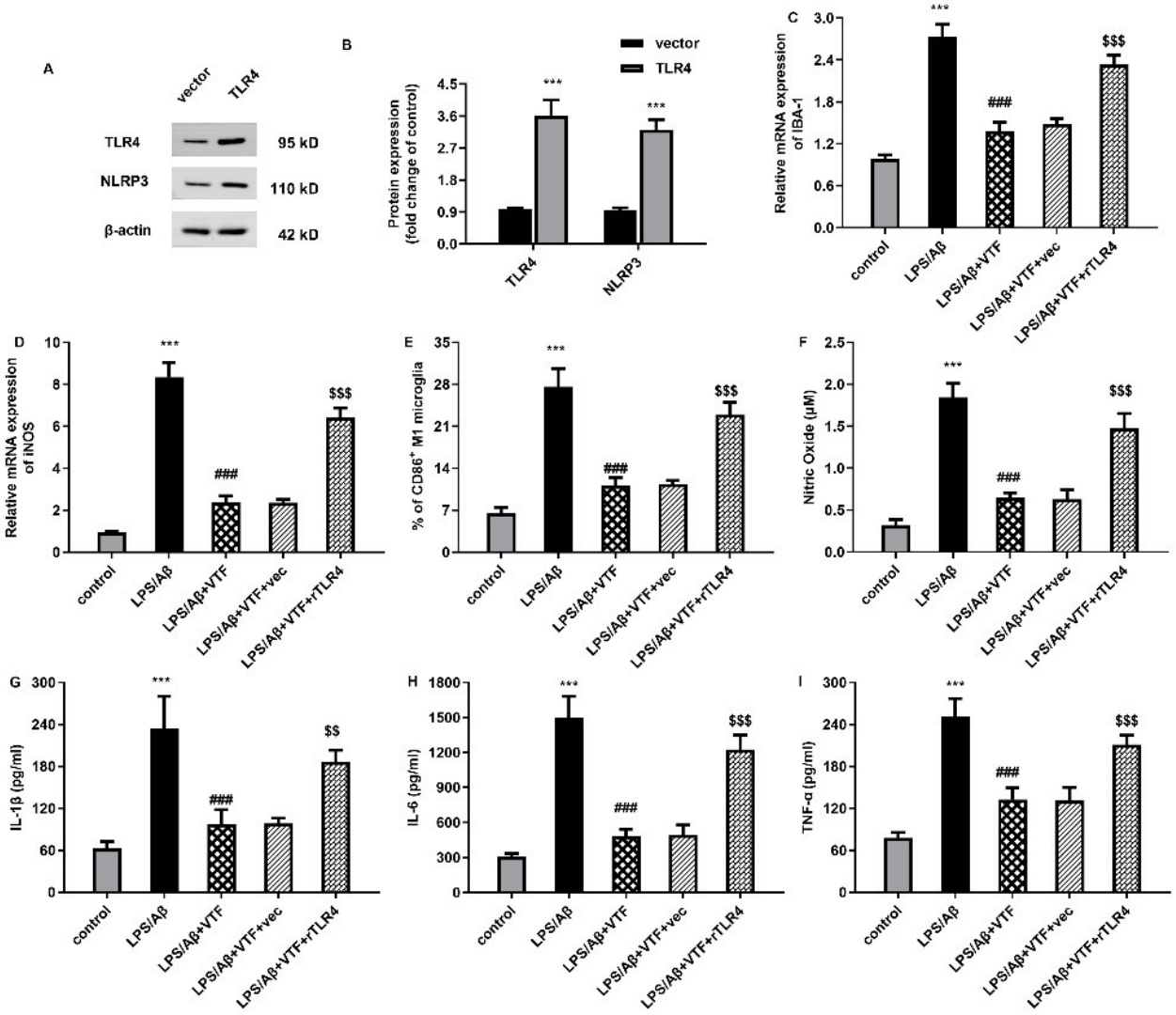


Fig. 3. The TLR4-NLRP3 inflammasome axis is responsible for the protective efficacy of VTF in microglial M1 polarization-evoked neuroinflammation. (**A, B**) Effects of TLR4 plasmid transfection on activation of the TLR4-NLRP3 pathway were analyzed. (**C, D**) Microglial cells were treated with TLR4 plasmid t and LPS/Aβ. Subsequently, the mRNA levels of IBA-1 (**C**) and iNOS (**D**) were determined by qRT-PCR assay. (**E-I**) the effects on percentage of $CD86^+$ M1 microglia (**E**), NO (**F**), IL-1β (**G**), IL-6 (**H**) and TNF-α (**I**) levels were also detected. *** P<0.001 vs. control group, ### P<0.001 vs. LPS/Aβ group, $$$ P<0.001 vs. LPS/Aβ and VTF group.

increased mRNA levels of microglia marker IBA-1 (Fig. 5A) and iNOS (Fig. 5B) in model mice relative to the control group. Moreover, the percentage of $IBA\text{-}1^+CD86^+$ M1 microglia was also elevated in the model group (Fig. 5C, D). Intriguingly, VTF inhibited activation of M1 microglia in model mice, which was reversed upon TLR4 reactivation by LPS. Moreover, VTF administration also attenuated the increased levels of pro-inflammatory cytokine IL-6 (Fig. 6A), IL-1β (Fig. 6B), and TNF-α (Fig. 6C), and reversed the decreased levels of anti-inflammatory IL-10 (Fig. 6D) in the model mice. However, these changes were reversed by LPS, indicating that VTF may alleviate M1 microglia-driven neuroinflammation in AD mice primarily by inhibiting the TLR4-NLRP3 axis.

Discussion

AD represents the leading cause of dementia and imposes a heavy economic and health burden worldwide, particularly among the aging population [1,2]. Our previous research confirmed the neuroprotection effects of VTF on Aβ-induced model of AD *in vitro* [19]. However, the therapeutic potential and underlying molecular mechanism of VTF in AD remain unclear. In this study, we confirmed that VTF inhibited microglia M1 activation-mediated inflammatory response under Aβ/LPS conditions by suppressing the TLR4-NLRP3 inflammasome signaling. Importantly, VTF administration alleviated cognitive deficits, Aβ levels and M1 microglia polarization-evoked neuroinflammation in APP/PS1-

