Attenuation of microglia-driven neuroinflammation by Tinosinenside A via TLR4 signaling protects against Alzheimer's disease in vivo

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Abstract

Tinospora sinensis (Lour.) Merr. is traditionally utilized in folk medicine for its heat-clearing and potent anti-inflammatory properties. Tinosinenside A (Tis A), a major bioactive sesquiterpene glycoside isolated from this plant, has demonstrated neuroprotective potential, yet its efficacy against neuroinflammation in Alzheimer's disease (AD) remains elusive. This study investigated whether Tis A ameliorates AD-related cognitive deficits and neuroinflammation by modulating microglial activation and phenotypic polarization via the TLR4-mediated signaling pathway. Eleven-month-old male APP/PS1 transgenic mice were treated with Tis A, and cognitive performance was assessed using the Morris water maze and open field tests. Neuroinflammatory cytokines, microglial activation states (M1/M2), and amyloid-β (Aβ) plaque deposition were evaluated using qPCR, immunofluorescence, and ELISA, while the TLR4/NF-κB/NLRP3 signaling cascade was analyzed via Western blot. Results showed that in vivo administration of Tis A significantly improved cognitive function and reduced Aβ plaque burden in APP/PS1 mice. It profoundly suppressed pro-inflammatory cytokines, attenuated microglial overactivation, and promoted a phenotypic shift from the pro-inflammatory M1 state toward the anti-inflammatory M2 state. Mechanistically, these neuroprotective effects were associated with the targeted inhibition of the TLR4/NF-κB/NLRP3 signaling pathway, which subsequently mitigated neuronal apoptosis and histological damage. These findings substantiate the traditional anti-inflammatory application of Tinospora sinensis and highlight Tis A as a promising natural therapeutic candidate for mitigating AD-related neuroinflammation and neuronal injury by modulating the TLR4-mediated signaling axis.

Introduction

As a chronic and fatal neurodegenerative condition, AD represents a major cause of elderly mortality, largely attributable to complications such as infection and malnutrition. According to World Health Organization statistics, AD ranks among the top underlying causes of death in older populations globally and is thus considered a public health priority(Ding et al. 2022). Since the beginning of this century, hundreds of compounds intended to cure AD have entered Phase II clinical trials. However, most were terminated in Phase III due to side effects and serious adverse reactions(Congdon et al. 2023). Although humanized IgG1 monoclonal antibodies targeting Aβ, such as Lecanemab, have received FDA approval, their efficacy and safety remain controversial(van Dyck et al. 2023). Current medications—including the AChE inhibitor donepezil and the NMDA receptor antagonist memantine—offer limited benefits and fail to halt disease progression(Hung and Fu 2017). Mounting evidence indicates that AD patients exhibit elevated inflammatory markers, while the discovery of genetic risk factors linked to innate immunity further highlights the involvement of neuroinflammatory processes in disease development(Leng and Edison 2021). Epidemiological findings from clinical and population studies demonstrate that NSAIDs use is linked to a reduced incidence of AD, supporting the strategy of targeting neuroinflammatory pathways as a viable therapeutic intervention(McGeer et al. 2018). The clinical utility of NSAIDs for long-term Alzheimer's treatment is limited by their gastrointestinal toxicity, thus making the identification of effective and tolerable agents an urgent public health priority.

It is now established that microglia-mediated neuroinflammatory responses persist throughout the progression of AD. Neuroinflammation is not only a key component of AD pathology but may also drive the development of other pathological features(Brett et al. 2022; Cousins et al. 2023; Mary et al. 2024; Reiss et al. 2023; Sudwarts et al. 2022; Zilka et al. 2012). Therefore, suppressing neuroinflammation represents a critical therapeutic strategy for AD. Emerging evidence implicates changes in microglial function and phenotype—a core feature of neuroinflammation—as pathogenic contributors to neurodegenerative disorders, and these alterations are closely linked to Aβ pathology(Beuker et al. 2022). Activated microglia exacerbate disease progression through dual mechanisms: they trigger immune-mediated neuroinflammation leading to neuronal apoptosis or death, while their impaired ability to phagocytose and clear Aβ results in increased Aβ deposition. This contributes to the formation of senile plaques and neuronal damage, hallmark pathological features of AD(Heppner et al. 2015; Nizami et al. 2019; Sebastian Monasor et al. 2020).Toll-like receptor 4 (TLR4) is a key pattern recognition receptor (PRR) on microglia that can detect Aβ. Its activation triggers the phosphorylation of IκB—the endogenous inhibitor of nuclear factor-κB (NF-κB)—leading to its release from the complex(Bauernfeind et al. 2009). Following nuclear translocation, the activated NF-κB p65-p50 heterodimer functions as a pleiotropic transcription factor that upregulates a suite of pro-inflammatory mediators (e.g., TNF-α, IL-1β, IL-6, IL-18) and promotes NLRP3 inflammasome assembly(Chen and Xu 2022). Upon its activation, the NLRP3 inflammasome processes the precursor molecules pro-caspase-1 and pro-IL-18, an event which in turn enhances microglial production of IL-6 and TNF-α, alongside the upregulation of inducible nitric oxide synthase (iNOS) and COX2(Fu and Wu 2023; Lu et al. 2014; Xu et al. 2025). Such sequential events establish a self-reinforcing cycle that intensifies the neuroinflammatory cascade.

In Tibetan medicine, AD falls under the category of "white channel disease." Traditional theory posits that dietary and behavioral imbalances, toxic heat invasion, or internal and external pathogens can cause hyperactivity, hypoactivity, or functional disorder of "rLung," which damages the white channels and leads to the disease(Haiping et al. 2012). Tibetan medicinal herbs can alleviate AD symptoms by regulating rLung function, offering a distinctive therapeutic advantage(Ai-qin et al. 2009). *Tinospora sinensis (Lour.)* Merr., known as "LeZhe" in Tibetan, is the stem of the Tinospora plant. It is documented in the "Four Medical Tantras" for its ability to clear heat disorders of rLung nature, and the "Treasury of Tibetan Medicinal Formulations" records its use in treating seasonal febrile diseases, rheumatism, and rLung-heat disorders(Liang et al. 2024). Primarily distributed in southeastern Tibet, T. sinensis is abundant in resources. It functions to clear heat, detoxify, dispel wind, unblock collaterals, and calm the spirit. It is commonly used in Tibetan regions to prevent and treat aging-related diseases and AD, and serves as a key component in the foundational formula "Manu Xitang," which is included in over 80 Tibetan prepared medicines(Mei et al. 2013).

Our previous study demonstrated that the ethanol extract of Tinospora sinensis (a Tibetan medicine) effectively improved cognitive performance in AD model rats induced by hippocampal $A\beta_{25-35}$ injection. The treatment significantly reduced the levels of pro-inflammatory cytokines, diminished Aβ accumulation and Tau protein hyperphosphorylation, enhanced cholinergic function, and mitigated hippocampal neuronal injury (Jiao et al. 2019; Sun et al. 2020), suggesting its potential as a therapeutic

candidate for AD. Furthermore, the active fraction of T. sinensis markedly decreased levels of inflammatory mediators such as IL-1β, TNF-α, NO, and iNOS in the brains of AD model rats, indicating a possible mechanism through suppressing neuroinflammation(Jiao et al. 2019).Recently, we confirmed that the T. sinensis extract effectively alleviates lipopolysaccharide-induced neuroinflammation in vivo by specifically regulating the TLR4/NF-κB/NLRP3 signaling pathway(Xie et al. 2025a). Concurrently, our earlier phytochemical studies led to the discovery of a novel sesquiterpene glycoside, Tis A(Huang et al. 2019)obtained from the n-butanol-extracted portion of T. sinensis. Pharmacokinetically, Tis A exhibits favorable drug-like properties: it is rapidly absorbed into the bloodstream and distributes into brain tissue at therapeutically relevant concentrations(Chen et al. 2025). Mechanistic studies revealed that, in vitro, Tis A suppresses this exact TLR4/NF-κB/NLRP3 axis, thereby curtailing microglial activation, promoting a shift from the M1 to the M2 phenotype, and indirectly inhibiting neuronal apoptosis(Xie et al. 2025b). Despite these promising findings, previous work on Tis A has been limited exclusively to cellular models. Its anti-neuroinflammatory efficacy and precise mechanisms at the whole-organism level—particularly within a progressive, chronic neurodegenerative environment—remain entirely unexplored. Therefore, capitalizing on Tis A's excellent brain targeting and established in vitro anti-inflammatory profile, this study employs APPswe/PS1dE9 double transgenic AD model mice to systematically evaluate its ameliorative effects on cognitive behavior, Aβ deposition, and neuronal damage in vivo. We further investigate whether Tis A exerts these benefits by modulating microglial activation status and phenotypic polarization, thereby influencing the downstream TLR4/NF-κB/NLRP3 inflammatory signaling pathway to curb neuroinflammation. This work aims to identify potential AD therapeutic leads and provide guidance for developing Tinospora sinensis as a valuable Tibetan medicinal resource.

Materials and methods

Plant Material and Authentication

The material used in this study is the dried rattan stem of Tinospora sinensis (Lour.) Merr., known as "LeZhe" in Tibetan or "Kuanjinteng" in Chinese. The plant material was purchased and identified by Associate Professor Gesang Ciren from the Tibetan Traditional Medical College. A voucher specimen(accession number: YL 2022-01) has been deposited in the Department of Pharmacology, Jiangxi University of Chinese Medicine, for future reference. The plant nomenclature was verified using the World Flora Online (WFO) Plant List (https://wfoplantlist.org/, accessed May 20, 2026).

Animals and Study Design

Male APPswe/PS1dE9 transgenic mice at four months of age, along with wild-type (WT) littermates, were housed under standard conditions. Following a four-week acclimation period, the animals were allocated into five experimental cohorts for oral gavage: WT mice (n = 14), APP/PS1 model mice (n = 14), a positive control group receiving 3 mg/kg galantamine (n = 12), and two treatment groups administered Tis A at 1 mg/kg (n = 13) or 2 mg/kg (n = 13). The Tis A compound (purity > 98%) was kindly provided by

Prof. Huang Huilian's laboratory at the Key Laboratory of the Ministry of Education, Jiangxi University of Chinese Medicine(Huang et al. 2019). Galantamine (batch no. 202306003 A) was sourced from Zhejiang Jinhua Conba Pharmaceutical Co., Ltd. Both compounds were suspended in 0.5% CMC-Na solution. Non-treated groups received the vehicle alone. All treatments were administered once daily for six months.During the final seven days of dosing, memory and spatial learning were assessed using the open field (OF) and Morris water maze (MWM) tests. After behavioral testing, all mice were humanely euthanized (Fig. 1).

