

Microglial ApoD Induced NLRC4 Inflammasome Activation Promotes Alzheimer's Disease Progression

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease with no effective therapies. It's well-known that chronic neuroinflammation plays a critical role in the onset and progression of AD. Proper neuronal-microglial interactions are essential for brain functions. However, as the main existence of immune cells, determining the role of microglia in Alzheimer's neuroinflammation and the associated molecular basis has been challenging. Herein, the inflammatory factors in the sera of AD patients were detected and the association with microglia activation was analyzed. The mechanism regarding the microglial inflammation was investigated. The IL6 and TNF- α were found to be significantly increased in the AD stage. Further analysis revealed microglia were extensively activated in AD cerebra releasing mounts of cytokines to impair the neural stem cells (NSCs) function. Moreover, ApoD induced NLRP4 inflammasome was activated in microglia, which gave rise to the proinflammatory phenotype. Targeting the microglial ApoD promoted NSCs self-renewal and inhibited neuron apoptosis. These findings demonstrate the critical role of ApoD in microglial inflammasome activation, and for the first time reveal that microglia-induced inflammation suppresses neuronal proliferation. Our studies establish the cellular basis for microglia activation in AD progression, and shed lights on cellular interactions important for AD treatment.

Introduction

Alzheimer's disease (AD) is a progressive disorder causing neurons to degenerate, which is the most common cause of dementia (1). The AD patients present an age-related continuous decline in memory and cognition that disrupts the ability to function independently. As disease progresses, AD patients will develop severe memory impairment without ability to carry out daily works (2). In advanced stages, complications, such as dehydration, malnutrition or infection, result in the deaths. There is no effective treatment to cure AD or protect neurons from constant injuring. Current medications for AD may temporarily improve symptoms or ease down the rate of decline (3). Therefore, a deeper understanding of the biological behaviors and molecular basis of AD is required for generating the therapeutic strategies.

AD has been manifested histologically by the parenchymal deposition of amyloid-beta (A β) plaques and neuroinflammation (4). Recently, many studies have reported immunity derangement contributed to AD onset and disease progression (5). Under such conditions, brain-resident microglia acquire proinflammatory activity, which has been identified to correlate with disease escalation (6). Microglia, the resident myeloid cells in central nervous system (CNS) and a major component of brain immune system, play an essential role in neuronal homeostasis and regulate multiple pathogeneses of disorders, such as neurodegenerative diseases and tumors (7). Recently, numerous studies have reported that brain-resident microglia distinguish from bone marrow derived macrophages (8, 9). Under homeostatic conditions, microglia originate from hematopoietic stem cells in the yolk sac but not from bone marrow (10). In the pathogenesis of AD, microglia are concentrated around amyloid plaques, which affects the function of neurons via releasing a wide range of cytokines (11). Therefore, targeting the microglia has represented a novel therapeutic approach to this difficult to treat disorder.

Recently, inflammasomes have been recognized for their roles in neurodegenerative disorders, neuroinflammation, tumorigenesis and host defense against pathogens invasion (12-14). Inflammasome assemble in response to pathogenesis or tissue damages by the Nod-like receptor protein (NLR) or absent in melanoma 2 (AIM2)-like receptors (ALR) (15). Lots of proinflammatory cytokines such as IL1 β , IL18, IL33 and pyroptosis are induced by inflammasome activation (16). They regulate innate immunity particularly by acting as the platforms for activation of Caspase-1, Caspase-8, Caspase-11 and IL-1R-associated kinases (IRAK). The role of the innate immune system has been identified in the etiology of AD driving neuroinflammation. Previously, the best-studied inflammasome, NLRP3 inflammasome was activated in neurons of AD brain, which produced a set of cytokines to promote inflammatory reactions (17). However, it's poorly understood whether microglia undergo inflammasome activation involving the AD progression. Recent studies indicate that lipid metabolism is linked with inflammasome functions. Krasemann *et al.* identified TREM2-ApoE pathway as a major regulator of microglia phenotype in neurodegenerative diseases and suggested that targeting this pathway could restore homeostatic microglia (18). In addition to ApoE, our study reveals that ApoD induced NLRC4 inflammasome is extensively activated in microglia of AD patient brains, which releases mounts of IL6 and TNF- α impairing the proliferation of neural stem cells (NSCs) and promoting the apoptosis of neurons. Inhibition of ApoD induced inflammasome proves beneficial and protective effects on NSCs self-renewal, thereby opening a new avenue for therapeutic intervention.