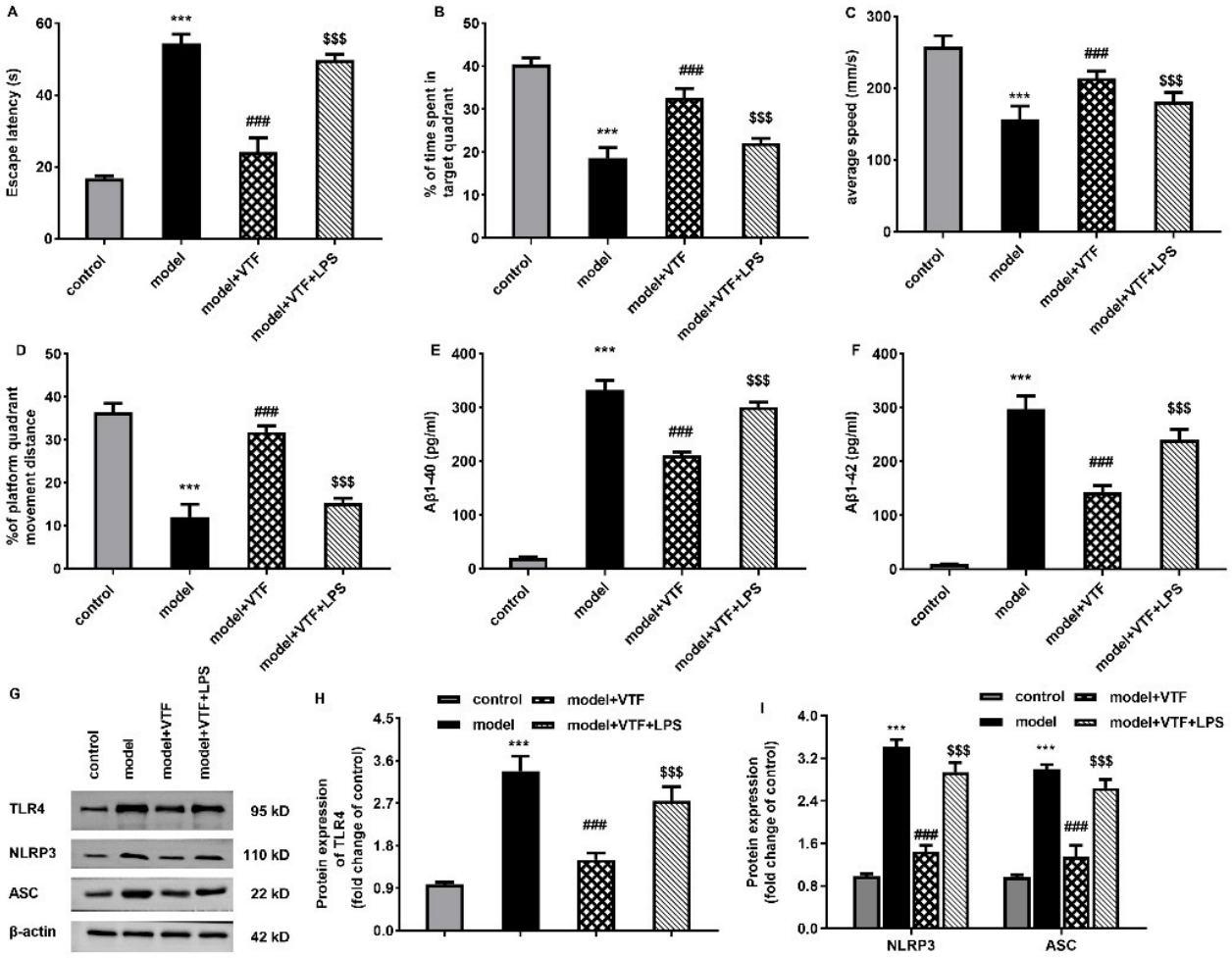


Fig. 4. VTF administration attenuates cognitive impairment and Aβ levels in AD model *via* the TLR4-NLRP3 axis. (**A-D**) C57BL/6 mice were used as the control group, APP/PS1 mice were used to construct AD model and treated with VTF and LPS. Then, the effects on escape latency (**A**), percentage of time spent in target quadrant (**B**), average speed (**C**) and percentage of platform quadrant movement distance (**D**) were analyzed by the Morris Water Maze Test. (**E, F**) The ELISA assay was carried out to determine the levels of Aβ1-40 (**E**) and Aβ1-42 (**F**). (**G-I**) Activation of the TLR4-NLRP3 inflammasome axis was analyzed by western assay. *** P<0.001 vs. control group, ### P<0.001 vs. model group, $$$ P<0.001 vs. model and VTF group.

constructed AD mice. Moreover, the TLR4-NLRP3 axis was accounted for the protective efficacy of VTF in AD. Thus, this study may support VTF as a promising candidate for AD intervention.

Convincing evidence indicates that microglial cells in central nervous system will be activated by Aβ accumulation and polarized toward a pro-inflammatory phenotype, thereby inducing release of abundant inflammatory cytokines to evoke neuroinflammation in AD [8,21]. Microglia-driven neuroinflammation contributes to neuronal death, ultimately leading to cognitive dysfunction and behavioral abnormalities in AD [2,6,9]. LPS and Aβ are well-established inducers of neuroinflammation and are widely used to mimic AD-like pathology *in vitro* [20,22]. Therefore, we stimulated microglial cells by Aβ/LPS and substantiated the M1-like activation of microglia. Similar with previous study [20,22], Aβ/LPS exposure enhanced M1 microglia-induced releases of inflammatory cytokines. Intriguingly, VTF inhibited M1 microglial polarization-evoked inflammation. Accumulating evidence confirms that *Vitis vinifera* L. exerts the important roles in multiple pathological progression by their bioactive constituents, such as anti-inflammation, anti-oxidant, analgesic, and age-related cognitive decline [14,15]. Of note, a previous study revealed that *Vitis Vinifera* L. could attenuate LPS-induced inflammation in macrophages based on their active compounds, including flavonoids [17]. Additionally, VTF has been shown to possess the immunoregulatory properties in LPS-induced immunological liver injury mice [18]. Thus, these data collectively support the possible anti-inflammatory efficacy of VTF in neuroinflammation, supporting the therapeutic potential of VTF in AD.