Behavioral tests

The open field test assesses an animal's behavior by observing its spontaneous exploration in a novel, open arena. In this procedure, each mouse was placed in the center of a square arena and permitted to explore autonomously for 5 minutes. The device was meticulously sanitized with a 75% ethanol solution between sessions to eradicate remaining smell cues. An automated tracking system was used to quantify the entire distance traveled and the duration spent in the center zone. Following a 5-day acquisition training in the MWM to measure spatial learning, an exploratory trial was executed by eliminating the hidden platform, during which the duration spent in the target quadrant and platform crossings were recorded to assess memory retention and the therapeutic potential of Tis A.

Tissue Preparation

Upon completion of all behavioral assessments, mice were subjected to transcardial perfusion with normal saline under deep anesthesia. Perfusion was continued until the liver turned pale and the effluent from the heart became clear. The brains were carefully extracted and divided for subsequent processing. One portion was fixed in 4% paraformaldehyde (PFA) for immunohistochemical and morphological analyses. The remaining hemispheres were rinsed in ice-cold PBS to remove blood contaminants. Samples were then placed in cryotubes and rapidly frozen at -80°C to prevent degradation and preserve tissue integrity.

Luminex Multiplex Assay

Brain tissue samples from the wild-type control, APP/PS1 model, galantamine, and both Tis A dose groups were processed for total protein extraction. Protein content was quantified via a BCA kit, and concentrations were normalized to 10 mg/mL using 1×PBS to ensure equal loading across all samples.A multiplex immunoassay was subsequently performed using the Luminex xMAP technology with a Mouse Th1/Th2 Cytokine 11-Plex Panel (Thermo Fisher Scientific, USA, Cat: EPX110-20820-901), as per the provided protocol. In this procedure, samples were incubated with antibody-bound magnetic beads. After thorough washing, the complex was sequentially exposed to biotinylated detection antibodies and a streptavidin–phycoerythrin conjugate. Signal acquisition was performed on a Luminex 200™ analyzer,

recording the median fluorescence intensity. Final cytokine concentrations (pg/mL) were derived from standard curves and used for analysis.

Immunofluorescence

Coronal brain sections (3 μm) were cleaned, hydrated again, and underwent extraction of antigen in a buffer with citrate (pH 6.0; G1201, Servicebio). Following permeabilization with 0.4% Triton X-100 (Solarbio, T8200) and subsequent blocking, slices were treated nightly at 4°C with secondary antibodies: anti-iNOS (Proteintech, 18985-1-AP; 1:500), anti-Iba1 (Abcam, ab178846; 1:500), and anti-Aβ (CST, 8243T; 1:100).After the initial antibody incubation, sections were subjected to a combination of secondary reagents: goat anti-rabbit IgG Alexa Fluor™ 488 (Invitrogen, A11008; 1:500), goat anti-rabbit IgG HRP (Servicebio, GB23303; 1:500), and iF555-Tyramide (Servicebio, G1233; 1:1000) for one hour at room temperature. Following the final washes, sections were mounted using a DAPI-containing medium.

Reverse transcription (RT)-qPCR

Total RNA obtained from tissues via an Animal Total RNA Extraction Kit (Foregene, RE-03,011/RE-03,111) was then transformed to cDNA utilizing 4× Hifair® III SuperMix plus (Yeasen, 11141ES60). Quantitative PCR was subsequently conducted with SYBR Green Master Mix (Yeasen, 11184ES08) and primers tailored to the gene (Table 1) under the following cycling conditions: 95°C for 30 s, then 40 cycles of 95°C for 3 s and 60°C for 20 s. Relative gene expression, normalized to GAPDH, was determined using the $2^{-\Delta\Delta CT}$ technique.

Table 1
Primer sequence

Gene	Forward primer (5'–3')	Reverse primer(5'–3')
iNOS	GTGGTGACAAGCACATTTGG	AAGGCCAAACACAGCATACC
TNF-α	AACCTCCTCTCTGCCGTCAAG	CCTCCCAGGTATATGGGCTCAT
IL-1β	AGCTTCAAATCTCGCAGCAG	TCTCCACAGCCACAATGAGT
IL-6	TTTCTCCACGCAGGAGACTT	AGACAGGTCTGTTGGGAGTG
COX-2	TCAATACTGGAAGC CGAGCA	CACCCCTTCACATTATTGCAGA
IL-4	AACGAGGTCACAGGAGAAGG	CTGCAGCTCCATGAGAACAC
IL-10	AGTACAGCCGGGAAGACAAT	TCTAGGAGCATGTGGCTCTG
Tgf-β	TTGCTTCAGCTCCACAGAGA	CAGAAGTTGGCATGGTAGCC
Arg-1	CTACCCACTGCCTTTGAAGC	CGGTGATAGGATGGGTGTCA
CD36	GCCAAGCTATTGCGACATGA	GGCATTGGCTGGAAGAACAA
CD206	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
CD16	CTAGGAAGGACACTGCACCA	ATGGGCAGTAGGCATCTTGT
CD32	CTAGGAAGGACACTGCACCA	TCCCTGTGATCAGGGTTTCC
GAPDH	AGGCCGGTGCTGAGTATGTC	TGCCTGCTTCACCACCTTCT

Western blotting

Total protein concentrations were quantified with a BCA assay kit (Solarbio, China) in accordance with the manufacturer's instructions. The protein specimens were resolved using SDS-PAGE and subsequently electroblotted onto PVDF membranes (Merck Millipore, USA). Following a blocking step with 5% BSA, the membranes were probed with primary antibodies through an overnight incubation at 4°C. NLRP3 (1:1000, ab263899, Abcam), Caspase-1 (1:1000, 24232S, CST), ASC (1:1000, 67824S, CST), TLR4 (1:1000, ab13556, Abcam), p-NF-κB p65 (1:1000, 3033S, CST), NF-κB p65 (1:1000, 10745-1-AP, Proteintech), p-IκBα (1:1000, 2859S, CST), IκBα (1:1000, 4812S, CST), COX-2 (1:1000, 12375-1-AP, Proteintech), IL-1β (1:1000, ab234437, Abcam), Arg-1 (1:1000, GB11285, Servicebio), IL-10 (1:1000, GB11108, Servicebio), caspase-3 (1:1000, 66470-2-Ig, Proteintech), Bcl-2 (1:1000, ab182858, Abcam), Bax (1:1000, 2772S, CST), and β-actin (1:5000, 20536-1-AP, Proteintech).Followed by an HRP-conjugated secondary antibody (1:5000, SA00001-2, Proteintech) for 1h.Signals were developed with ECL substrate (BMU102-CN, Abbkine) and quantified using ImageJ.

H&E and Nissl Staining

Three 4% PFA-fixed brain tissues containing the hippocampal region were randomly selected from each group. Following an 8-hour rinse under running water, the tissue specimens were processed through a series of steps for paraffin embedding. Following dehydration, clearing, and paraffin embedding, 3-μm tissue sections were prepared and affixed to slides. After baking, deparaffinization, and rehydration, consecutive sections were stained using commercial H&E (Servicebio, G1076) and Nissl (Servicebio, GP1043) staining kits according to standard protocols. All stained sections were coverslipped utilizing neutral resin and photographed under a fluorescence microscope.

ELISA

Soluble and insoluble brain proteins were extracted (Kit: C500071, Sangon Biotech) and quantified by BCA assay (Solarbio, China).Soluble Aβ40 and Aβ42 concentrations were quantified in the supernatant using human-specific ELISA kits (CUSABIO; Aβ40: U08025489, Aβ42: U09025490). The insoluble pellet was dissolved in pre-cooled extraction buffer for analysis of insoluble Aβ40 and Aβ42.

TUNEL Staining

Tissue pretreatment was carried out as previously outlined for the immunofluorescence procedure. Following the removal of the blocking solution, apoptosis was assessed via the TUNEL assay, employing an In Situ Cell Death Detection Kit (Roche, TMR red, 12156792910) in accordance with the manufacturer's protocol.Following permeabilization, brain sections were processed for TUNEL assay to label apoptotic cells. Nuclei had been stained with DAPI (Solarbio, P0131), and samples were examined under a Leica DM2000 LED fluorescence microscope to visualize TMR red-positive apoptotic signals.

Data Analysis

Data are expressed as mean ± SEM and were analyzed with IBM SPSS Statistics 21.0. Figures were created using GraphPad Prism 9.0. We employed repeated-measures ANOVA (water maze), one-way ANOVA with post-hoc tests (LSD or Tamhane's T2; multiple groups), unpaired Student's t-test (two groups), and Pearson correlation. A p-value < 0.05 was deemed statistically significant.

Results

Effects of Tis A on Cerebral Inflammatory Cytokine Levels in APP/PS1 Mice

Prior research has demonstrated that neuroinflammation is pivotal in the central nervous system and facilitates the advancement of AD. Although Tis A has demonstrated anti-inflammatory properties, its specific effects within the CNS remain unclear. To this end, we quantified a panel of 11 pivotal inflammatory cytokines in mouse brain lysates by employing Luminex technology integrated with a high-throughput liquid-phase protein microarray (Fig. 2A–K). A significant elevation in key pro-inflammatory mediators (IL-12p70, IL-2, IL-1β, IL-6) was detected in APP/PS1 mouse brains relative to WT controls. (Fig. 2B, E, D, H). Administration of Tis A significantly mitigated the elevated expression of these

cytokines in the APP/PS1 model. Additionally, levels of IFN-γ, IL-5, TNF-α, and GM-CSF, while showing an upward trend inAPP/PS1 mice relative to WT animals (Fig. 2A, G, I, J), were effectively counteracted by Tis A treatment.These results demonstrate that Tis A potently attenuates a wide range of inflammatory cytokines in the APP/PS1 mouse brain.

Effects of Tis A on Pro- and Anti-inflammatory Marker Expression in Brain Tissue

To further validate the Luminex findings, we analyzed pro- and anti-inflammatory markers using immunofluorescence and quantitative PCR. Relative to WT controls, the APP/PS1 mice exhibited a marked increase in the mean fluorescence intensity of iNOS across hippocampal subregions (CA1, CA3, DG) and the cerebral cortex, as determined by immunofluorescence analysis. Tis A treatment markedly reduced iNOS intensity in these regions, indicating effective suppression of iNOS expression (Fig. 3A–E).qPCR results further confirmed the Luminex data. Tis A treatment significantly counteracted the elevated mRNA levels of pro-inflammatory mediators (iNOS, COX-2, IL-1β, IL-6, TNF-α) observed in APP/PS1 animals relative to WT controls (Fig. 3F–J).In contrast, the mRNA released by anti-inflammatory factors (IL-10, TGF-β, Arg-1) was markedly reduced in APP/PS1 mice, and this decrease was reversed by Tis A administration.(Fig. 3K–N).Collectively, our results reveal a dual function of Tis A in neuroinflammation: it concurrently dampens key genes that promote inflammation while enhancing the production of anti-inflammatory, indicating a coordinated regulatory function.