Materials And Methods

Patients and samples

The serum specimens were collected from 62 AD patients (81.2 years, age range 57 ~ 93 years) and 6 health controls at the Department of Neurology in the Second Affiliated Hospital of Henan University of Science and Technology between October 2013 and September 2019. The diagnosis of AD was based on the NINCDS-ADRDA research criteria (19). None of patients had received the associated treatment before blood collection. The patient information including the general characters, stages and prognosis was obtained from the medical records or outpatient follow-up records and listed in Table 1. All of brain tissues of AD patients or health controls were harvested from six donors. Subsequently the samples were immediately frozen with liquid nitrogen and stored until further study. The aforementioned procedures were approved by the Research Ethics Committee of The Second Affiliated Hospital of Henan University of Science and Technology. All the methods were carried out in accordance with relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines. All patients provided written informed consent according to the Declaration of Helsinki.

Mice

The C57/BL6 wild type (WT) mice were acquired from Jackson Laboratory. The 3 \times Tg AD mouse models were purchased from Beijing Vitalriver Co., Ltd. (Beijing, China). All animals were maintained in the Laboratory Animal Facility at The Second Affiliated Hospital of Henan University of Science and

Technology. Experiments were performed in accordance with procedures approved by the Animal Care Committee.

Cells

The microglia cell line, BV2, was purchased from the Institute of Basic Medical Sciences Peking Union Medical College. The BV2 cells were authenticated using cellular morphology and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and penicillin/streptomycin (1%) at 37°C in the humidified incubator with 5% CO₂. To obtain the NSCs, murine hippocampal tissues were dissociated into the single cell suspension for further FACS sorting using the CD133 surface marker. The harvested cells were cultured in Neurobasal medium (Gibco) containing B27 (2%), EGF (50 ng/mL), bFGF (20 ng/mL), L-glutamine (1%) and penicillin/streptomycin (1%) (20). To collect the neurons, the cortexes of fetal or adult mice were digested with 0.25% trypsin and resuspended at 1×10^7 /ml in Neurobasal medium supplemented with B27 (2%), L-glutamine (1%) and penicillin/streptomycin (1%) to isolate the neurons (binding part) and glia (suspended part).

Immunohistochemistry

Tissues were dehydrated by utilizing Ethanol alcohol gradient followed by incubation with 100% alcohol three times for 5 minutes. The paraffin-embedded block was sliced into 5 µm sections and washed in the 40 °C water bath for 15 minutes. Sections were fixed in 4% PFA (Invitrogen) at 4°C overnight. The sections were heated at 5% FBS (Gibco) and 75°C for 30 minutes to reduce unspecific background. Briefly, to investigate the expression of Aβ, IBA1 and NLRC4 in brain tissues, the prepared sections were stained with antibodies against Aβ (Ab120851, Abcam, 1:100), IBA1 (Ab5076, Abcam, 1:100), NLRC4 (CST12421, Cell Signaling Tech, 1:100), Caspase-1 (CST24232, Cell Signaling Tech, 1:100) and ApoD (Ab191275, Abcam, 1:200) at 4°C overnight. Then sections were washed with PBS and incubated with the secondary antibodies for 1 hour at room temperature. The scores of stained cells were calculated by counting the average number of positive cells in 500 nuclei in four random light high-power magnifications (400 X). The average IHC score of Aβ, IBA1 and NLRC4 was 3, 4 and 4, respectively. Histopathological diagnoses were independently confirmed by two pathologists based on the WHO criteria (21).

in situ hybridization

DIG-UTP-labeled probes for *APOD* were added to this mixture on the tissue frozen sections, and then hybridization was performed at 60 °C for 48 hours. After hybridization, sections were incubated overnight at 4 °C with the alkaline phosphatase-conjugated antibody against DIG. Bound probe was visualized by incubating sections in NBT/BCIP at 4 °C overnight in the dark.

ELISA

The concentration of IL1β, IL6, IL18 and TNF-α was examined by utilizing the ELISA Kit (Sigma). The 100 µL of cell culture supernatant or sera was added into duplicates and incubated at 37 °C for 90 minutes.