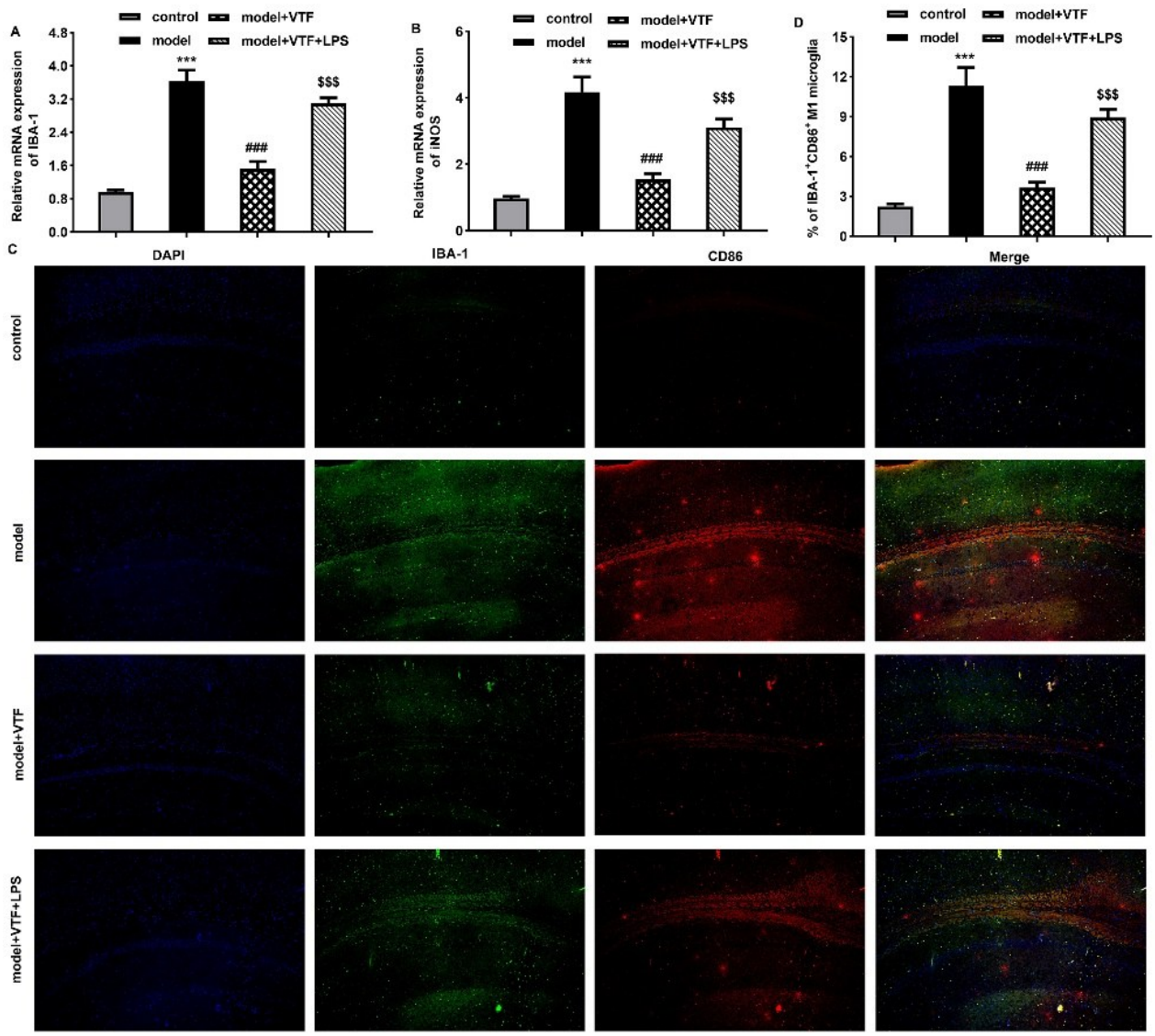


Fig. 5. VTF alleviates M1 microglial polarization in AD model by inhibiting the TLR4-NLRP3 axis. (**A, B**) APP/PS1-constructed AD mice were treated with VTF and LPS. C57BL/6 mice were used as the control group. Then, the mRNA levels of IBA-1 (**A**) and iNOS (**B**) were determined. (**C, D**) The levels of IBA-1+CD86+ M1 microglial cells were evaluated by immunofluorescence analysis. *** P<0.001 vs. control group, ### P<0.001 vs. model group, $$$ P<0.001 vs. model and VTF group.