Impact of Tis A on Microglial Activation

We assessed microglia activity by analyzing the amount of production of Iba-1, a particular marker for microglia, in APP/PS1 mice employing immunofluorescence. A marked enhancement of Iba-1 immunoreactivity, reflected by both increased positive area and fluorescence intensity, was observed in the hippocampal CA1, CA3, and DG subfields as well as the cortical regions of APP/PS1 mice relative to WT controls (Fig. 4A). Treatment with 3 mg/kg galantamine, 1 mg/kg Tis A, or 2 mg/kg Tis A significantly reduced the Iba-1-labeled area and fluorescence intensity (Fig. 4B–E), demonstrating that Tis A suppresses microglial activation in APP/PS1 mice.In order to investigate the impact of Tis A on microglial phenotype, we measured mRNA levels for M1 indicators (CD16, CD32) and M2 indicators (CD206, CD36) by qPCR (Fig. 4F–I).APP/PS1 mice exhibited elevated CD16 and CD32 mRNA levels and reduced CD206 and CD36 levels compared to WT mice. Treatment with 2 mg/kg Tis A significantly increased CD206 and CD36 expression while decreasing CD16 and CD32 expression. These results suggest that Tis A suppresses microglial activation and facilitates their functional repolarization into the M1 to the M2 state in the brains of APP/PS1 mice.

Effects of Tis A on microglial function

Neuroinflammation is intimately associated with AD pathogenesis, in which the amyloid-β (Aβ) peptide serves as a pivotal driver. Chronically activated microglia undergo phagocytic exhaustion, leading to

impaired Aβ clearance and disrupting the balance between Aβ production and removal. Consequently, Aβ buildup and deposition in the cerebral tissue are promoted.To investigate whether Tis A modulates microglial function and suppresses excessive activation, we performed double immunofluorescence staining using antibodies against Iba-1 and Aβ to assess changes in the quantity of microglia co-localized with plaques expressing A (Fig. 5A). Compared with WT controls, APP/PS1 transgenic mice exhibited a significant elevation in both the total number of microglia and the proportion of $Aβ^+$ area co-localized with Iba-1^+ cells.Tis A treatment markedly reduced both microglial number and the extent of Aβ co-localization with Iba-1^+ cells (Fig. 5B–C).The findings indicate that Tis A may protect neurons from neuroinflammatory damage by suppressing microglial overactivation and enhancing their ability to phagocytose and clear Aβ.

Effects of Tis A on the TLR4/NF-κB/NLRP3 Signaling Pathway in APP/PS1 Mice

Microglial function and phenotype are closely associated with TLR4/NF-κB/NLRP3 signaling. While resting microglia exhibit low expression of these pathway components, activation upregulates their levels. M1-polarized microglia drive pro-inflammatory cytokine production through NF-κB activation. Conversely, the M2 phenotype promotes IκB activity, which inactivates NF-κB, consequently suppressing pro-inflammatory genes and upregulating anti-inflammatory mediators like TGF-β. To investigate Tis A's role in this context, we performed Western blot analysis. Results demonstrated a marked upregulation of key NLRP3 inflammasome constituents (NLRP3, caspase-1, ASC) in APP/PS1 mice compared to WT controls (Fig. 6A–D). Concurrently, phosphorylation levels of TLR4, NF-κB p65, and IκBα were also markedly increased in the model group (Fig. 6E–H). These alterations were effectively reversed by Tis A treatment.Moreover, Tis A downregulated the downstream inflammatory effectors IL-1β and COX-2 (Fig. 6I–K), while concurrently enhancing the expression of anti-inflammatory mediators Arg-1 and IL-10 (Fig. 6I, L–M). In conclusion, Tis A alleviates neuroinflammation in AD mice by suppressing the TLR4/NF-κB/NLRP3 signaling axis.

Effects of Tis A on Behavioral Performance in APP/PS1 Mice

To evaluate the effect of Tis A in counteracting neuroinflammation during AD progression, we assessed its effects on motor deficits and cognitive function using open field and MWM tests. APP/PS1 model mice exhibited reduced the overall distance walked and prolonged immobility uration during an open field test relative to WT controls (Fig. 7A–C). Beginning on the following day, escape latency in the MWM was significantly prolonged in APP/PS1 mice (Fig. 7D), followed by an augmented area under the latency curve (AUC, Fig. 7F). In the probing trial, APP/PS1 mice demonstrated a notable decrease in platform crossings and duration allocated to the target quadrant (Fig. 7G–H).In contrast, both galantamine- and Tis A-treated groups traveled longer distances with shorter immobility times in the open field. They also displayed gradually shortened escape latencies, reduced Area under the curve (AUC), and increased platform crossings and target quadrant duration.These results indicate that Tis A does not induce

neurological excitation or suppression but effectively ameliorates both motor and cognitive deficits in AD model mice.

Effects of Tis A on Neurohistopathology in APP/PS1 Mice

Staining with H&E of hippocampal regions revealed that APP/PS1 mice displayed disrupted neuronal organization, enlarged interstitial gaps, and pronounced nuclear dysfunction in the CA1, CA3, and DG subregions relative to WT control. Both galantamine and Tis A treatment groups showed amelioration of these histopathological alterations (Fig. 8A).Nissl staining further evaluated neuronal integrity. WT mice exhibited clear Nissl staining, high neuronal density, and well-aligned cell arrangement without obvious damage. In contrast, APP/PS1 mice displayed paler staining and reduced neuronal density in the CA1, CA3, and DG regions, indicating neuronal loss or injury. Tis A and galantamine treatment improved neuronal morphology, staining intensity, and cell density compared to the APP/PS1 group (Fig. 8B–E).These results demonstrate that Tis A significantly ameliorates abnormalities in neuronal arrangement and morphology while reducing neuronal loss in the hippocampus of AD model mice, indicating a protective effect on neurons in these regions.

Effects of Tis A on Aβ Levels and Deposition in APP/PS1 Mice

We next determined if Tis A affects Aβ aggregation and amyloid plaque deposition. Immunofluorescence staining with an Aβ-specific antibody revealed significantly increased Aβ-positive areas in the hippocampal CA1, DG, and cortical areas of APP/PS1 animals relative to WT controls(Fig. 9A). By contrast, Tis A treatment markedly reduced Aβ plaque deposition in these regions (Fig. 9B–E).We further quantified PBS-soluble and -insoluble Aβ40 and Aβ42 levels in brain homogenates using ELISA. APP/PS1 mice demonstrated markedly increased concentrations soluble Aβ40, soluble Aβ42, and insoluble Aβ40 compared to WT mice (Fig. 9F–G). Tis A treatment significantly reduced soluble Aβ40, soluble Aβ42, and insoluble Aβ40 levels, while insoluble Aβ42 levels remained unchanged across groups.Together, our findings indicate that Tis A primarily reduces the more toxic soluble Aβ oligomers and inhibits early deposition, thereby attenuating neurotoxicity and delaying disease progression.

Neuroprotective Effects of Tis A in APP/PS1 Mice

The direct neurotoxicity of Aβ underlies neuronal dysfunction and apoptosis. To assess whether Tis A confers neuroprotection, we analyzed apoptosis and the expression of associated proteins in APP/PS1 mice via TUNEL staining and WB. Relative to WT controls, APP/PS1 mice demonstrated a marked pro-apoptotic shift, characterized by elevated caspase-3 and Bax alongside reduced Bcl-2 expression. (Fig. 10B–E). This was corroborated by increased apoptotic cells in the hippocampal DG and cortex (Fig. 10A,G–H). Thus, Tis A counteracted apoptotic alterations and alleviated neuronal death in the AD model via modulation of key apoptotic regulators, including suppressed caspase-3/Bax and enhanced

Bcl-2.We subsequently employed Pearson correlation analysis to assess the association between Tis A-mediated suppression of neuroinflammation and its therapeutic effects in AD (Fig. 10F). From a microglial perspective, the results clarify the pathway through which Tis A modulates the TLR4/NF-κB/NLRP3 axis to alleviate neuroinflammation.Tis A-induced changes in microglial function and phenotype showed significant positive correlations with:TLR4 (R = 0.578) and NF-κB (R = 0.655) expression,Neuroinflammatory factors TNF-α (R = 0.558), IL-1β (R = 0.734), and COX-2 (R = 0.594),AD efficacy markers: Aβ40 (R = 0.75), Aβ plaque load (R = 0.844), and TUNEL-positive cells (R = 0.798).Positive but weaker correlations were observed with NLRP3 (R = 0.402) and IL-6 (R = 0.417).Taken together, these results demonstrate that the beneficial disease properties of Tis A are facilitated by the modulation of TLR4/NF-κB/NLRP3 signaling, which curtails microglial neuroinflammation and subsequent neuronal damage.

Discussion

AD poses a severe threat to the health and lives of the elderly, while current therapeutic options remain limited. Neuroinflammation represents a core pathological feature of AD, persisting throughout its progression and actively contributing to disease aggravation. The TLR4/NF-κB/NLRP3 signaling axis plays a pivotal role in driving neuroinflammatory processes. Inhibiting Aβ-induced microglial activation and subsequent neuroinflammation offers a promising therapeutic strategy and represents an emerging breakthrough in AD treatment. Recent studies have demonstrated that the crude extract of Tinospora sinensis effectively alleviates LPS-induced acute neuroinflammation in mice by regulating the TLR4/NF-κB/NLRP3 signaling pathway(Xie et al. 2025a). Furthermore, Tinosinenside A (Tis A), a novel sesquiterpene glycoside isolated from this Tibetan medicinal plant through our National Natural Science Foundation-supported project (81660713), has been identified as a key active monomer. In vitro evaluations revealed that Tis A inhibits neuroinflammation and protects HT22 cells by suppressing this same signaling axis in BV2 microglia, and molecular docking studies further predicted stable binding between Tis A and multiple inflammatory targets within this pathway(Stancu et al. 2019; Xie et al. 2025b). However, these previous findings were limited to crude extracts in vivo or isolated monomers in vitro. The in vivo anti-neuroinflammatory efficacy of the purified monomer Tis A, particularly in the context of a chronic, progressive neurodegenerative model like APP/PS1 transgenic mice, and its specific mechanistic basis had not been evaluated and remained to be elucidated. This study bridges this gap, extending previous in vitro findings to confirm Tis A's therapeutic potential as a targeted molecular treatment for AD in vivo.