After washing 3 times, indicated antibodies was added and incubated at 37 °C for 60 minutes. After washing three times, 100 µL indicating antibodies from ELISA kits were added and incubated at 37°C for 60 minutes. After color reaction with TMB substrate for 15 minutes, absorbency was detected at 450 nm in microplate reader.

Western blot analysis

Tissues and cells were lysed in splitting buffer (pH = 7.4) containing protease-inhibitor cocktail. Whole proteins were separated in 10% SDS-PAGE and transferred on PVDF membrane. Membranes were subsequently incubated with antibodies against IL1 β , IL6, IL18, TNF- α , ApoD, Caspase-1, NLRC4 and β -actin overnight at 4 °C, and then probed with secondary antibodies for 1 hour at room temperature. All of first antibodies were diluted at 1:500 except for β -actin at 1:3000.

RT-PCR

RNA was isolated using Trizol reagent according to the manufacture's procedures (Invitrogen). cDNA was synthesized by using iScript reverse transcription brand. Quantitative levels of mRNA were determined by using SYBR-green on the Mx3000P QPCR system and normalized for expression of GAPDH mRNA.

Flow cytometry analysis

Flow cytometry (FCM) analysis was performed based on the manufacture instructions. Briefly, cells were diluted to 1×10^5 /mL, dissociated in 1 X binding buffer, subsequently stained with Annexin V-FITC (Biolegend) and propidium iodide (PI) in the dark at room temperature for 20 minutes and then analyzed by utilizing FCM. The apoptosis rate was calculated as the percentage of double Annexin V-FITC⁺/PI⁺ cells in total cells.

Lentivirus production

Lentivirus was prepared by using shRNA of NLRC4, shRNA of ApoD and scrambled shRNA (Sigma), pMD2.G, and psPAX2 (Addgene) plasmids according to the standard protocol. Briefly, 293T packaging cells were transfected with 2M CaCl₂ and incubated for 8 hours before replacing the culture media with 10 mL of DMEM complete medium with 2-5 mM sodium butyrate. The virus was collected at 48 and 72 hours following the transfection.

MTT assay

NSCs (1,000 cells/well) were seeded into 96-well plates for 24 hours (37 °C, 5% CO₂). Cell viability was evaluated by performing the MTT assay. Briefly, 0.5 mg MTT reagent was added to each well for 4 hours of incubation. After removing the supernatant, 100 µL DMSO (J&K) was added to each well and incubated for 10 min. The purple mixture in each well was then measured at 490 nm using the Polarstar Optima microplate reader. The absorbance is tested at 660 nm to measure background signals due to cell debris and excess coupling reagent.

Statistical analysis

Data were presented as the means \pm standard deviation, which were analyzed by Student's *t*-test and Mann-Whitney test using SPSS19.0 and GraphPad Prism8.0. The unpaired *t* test was used for comparison between two groups, and comparison of mean values between multiple groups was evaluated by one-way ANOVA followed by Student-Newman-Keuls post hoc test. The functional experiments were performed at least three times (22). The correlation between *NLRC4* mRNA and *IL6* or *TNFA* mRNA expression was analyzed by Pearson correlation. The Logistic regression was performed to analyze the clinical risk factors. $P < 0.05$ was considered as the significant difference.

Results

Microglia is activated in AD brains resulting in neuroinflammation.