The APP/PS1 mice are widely recognized as the classic AD mouse model to explore the pathological characterizes of AD [23,24]. To further elucidate the function of VTF in AD, APP/PS1 mice were employed in this study. Moreover, compared with the control C57BL/6 mice, APP/PS1 mice exhibited the obvious cognitive impairment concomitant with increased accumulation of Aβ. Deposition of Aβ, particularly the highly neurotoxic Aβ40 and Aβ42, represents a major pathological hallmark driving AD progression [1,9]. Notably, VTF administration improved learning and memory ability in APP/PS1-constructed AD mice. Simultaneously, a previous study also revealed the beneficial effects of VTF on cognitive impairment in AD rats by regulating synaptic plasticity and cholinergic neurotransmitters [25]. Importantly, VTF treatment reduced Aβ levels in AD mice. Thus, these data may support VTF as a promising agent for AD by improving cognitive injury and reducing Aβ deposition.

Several lines of evidence indicate that deposition of Aβ is a proverbial pathological feature of AD [10,26]. Notably, in this study, VTF treatment obviously reduced Aβ levels in APP/PS1-constructed AD mice. Intriguingly, increasing reports has corroborated that excessive Aβ accumulation will trigger microglial activation, which in turn drives persistent neuroinflammation by secreting abundant pro-inflammatory cytokines, particularly TNF-α and IL-1β [9]. Furthermore, proinflammatory microglial-induced neuroinflammation will impair Aβ clearance, thereby exacerbating the pathological progression of