Acute neuroinflammation serves as an adaptive response to neurological injury, infection, and other stimuli. However, when inflammation persists, it transitions into a chronic state that continuously stimulates the nervous system. Chronic neuroinflammation drives the persistent secretion of inflammatory cytokines, resulting in a pathological disruption of immune balance that favors pro-inflammatory responses over anti-inflammatory mechanisms. This exacerbates inflammatory damage and ultimately drives neuronal injury and diverse pathological alterations(Mantovani et al. 2002).In APP/PS1 mice, Tis A ameliorated neuroinflammatory imbalance by downregulating pro-inflammatory

mediators (e.g., IL-1β, IL-6, TNF-α) and upregulating anti-inflammatory factors (e.g., IL-10, TGF-β), as validated by Luminex assay, immunofluorescence, and qPCR.

In AD, neuroinflammation escalates with disease progression(Fan et al. 2017). Specifically, neuroinflammatory levels peak initially during early AD development due to initial anti-inflammatory responses, followed by a second peak during the transition from mild cognitive impairment to dementia. The pro-inflammatory M1 phenotype, exacerbates neuroinflammation by secreting a suite of cytokines—namely TNF-α, IL-4, IL-6, IL-12, and IL-18—concurrently with a decline in phagocytic efficiency(Hamelin et al. 2016; Michaud et al. 2013). Moreover, microglia in a state of chronic activation around Aβ plaques attempt phagocytic clearance but simultaneously release inflammatory mediators, resulting in a detrimental overactivation cycle that worsens the pathology(Yu et al. 2013).Consistent with these findings, our study detected a significant presence of microglia that were activated throughout the brain's hippocampus and cortical areas of APP/PS1 animals subjected to inflammatory stimulation. Tis A treatment effectively reduced microglial activation, suppressed M1 marker CD32 mRNA expression, and elevated M2 markers CD206 and CD36. Collectively, these findings indicate that Tis A suppresses M1 microglial polarization and concurrently fosters the anti-inflammatory M2 phenotype in the APP/PS1 mouse brain, concomitant with improvements in microglial morphology and a marked enhancement of phagocytic function.

Within the central nervous system, TLR4 exhibits broad expression on macrophages and is involved in recognizing Aβ. Accumulated evidence underscores its pivotal function in inflammatory regulation(Lee et al. 2021). The interaction of TLR4 with Aβ stimulates the pathway of NF-κB signaling, initiating the DNA synthesis of NLRP3 and pro-IL-1β. TLR4 activation initiates the canonical NF-κB pathway: IκB phosphorylation and degradation release the p65 subunit, which then moves within the nucleus, binds to DNA κB motifs, and regulates the gene expression of pro-inflammatory mediators(Zhang and Ghosh 2001; Zhang et al. 2023).This cascade promotes NLRP3 inflammasome assembly to drive the maturation of IL-1β and IL-18 from their pro-forms. The consequent substantial release of these cytokines aggravates AD pathogenesis(Dhapola et al. 2021).To evaluate the potential impact of Tis A on the TLR4/NF-κB/NLRP3 axis in vivo, we performed Western blotting. Our data indicate that in APP/PS1 mice, TLR4 activation initiates downstream NF-κB events, as evidenced by increased phosphorylation levels of both IκBα and the NF-κB p65 subunit (Fig. 11). Treatment with Tis A, however, markedly attenuated NLRP3 inflammasome activation, downregulated TLR4 protein levels, and impeded the phosphorylation and degradation of IκBα. These effects collectively resulted in the blockade of NF-κB p65 nuclear translocation. Thus, the anti-inflammatory property of Tis A is attributed, at least in part, to its inhibition of the TLR4/NF-κB/NLRP3 signaling cascade.

By suppressing microglial activation, anti-inflammatory drugs including celecoxib and NSAIDs confer neuroprotection and alleviate neuronal pathology in AD model mice(Chen et al. 2017).Collectively, suppressing neuroinflammation offers a viable therapeutic approach for AD. Based on this premise, we evaluated the potential for therapy of Tis A in mitigating neural inflammation and investigated its efficacy in APP/PS1 AD model mice. Using a comprehensive approach including behavioral tests,

histopathological examination, quantification of Aβ deposition, neuronal apoptosis assessment, and analysis of apoptosis-related protein expression, we demonstrated that APP/PS1 mice developed severe memory deficits with significantly increased neuronal death in cortical and hippocampal regions. Tis A treatment effectively ameliorated these spatial memory and learning impairments, and reversed inflammation-induced neuronal apoptosis, indicating substantial neuroprotection. As excessive accumulation of Aβ is considered one of the initial occurrences in AD pathogenesis, and enhanced neuroinflammation promotes Aβ production while aggregated Aβ conversely stimulates microglial proliferation and exacerbates inflammation(Novoa et al. 2022), we specifically examined Aβ levels. Our results showed significantly elevated Aβ in APP/PS1 mouse brains, while Tis A treatment markedly reduced Aβ deposition in both cortex and hippocampus, indicating its ability to suppress amyloid pathology. Furthermore, Pearson correlation analysis revealed significant positive correlations between Tis A-mediated suppression of neuroinflammation and its therapeutic effects in AD. These results collectively support the potential value of Tis A as a promising therapeutic candidate for AD.

Conclusion

Tis A significantly reduces Aβ deposition, reverses neuronal apoptosis, and ameliorates cognitive deficits in APP/PS1 mice, demonstrating notable anti-AD efficacy. These beneficial effects are likely mediated through suppression of the key neuroinflammatory TLR4/NF-κB/NLRP3 signaling pathway, by lowering pro-inflammatory cytokines, and restoration of microglial function. Our findings confirm its promise as an AD therapeutic, offering a novel strategy for AD treatment.

Abbreviations

Acetylcholinesterase (AChE); Alzheimer's disease (AD); Amyloid-β (Aβ); Apoptosis-associated speck-like protein containing a CARD (ASC); Area under the curve (AUC); B-cell lymphoma 2 (Bcl-2); Bcl-2-associated X protein (Bax); Dentate gyrus (DG); Deoxyribonucleic acid (DNA); Food and Drug Administration (FDA); Hematoxylin and eosin staining (HE); Inducible nitric oxide synthase (iNOS); Ionized calcium-binding adapter molecule 1 (Iba-1); Morris water maze (MWM); N-methyl-D-aspartate receptor (NMDA); NOD-like receptor protein 3 (NLRP3); Non-steroidal anti-inflammatory drugs (NSAIDs); Nissl staining (Nissl); Nuclear factor-kappa B (NF-κB); Open field test (OF); Paraformaldehyde (PFA); Pattern recognition receptor (PRR); Quantitative polymerase chain reaction (qPCR); Tau protein; Tinosinenside A (Tis A); Toll-like receptor 4 (TLR4); Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL); Wild-type (WT).

Declarations

Ethics statement All animal experiments complied with ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals. Only male APP/PS1 and wild-type (WT) mice were used to minimize the influence of hormonal fluctuations on study results. Animals were housed in an SPF facility at the Experimental Animal Center of Jiangxi University of Chinese Medicine (License: SYXK (Gan) 2022-

0002; SCXK (Su) 2021-0013; Certificate: 202353941). The study protocol was approved by the University's Animal Ethics Committee (JZLLSC-202100316; March 16, 2021), and all procedures followed strict ethical regulations.

Authorship contributions JW and CM performed the experiments and analysed data, meanwhile wrote original draft. YX, SW and ZH reviewed and discussed the data. HJ, JM and FD participated in the study and checked the format of References and tracing the source. LH conceived, designed and revised the manuscript. All authors have read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability All source data for this work (or generated in this study) are available upon reasonable request.

Competing interests The authors declare no competing interests.