Neuroinflammation has been identified as one important character of AD (23). To observe the inflammation in patients with AD, A total of 6 health controls and 62 cases were included in the current cohort: 28 (45.16%) patients suffered from mild cognitive impairment (MCI) and 34 (54.84%) were in AD stage (Table 1). Sera were collected from these patients to examine the levels of well-functioned inflammatory factors including IL1 β , IL6, IL18 and TNF- α . As shown in Figure 1A, all the cytokines were elevated in both MCI and AD patients, as well as progressively increased in the AD stage. Furthermore, the IL6 and TNF- α were most significantly increased in the AD stage (Figure 1A and Table 1). As evidenced by the Logistic regression analysis, the expression of IL6 and TNF- α , as well as the parameters of age and stage, affected the prognosis suggesting that they could be the valuable prognostic indicators for patients with AD (Table 1). Similarly, the immunoblotting assay indicated the higher levels of IL1 β , IL6, IL18 and TNF- α in AD brain tissues (Figure 1B). Previously, activated microglia have been reported to represent a common pathological character of neuroinflammation (24). Hippocampus is a critical region for memory affected by cognitive decline in old age persons as well as AD patients (25). Accordingly, to detect the activation of microglia in the hippocampus of AD brains, we examined the expression of IBA1 and A β by IHC in human donor samples. A large number of microglia (IBA1⁺) were present across the Alzheimer's hippocampus compared with the normal control, which correlated positively with the expression of A β (Figure 1C). To confirm the inflammation in brain tissues after stimulation, we used the well-verified lipopolysaccharide (LPS) to treat the mice for three days and harvested the cerebra to examine the mRNA levels of *IL6* and *TNFA*. As displayed in Figure 1D, *IL6* and *TNFA* mRNA expression were observed to be upregulated in the LPS-stimulated mice demonstrating LPS could induce the neuroinflammation. To further identify the role of microglia in production of IL6 and TNF- α , BV2 cells were cultured with LPS stimulation for 48 hours and the supernatant was collected to examine the concentration of IL6 and TNF- α . As expected, they were significantly increased in the LPS treatment group (Figure 1E), which suggested that activated microglia could provide the specific inflammatory factors. With the goal of identifying the effects of IL6 and TNF- α on the proliferation of NSCs, NSCs purified from hippocampus were cultured with the treatment of IL6 and TNF- α for 48 hours, respectively. Then MTT assays showed that the self-proliferation was dramatically impaired by the two cytokines (Figure 1F).

Taken together, these data suggest that activated microglia in AD hippocampus attenuate the self-renewal via IL6 and TNF- α .

NLRC4 inflammasome is activated in microglia of AD brains.

Having identified the microglia activation in AD brains, we next sought to detect the molecular mechanism regarding the microglial proinflammatory phenotype. Accumulating previous studies have reported that inflammasome activation contributed to releasing a wide variety of cytokines, such as IL1 β , IL18 and TNF- α in the absence of cell death (15, 26). To detect the specific activation of inflammasome during Alzheimer's progression, RT-PCR analysis was performed in the cerebra tissues of AD patients and health control donors, which indicated that *NLRP3* and *NLRC4* mRNA levels were significantly increased in contrast to the control group (Figure 2A). However, the best-studied inflammasome, NLRP3 inflammasome has been reported to be activated in neurons with α -synuclein pathology and dopaminergic neurodegeneration in mice (17). It's poorly understood whether microglia undergo NLRC4 inflammasome activation to promote neurodegeneration. To address this, microglia were collected from 3 \times Tg AD mice cerebra tissues and the mRNA levels of *NLRP3* and *NLRC4* were examined by RT-PCR test. As shown in Figure 2B, significant difference existed in *NLRC4* mRNA expression. Similar to the RT-PCR analysis, a significant population (31.2%) of microglia expressed NLRC4 in the hippocampus of AD mice (Figure 2C). As shown in Figure 1B, the expression of IL1 β and IL18, the marker of inflammasome activation, were also significantly increased in AD brain tissues. In order to link NLRC4 inflammasome activation with the proinflammatory phenotype, we found a positive correlation between *NLRC4* and *IL6/TNFA* mRNA expression by further analyzing RT-PCR data (Figure 2D). As shown in Figure 1E, microglia produced mounts of IL6 and TNF- α after LPS stimulation. To explore the role of NLRC4 in interleukin production, we generated BV2 cells by a lentiviral approach (shRNA) to reduce the expression of NLRC4. After NLRC4-deficient cells were treated with LPS for 72 hours, immunoblotting assay was performed showing the decreased levels of NLRC4, Caspase-1, IL1 β and IL18, which indicated the inactivation of microglial NLRC4 inflammasome (Figure 2E). Furthermore, the *IL6* and *TNFA* mRNA expression were observed to be downregulated demonstrating inflammation phenotype was attributed to NLRC4 inflammasome activation (Figure 2F). Collectively, these data highly suggest that microglial NLRC4 inflammasome activation contributes to the proinflammatory features.

ApoD is required for microglial NLRC4 inflammasome activation.