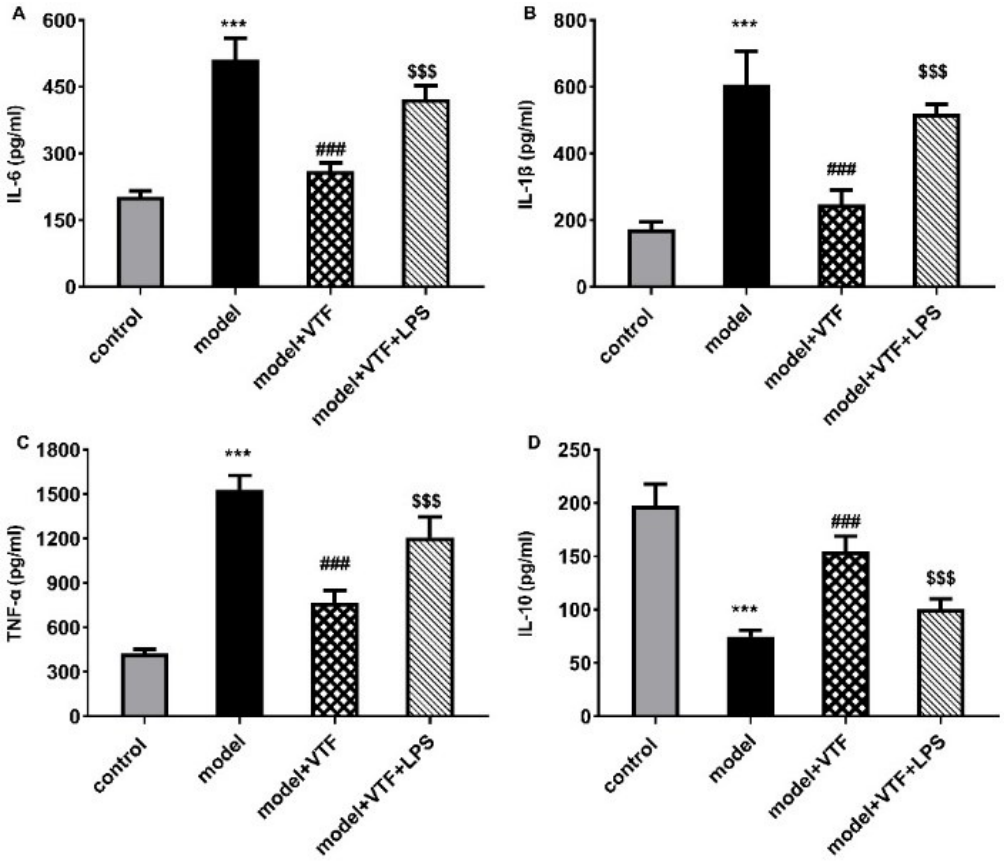


Fig. 6. VTF administration attenuates M1 microglial-evoked neuroinflammation in AD model. Following treatment with VTF and LPS in APP/PS1-induced AD model mice, the levels of pro-inflammatory IL-6 (**A**), IL-1β (**B**), TNF-α (**C**), and anti-inflammatory IL-10 (**D**) were determined in AD model and control C57BL/6 mice. *** P<0.001 vs. control group, ### P<0.001 vs. model group, $$$ P<0.001 vs. model and VTF group.

AD [6,9]. Moreover, inhibition of microglial M1 polarization can protect neuronal cells from microglia-driven neuroinflammation, supporting this approach as a promising therapeutic strategy for AD intervention [27]. Therefore, we next elaborated the function of VTF in microglia-mediated neuro-inflammation in AD model. Importantly, VTF administration suppressed microglial polarization towards a pro-inflammatory M1-like phenotype and attenuated the elevated levels of IL-6, TNF-α and IL-1β in AD mice. Intriguingly, a previous study highlighted that VTF could ameliorates immunological liver injury by regulating pro-inflammatory and anti-inflammatory imbalance [18]. Together, these data suggest that VTF may attenuate the progression of AD by inhibiting M1 microglia-induced neuroinflammation.

Further mechanistic investigations confirmed the activation of the TLR4-NLRP3 inflammasome pathway in LPS/Aβ-activated microglia and AD mice model. As a key and classic signaling, TLR4 can act as a sensor of Aβ to activate the NLRP3 inflammasome, a multiprotein complex comprising NLRP3, ASC, and CASP1 in microglia [28]. Accumulating evidence indicates that aberrant activation of the TLR4-NLRP3 inflammasome axis in AD can regulate Aβ-triggered pro-inflammatory microglial activation and play the important roles in driving neuroinflammation and neurodegeneration in AD [21,29]. Of note, inhibition of the TLR4-NLRP3 inflammasome pathway can shift microglial polarization from a proinflammatory M1 phenotype toward an anti-inflammatory M2 phenotype, thereby attenuating neuroinflammatory response and enhancing Aβ clearance [30,31]. Notably, the current study substantiated that VTF inhibited activation of the TLR4-NLRP3 inflammasome axis. Currently, increasing evidence confirms that targeting TLR4-NLRP3 axis is a new therapeutic strategy for AD [21,31]. Importantly, in this study, reactivating the TLR4 pathway reversed the protective effects of VTF against cognitive impairment, neuroinflammation and Aβ levels in AD mice. Thus, these data reveal that the TLR4-NLRP3 axis may account for VTF-mediated protection against AD.

Collectively, the current data highlight that VTF may alleviate the progression of AD by ameliorating

cognitive deficits and suppressing microglial M1 polarization-induced neuroinflammation. Mechanistically, the TLR4-NLRP3 inflammasome axis may be responsible for the protective efficacy of VTF in AD. Thus, these data may reveal a new mechanism by which VTF attenuates the progression of AD. Therefore, these findings may support VTF as a new promising therapeutic intervention for AD. However, in this study, reactivating the TLR4/NLRP3 axis could not completely reversed the protective effects of VTF against AD. Therefore, whether other pathways are involved in VTF-mediated protection in AD, such as the PI3K/AKT pathway. Moreover, does VTF work as desired in clinical practice? These questions will be addressed in future research.

Conflict of Interest

There is no conflict of interest.

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