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Figures

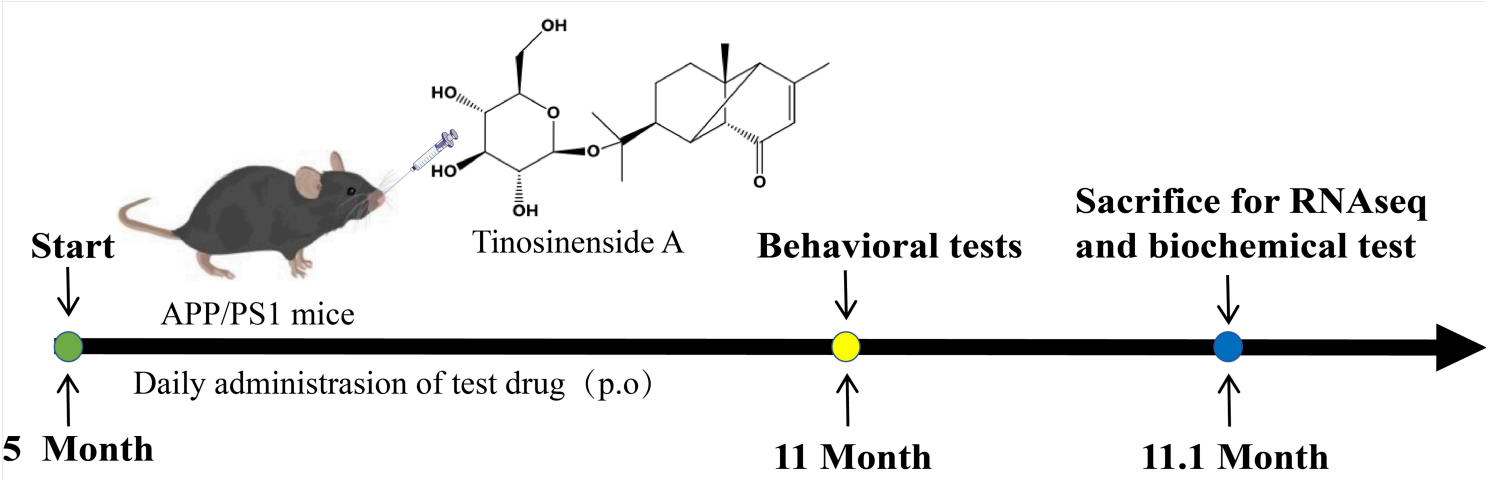

Figure 1

Schematic diagram of the experimental design

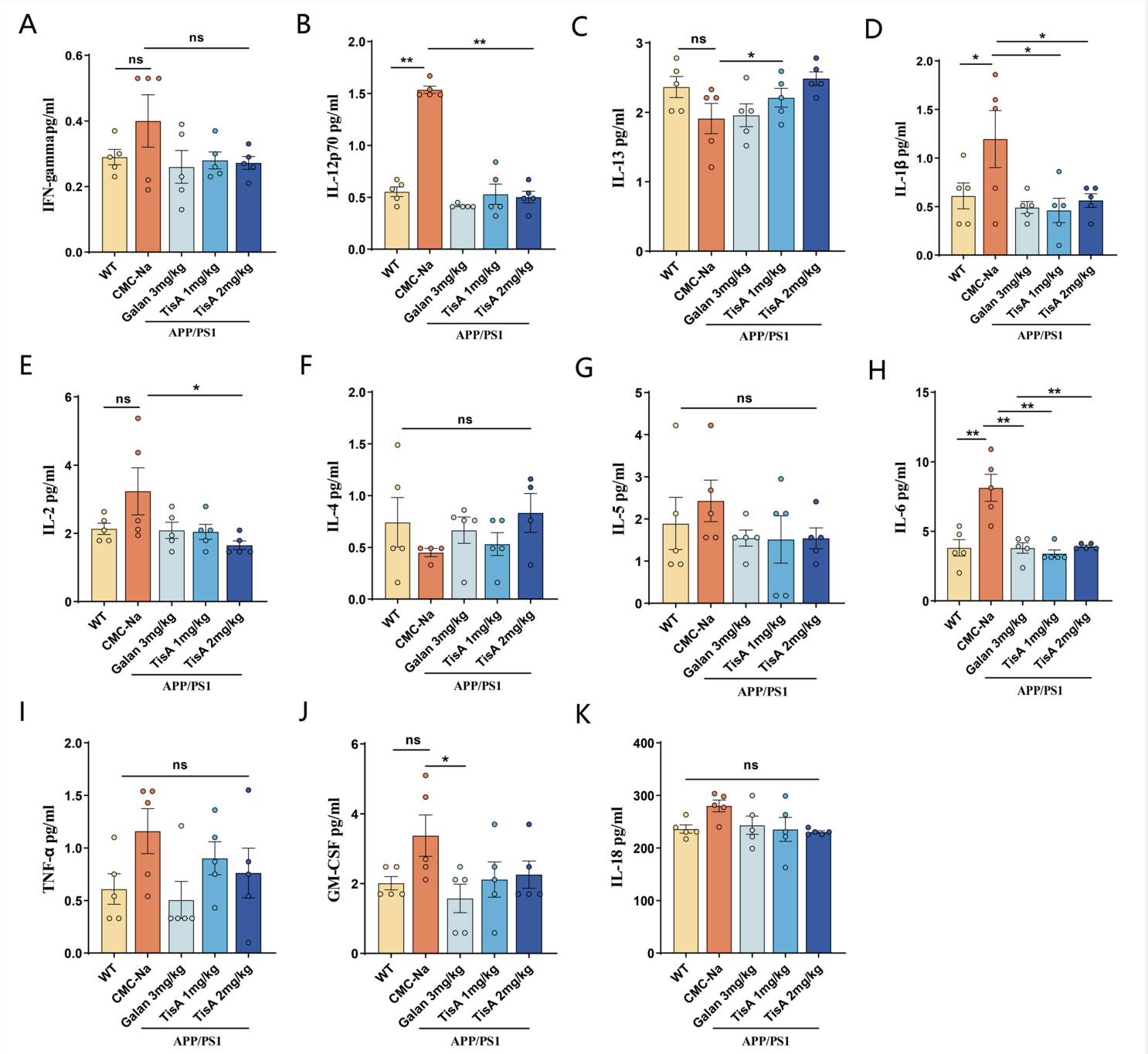

Figure 2

Effects of Tis A on cerebral inflammatory cytokine levels in APP/PS1 mice. (A-K) Levels of IFN-γ, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α, GM-CSF, and IL-18 in brain lysates were measured by Luminex assay. Data are presented as mean ± SEM; n = 5 mice per group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, ns $p > 0.05$.

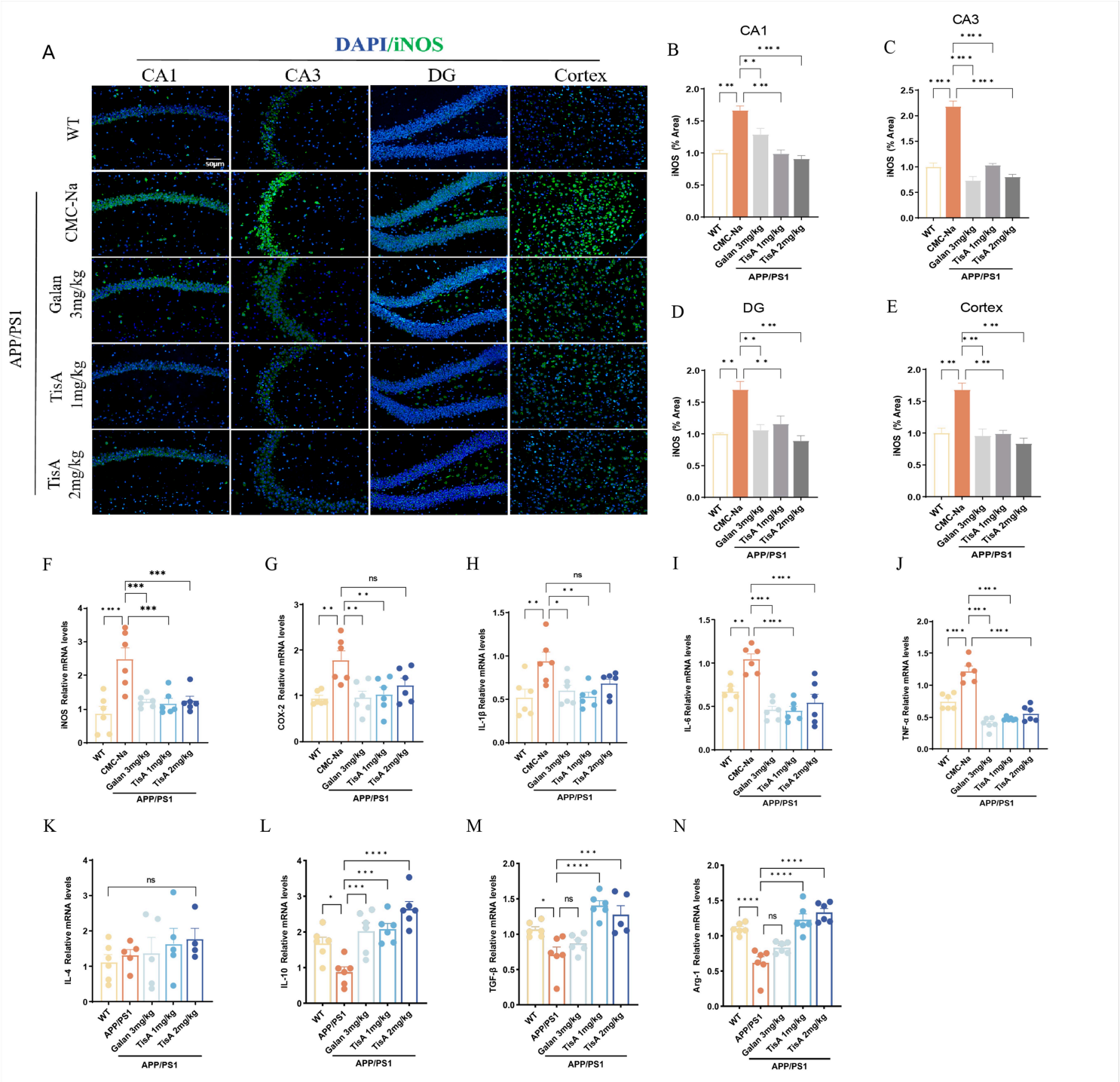

Figure 3

Effects of Tis A on the expression of pro- and anti-inflammatory markers in brain tissue. (A) Immunofluorescence microscopy images of iNOS (green) in hippocampal areas (CA1, CA3, DG) and the cortex. Scale bar measures 50 μm. (B-E) Corresponding quantification of the iNOS-positive area in each region. (F-J) The relative mRNA abundance of pro-inflammatory genes (iNOS, COX-2, IL-1β, IL-6, TNF-α) was measured by qPCR. (K-N) Similarly, the mRNA levels of anti-inflammatory genes (IL-4, IL-10, TGF-β, Arg-1) were detected. Data are presented as mean ± SEM; n = 6 mice per group. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$, ns $p > 0.05$.

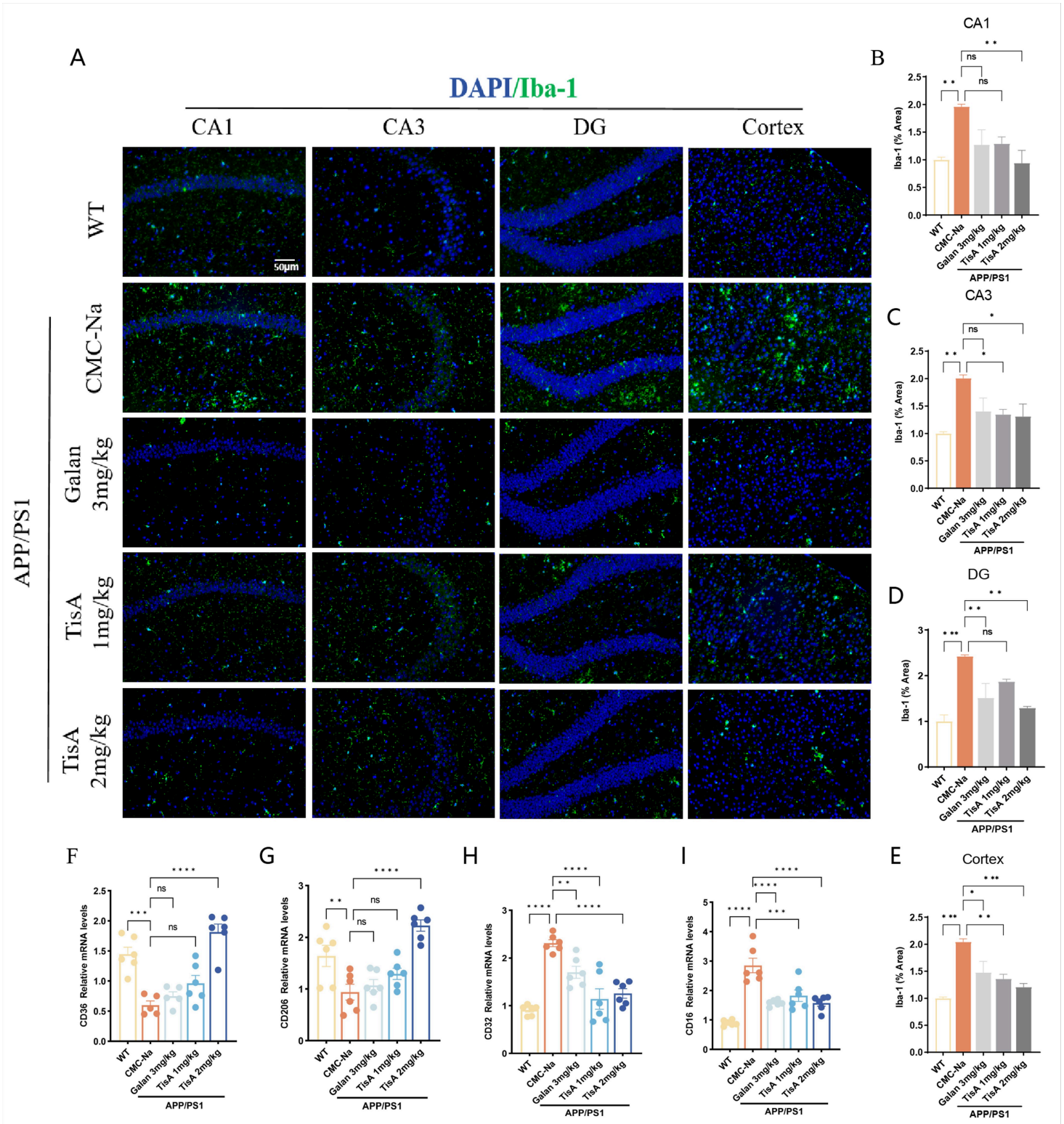

Figure 4

Impact of Tis A on microglial activation. Sample fluorescence micrographs depicting Iba-1-positive microglial (green) across the hippocampus formation (CA1, CA3, DG) and cortex in several groups. Scale bar measures 50 μm. (B-E) Quantification of the Iba-1-immunoreactive area within these specified brain regions. (F-G) Relative mRNA abundance of the M2 polarization markers CD36 and CD206 in brain tissue, as determined by qPCR. (H-I) Brain transcript concentrations of the M1 biomarkers CD32 and

CD16, measured via qPCR. Data are presented as mean ± SEM; n = 6 mice per group. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$, ns $p > 0.05$.