Previously, ApoD has been identified to be downregulated in microglia during development. Upon the neurodegeneration or neuroinflammation, ApoD as a key molecule broadly activates diverse pathways (27, 28). The expression of microglial ApoD prompted us to ask whether it could induce NLRC4 activation. The ISH staining of *APOD* was diffusely observed in cerebra of AD patients, supporting the evidence linking ApoD with Alzheimer's pathogenesis (Figure 3A). Moreover, the strong correlation between *APOD* and *NLRC4* expression was determined based on the RT-PCR and immunoblotting analysis of human AD brain tissues (Figure 3B and 3C). To further detect the role of ApoD in NLRC4 inflammasome activation, BV2 cells were infected with shApoD or scrambled shRNA to delete the

expression (Figure 3D). When treating the ApoD-deficient BV2 cells with LPS, RT-PCR assay showed the suppression of *NLRC4*, *IL6* and *TNFA* compared with the controls (Figure 3E). Thus, the above data indicate that ApoD confers proinflammatory signatures to microglia through NLRC4 inflammasome.

Targeting the ApoD improves the neuronal function.

The above data have figured out that activated microglia contributed to AD progression. Finally, we investigated whether neuronal injury could be inhibited by targeting ApoD induced NLRC4 inflammasome. Receptor-associated protein (RAP) treatment (20 $\mu\text{g}/\mu\text{L}$) for 48 hours was utilized to block the ApoD in BV2 cell cultures. Following the LPS (1 $\mu\text{g}/\text{mL}$) stimulation, the *NLRC4*, *IL6* and *TNFA* mRNA expression was dramatically suppressed by blockage of ApoD (Figure 4A). Then harvesting the supernatant of LPS-stimulated BV2 cell cultures to mix it with NSCs cultures at 1 : 1, MTT assay displayed that NSCs was powerfully suppressed with 70% expansion inhibition without RAP treatment compared with the RAP treatment group (Figure 4B). Furthermore, we co-cultured the NSCs with LPS-stimulated BV2 cells or primary microglia collected from P4 mice with or without RAP treatment (20 $\mu\text{g}/\mu\text{L}$) for 48 hours. Ki-67 index was examined by immunostaining assay showing the decreased level in the control group (Figure 4C and 4D). Most NSCs were actively proliferating (Ki-67⁺) after RAP treatment (Figure 4C and 4D). These data suggest that inhibiting the neuroinflammation provides beneficial effects on NSCs self-proliferation by targeting the ApoD. In addition, when neurons cultured in the media with half of supernatant of LPS-stimulated BV2 cells or microglia from AD mouse models for 48 hours, FCM analysis showed that RAP treatment (20 $\mu\text{g}/\mu\text{L}$) dramatically inhibited the apoptosis of neurons in contrast to the control (Figure 4E and 4F). In sum, these data demonstrate that activated microglia construct a NLRC4 inflammasome-associated cytokine microenvironment promoting AD progression.

Discussion

AD has been identified as the most common cause of dementia, which is a general condition for memory impairment and other cognitive dysfunction serious enough to affect the daily life (3). The patients with AD account for 65 ~ 80% of dementia cases. The well-known risk factor is the increasing age, and a majority of patients with AD are 65 years old and even older. However, Alzheimer's isn't either a normal part of aging nor a disease of old age (29). AD has emerged as the sixth leading cause of death in the United States (30). Approximately 200,000 Americans under the 65 years old have suffered from the early-onset Alzheimer's (31). Then, this detrimental disorder worsens over time. AD has been recognized as a progressive disease, where dementia symptoms gradually worsen over several years. In the early stage, MCI stage, the patients present mild memory loss. Nevertheless, with the AD stage, patients would significantly lose the ability to carry on a conversation and respond to their environments (2, 32). On average, patient with AD live 4 ~ 8.5 years after first diagnosis, but they can also live as long as 20 years depending on other factors. Consistent with these previous findings, our current study reported that both age and risk stages mainly affected the prognosis of AD patients. However, in China, there is still a shortage of big data or thorough databases to comprehensively analyze the Chinese patient features, which promotes us to develop the associated system for improving the diagnosis and treatment.