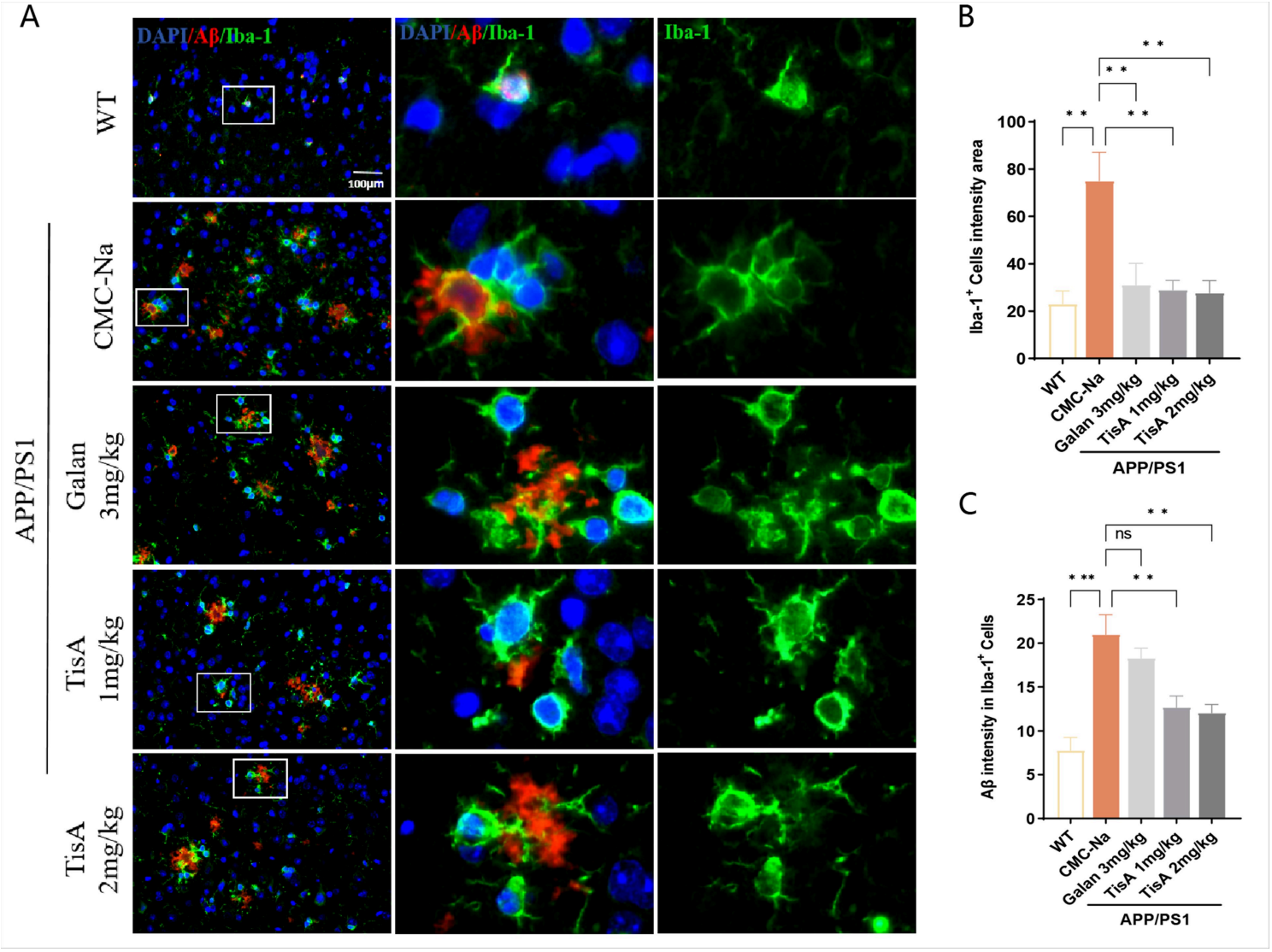

Figure 5

Effects of Tis A on microglial function. (A) Representative photos illustrating co-localization of Aβ (red) and Iba-1 (green) by immunofluorescence in the cortical regions across groups. Scale bar measures 100 μm. (B) Statistical assessment of Iba-1⁺ cell numbers. (C) Percentage of Aβ⁺ area overlapping with Iba-1⁺ cells. Data are expressed as mean ± SEM; n = 3 mice per group. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, ns $p > 0.05$.

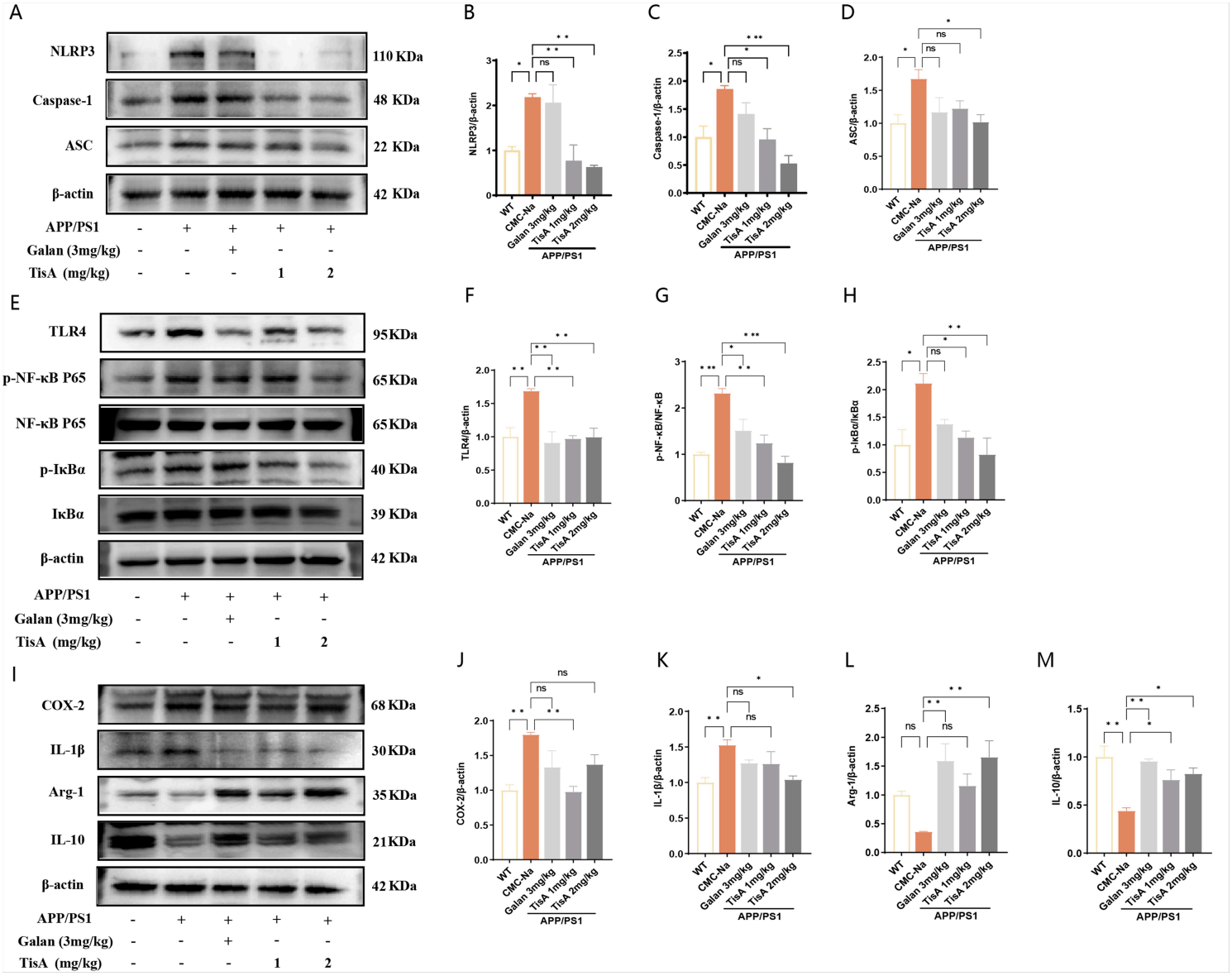

Figure 6

Effects of Tis A on the TLR4/NF-κB/NLRP3 Signaling Pathway in APP/PS1 Mice. (A-D) Protein expression and quantification of NLRP3 inflammasome components (NLRP3, caspase-1, ASC). (E-H) WB analysis and quantification of proteins involved in the TLR4/NF-κB signaling pathway. (I) WB analysis of pro-inflammatory proteins (IL-1β, COX-2) and anti-inflammatory proteins (Arg-1, IL-10). (J-M) Quantification of the blots shown in (I). Data are presented as mean ± SEM; n = 3 mice per group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, ns $p > 0.05$.

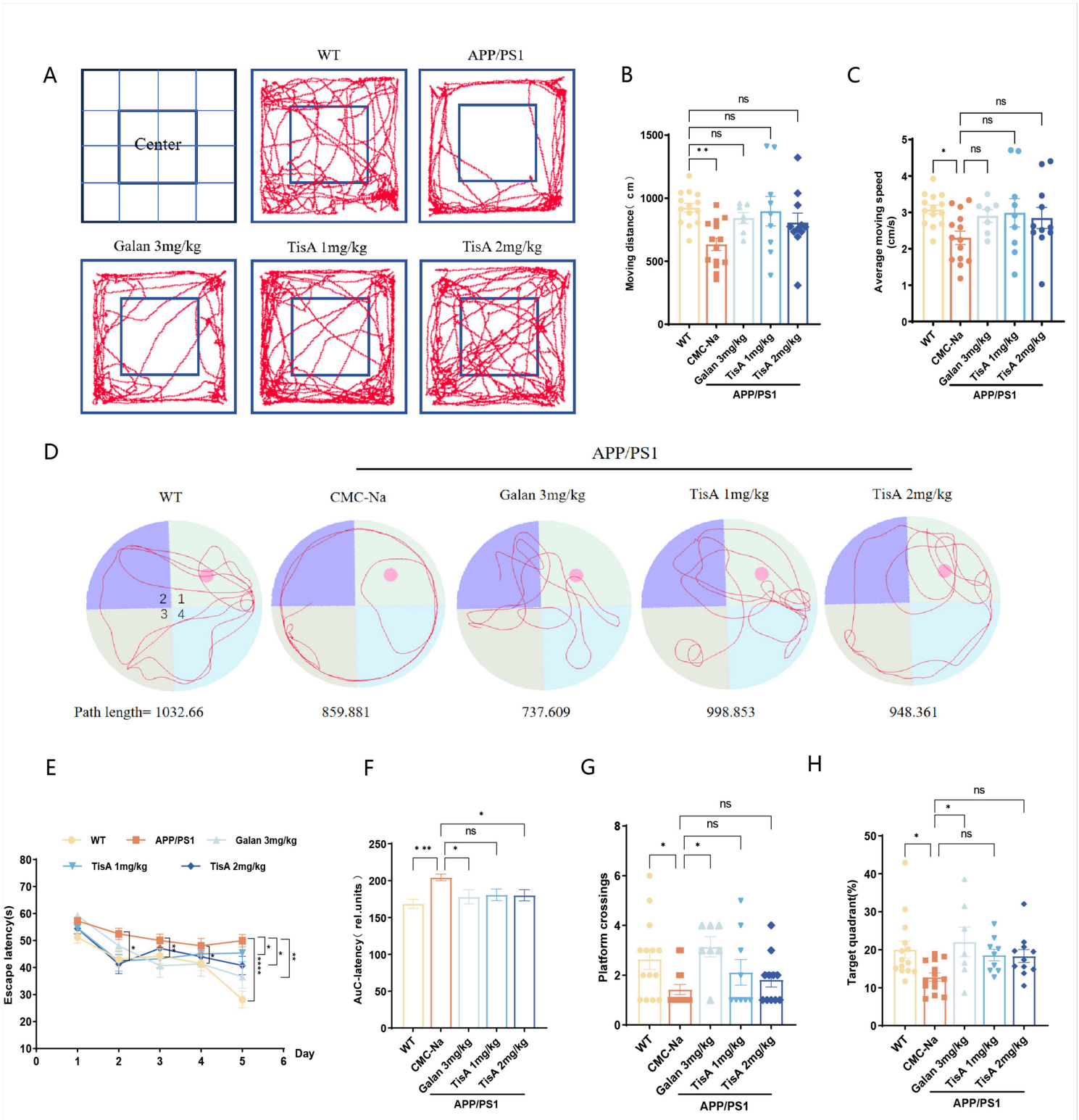

Figure 7

Effects of Tis A on behavioral performance in APP/PS1 mice. (A) Sample locomotion traces of mice in the open field. (B-C) Ambulatory distance and mean velocity quantified in the open field. (D) Typical paths of mice during the water maze training phase. (E) Time taken to identify the concealed platform during training.(F) AUC of escape latency.(G) Quantity of crossings over the prior platform location during probe testing.(H) Percentage of time allocated to the target quadrant.Data are expressed as mean

± SEM. Group sizes: WT and CMC-Na groups (n=14), Galan 3 mg/kg (n=8), Tis A 1 mg/kg (n=9), Tis A 2 mg/kg (n=12). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, ns $p > 0.05$.

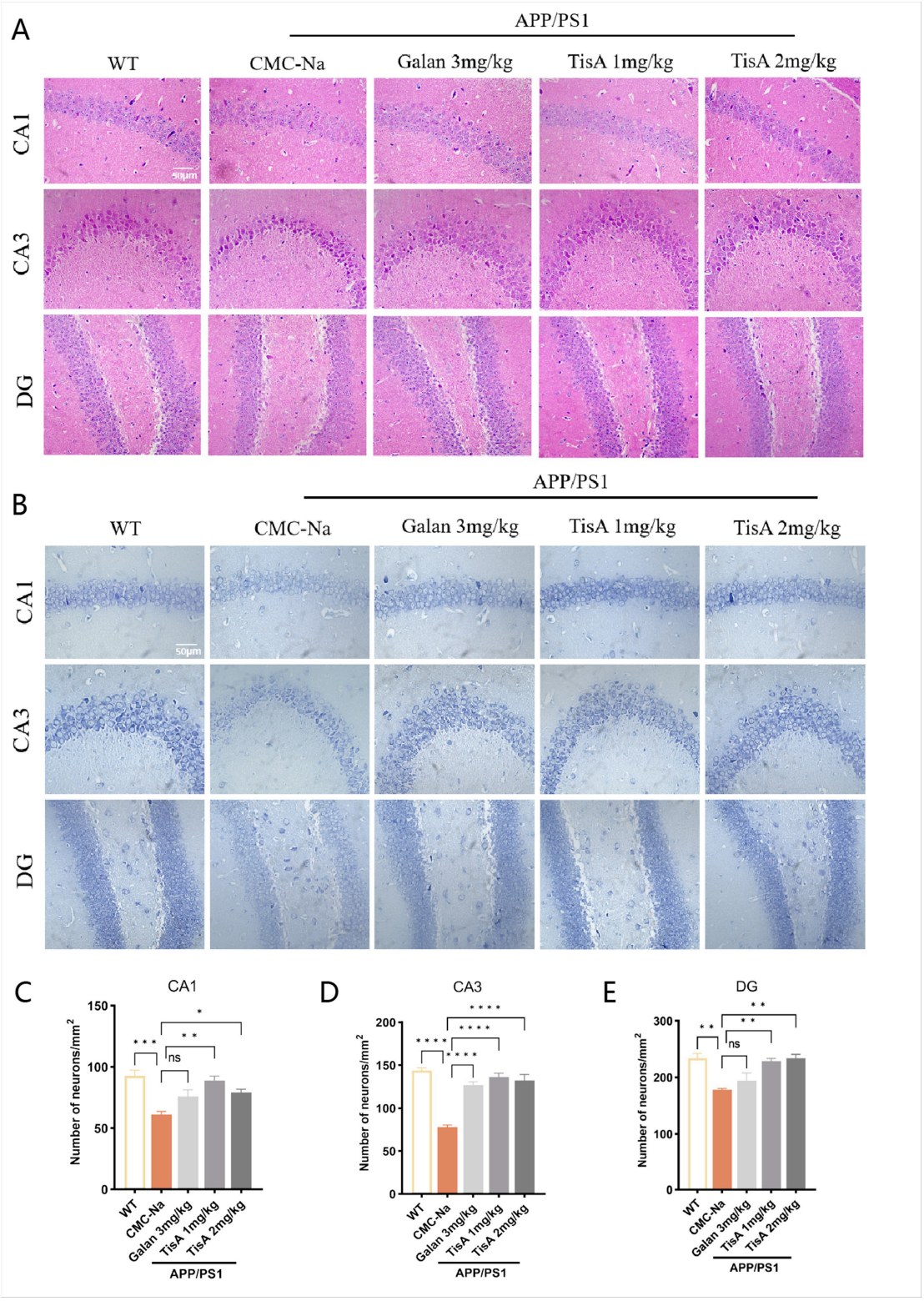

Figure 8

Effects of Tis A on neurohistopathology in APP/PS1 mice. (A) Microphotographs of H&E-stained hippocampal subregions (CA1, CA3, DG) from each experimental group. (B) Parallel sections visualized with Nissl staining. Scale bar = 50 μm. (C-E) Quantitative assessment of neuronal density based on Nissl

staining. n = 3 mice per group with 6 randomly selected fields analyzed. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, ns $p > 0.05$.