Nowadays, AD has been located at the forefront of biological medical research. Scientists have discovered lots of aspects of AD and other dementias as possible. Some of most remarkable advances have shed light on how Alzheimer's pathogenesis affects brain function. Numerous potential approaches are currently under investigation around the world with the hope of better understanding the molecular basis. Potentially, there are two abnormal structures named plaques and tangles damaging the neurons (33). The plaques are deposits of a protein fragment building up in the spaces between neurons and other stromal cells. Tangles are twisted fibers of another protein mainly assembling inside neurons (34). These two structures will lead to chronic inflammation, which is the serious reason for AD progression. In our study, with the goal of studying the proinflammatory phenotype of microglia, we used LPS to stimulate the cells and mice. Additionally, microglia activation can be also induced by a 40-42 amino acids peptide (β -amyloid peptide, A β), arising from the sequential proteolytic processing of the Amyloid Precursor Protein (APP) by beta- and gamma-secretases, especially in AD models (35). Although the studies regarding Alzheimer's neuroinflammation show that many cases develop some plaques and tangles as they age, those with AD tend to develop far more and in a predictable pattern, beginning in the areas important for memory before spreading to other neural functional areas (25, 34). Even researchers don't have the exact knowledge about the role of neuroinflammation in AD, they believe that it will block the communications among nerve cells and disrupt the processes of proliferation and survive.

Microglia represent an intrinsic and dynamic immune cell population in CNS, which commonly secret cytokines and phagocytose the pathogenic substances. Microglia activation is the principal component as a double entendre that executes both detrimental and beneficial influences on disorders (36, 37). Proper neuronal-microglial interactions are essential for brain functions. By affecting the stroma components, microglia are also involved in brain tumor proliferation and migration (38). Related to this, identification of microglial features will provide a systematic knowledge of modulation in treatment of CNS diseases. Activated microglia have represent a common pathological feature of AD. Cumulative evidence has suggested that microglial inflammation in AD brain tissues is increased while microglial-mediated clearance mechanisms are compromised (24). Recently, Amit I group described a novel microglia type associated with the neurodegenerative diseases by using the single-cell transcripts (39). In the current study, we demonstrated that microglia were activated in AD brain tissues. The specifically activated microglia presented the proinflammatory phenotype secreting NLRC4 inflammasome induced IL6 and TNF- α to promote the Alzheimer's progression. These findings highlight the essential roles of microglia in neuroinflammation, and further offer more opportunities for therapeutic strategy.

In the current study, to investigate the mechanism regarding microglial proinflammatory phenotype, we found the activation of NLRC4 inflammasome by analyzing the mRNA and protein expression. To further detect the molecular basis of inflammasome activation, we manipulated the microglia by shRNA technology determining the critical role of ApoD in triggering inflammasome-mediated cytokine production. An inflammatory microenvironment is considered as a hallmark of AD (40). Since our findings indicate the important effects of ApoD on controlling the microglial inflammasome activation during AD progression, it implies a general orderliness managing the inflammatory reactions in other pathogenic conditions. However, it is still under debate whether inflammasome activation can benefit or

impair the brain functions. NSCs in hippocampus proliferate extensively supported by other special nervous populations, such as microglia, astrocytes and even mature neurons (41). Nonetheless, molecular basis underlying the interactions and the cell activation are not well defined. Recently, an explosion of studies gives us insights into the involvement of newly discovered non-pathological "functional" signalosome complexes in microenvironmental inflammation, such as inflammasome, necrosome and innateosome (42, 43). In conclusion, our studies indicate the crucial role of ApoD in microglial inflammasome activation, and demonstrate that microglia-induced inflammation promotes the AD progression. The cellular basis for microglia activation in AD progression is established well, which sheds lights on cellular interactions important for AD treatment.

Declarations

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Authors' contribution statement

Drs. Lv JZ and Yu YL contributed to the whole conception and design of this project. Drs. Yu YL, Ma D and Zhang YH were responsible for the details of experimental performance. Drs. Han Y, Wang ZT and Lv JZ made much efforts to the pathological estimates. Drs. Yu YL, Xu PL and Wang SL contributed to the analysis and interpretation of data. Drs. Yu YL and Wang ZT analyzed the clinical data. Drs. Yu YL and Lv JZ completed the manuscript, figures and tables.