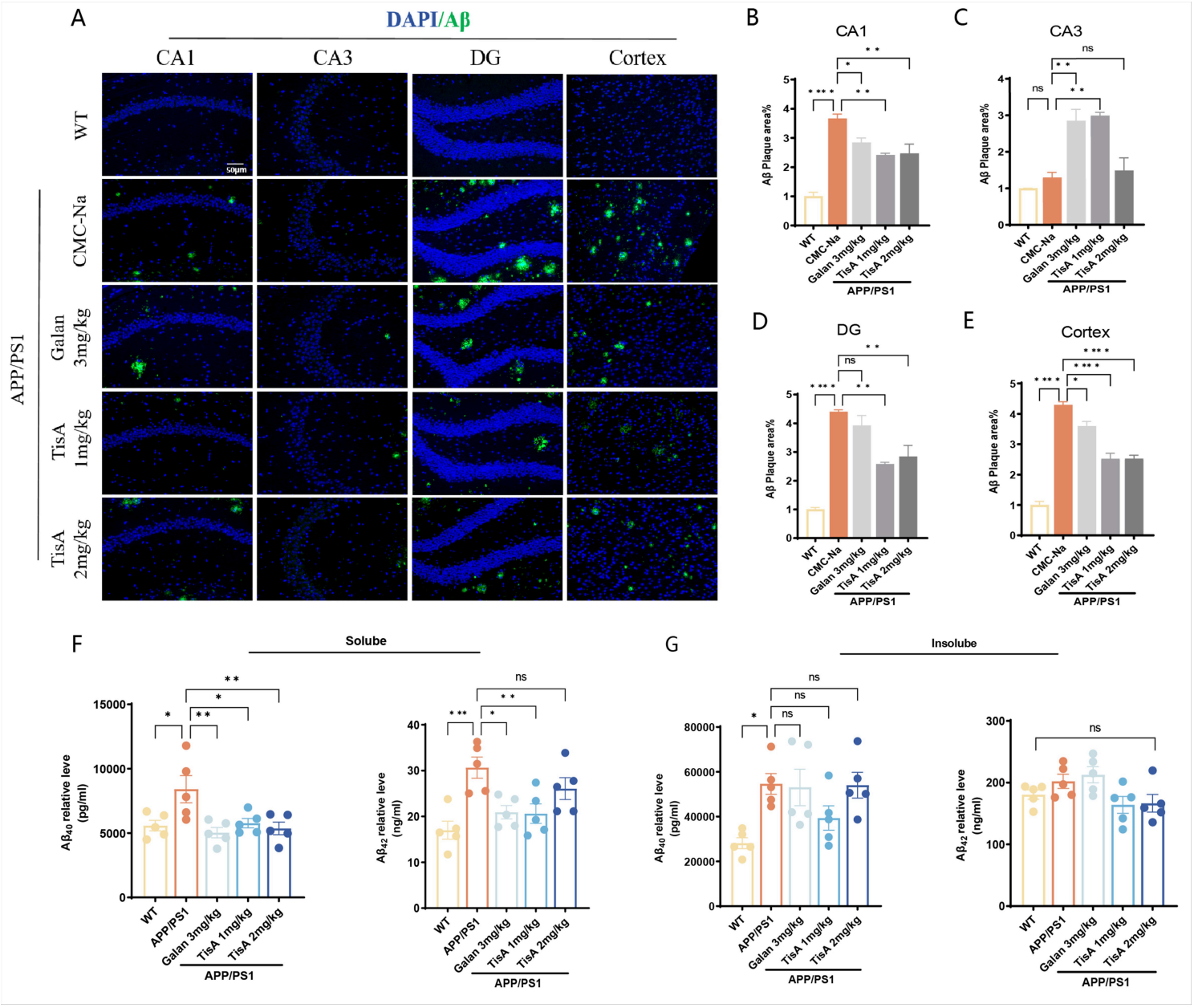

Figure 9

Effects of Tis A on Aβ levels and deposition in APP/PS1 mice. (A) Microscopy pictures of deposits of Aβ (green) in hippocampal subregions (CA1, CA3, DG) and the cortex. Scale bar = 50 μm. (B-E) Quantification of Aβ plaque burden in the corresponding brain regions. Values represent mean±SEM (n=3).(F) Concentrations of PBS-soluble Aβ40 and Aβ42 in brain homogenates, as determined by ELISA. (G) Analysis of PBS-insoluble Aβ40 and Aβ42 conducted by ELISA. Data are expressed as mean ± SEM; n = 5 mice per group. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$, ns $p > 0.05$.

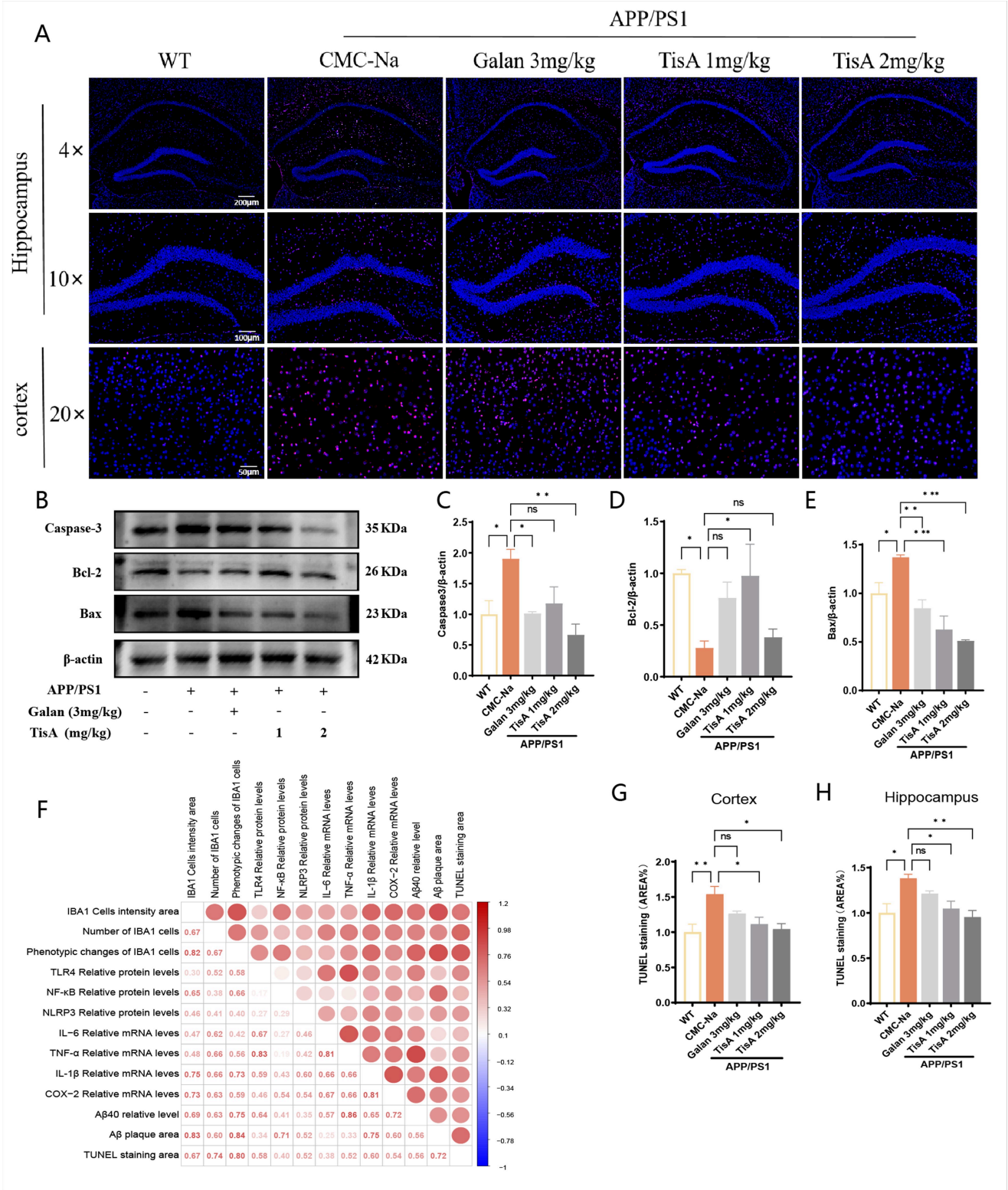

Figure 10

Neuroprotective effects of Tis A in APP/PS1 mice. (A) Assessment of key apoptosis regulators (caspase-3, Bcl-2, Bax) in brain tissue by WB. (B-D) Densitometric quantification of protein levels from (A). (E) Sample micrographs showing TUNEL (red) and DAPI (blue) co-staining in the hippocampal DG and cortex. (F-G) Quantification of apoptotic cells in the cortex (20×) and hippocampus (10×), respectively. Data are expressed as mean ± SEM; n = 3 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001,

ns p > 0.05. (H) (H) Pearson correlation analysis among microglial parameters, TLR4/NF-κB/NLRP3 pathway expression, neuroinflammatory markers, and AD efficacy indicators. The size and color intensity of the circles in the matrix correspond to the magnitude of the correlation coefficient, with warm colors (red) indicating positive associations and cool colors (blue) representing inverse associations.Corresponding R-values are shown on the right, with larger values indicating stronger correlations.

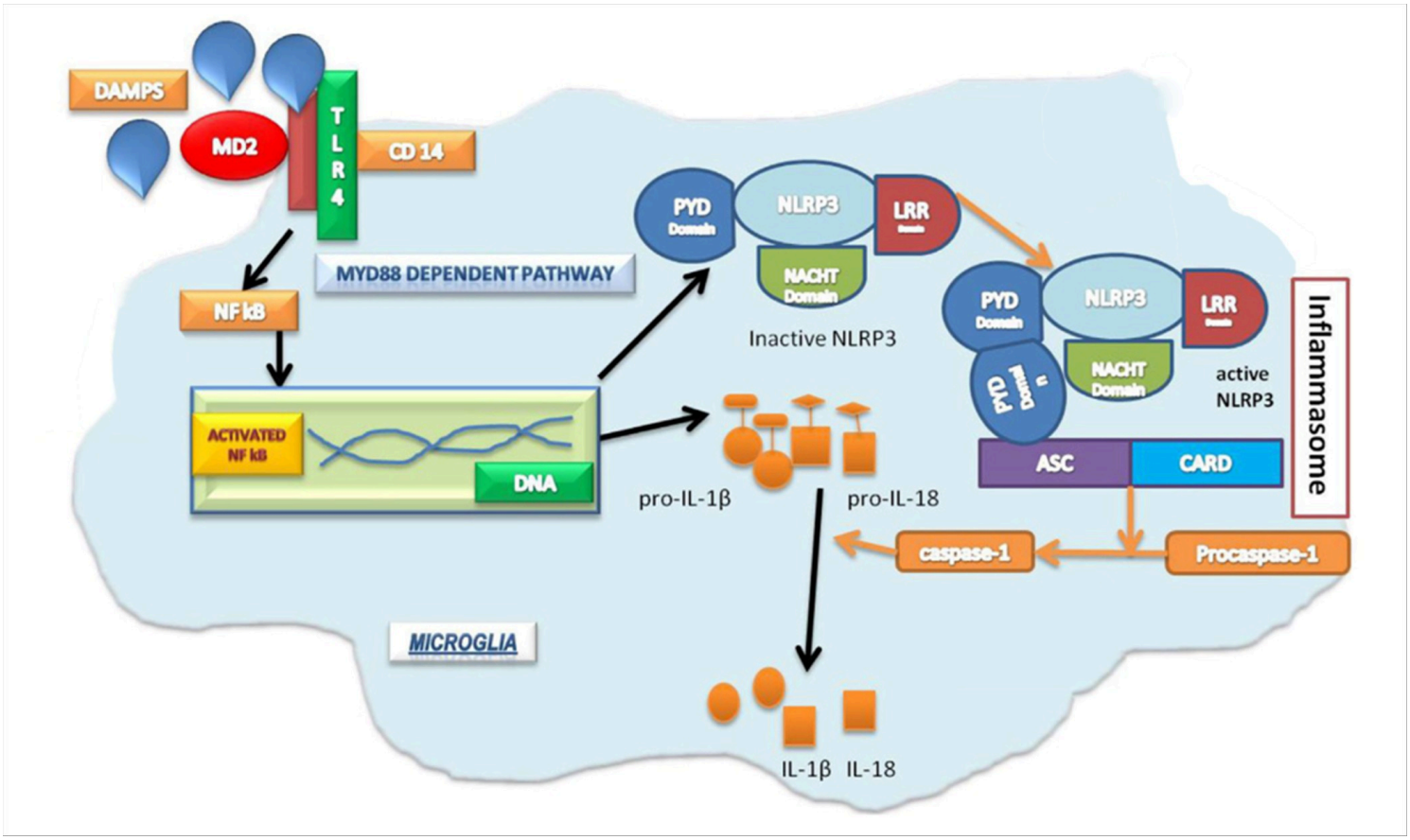

Figure 11

TLR4/NF-κB/NLRP3 signaling in neuroinflammation

Supplementary Files

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- Graphicalabstract.jpg