Declaration of Interests

The authors declare no competing interests.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

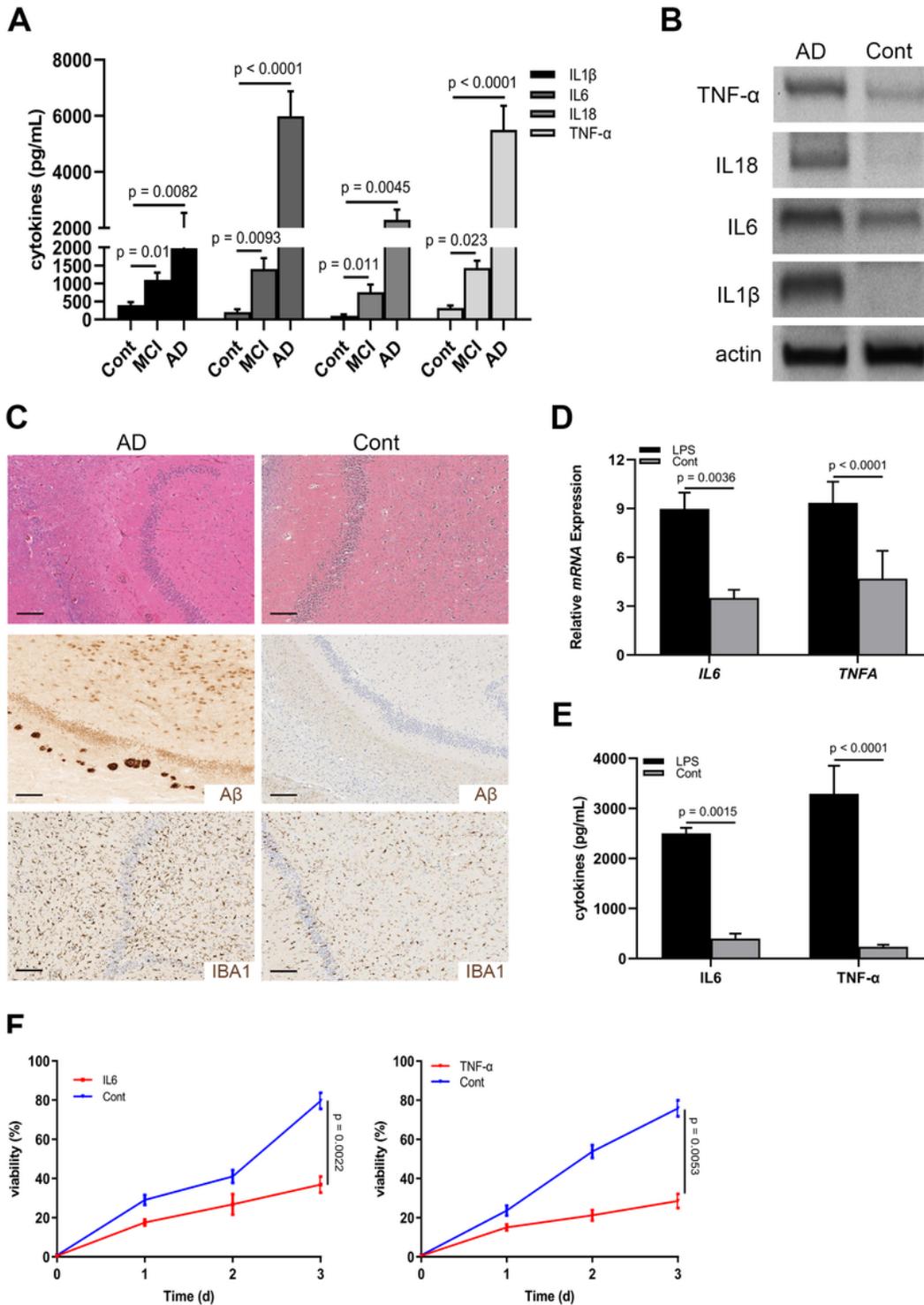


Figure 1

Figure 1

Microglia is activated in AD brains. **A** The expression of IL1β, IL6, IL18 and TNF-α in the sera from health control (n = 6), MCI (n = 28) and AD (n = 34) patients examined by ELISA. **B** The expression of IL1β, IL6, IL18 and TNF-α in human health brains and AD brains tested by western blot. The β-actin protein was chosen as the internal control. **C** Representative images of IHC staining of Aβ and IBA1 in hippocampus of AD patients (n = 3) and health controls (n = 3, bar, 10 μm). **D** The mRNA expression of IL6 and TNFA

in the cerebra tissues from LPS-treated mice (n = 6) and controls. E The expression of IL6 and TNF- α in supernatant of BV2 cells treated with LPS (n = 3). F MTT assay showing the proliferation of NSCs treated with IL6 and TNF- α (n = 6). All the experiments were performed at least three times.

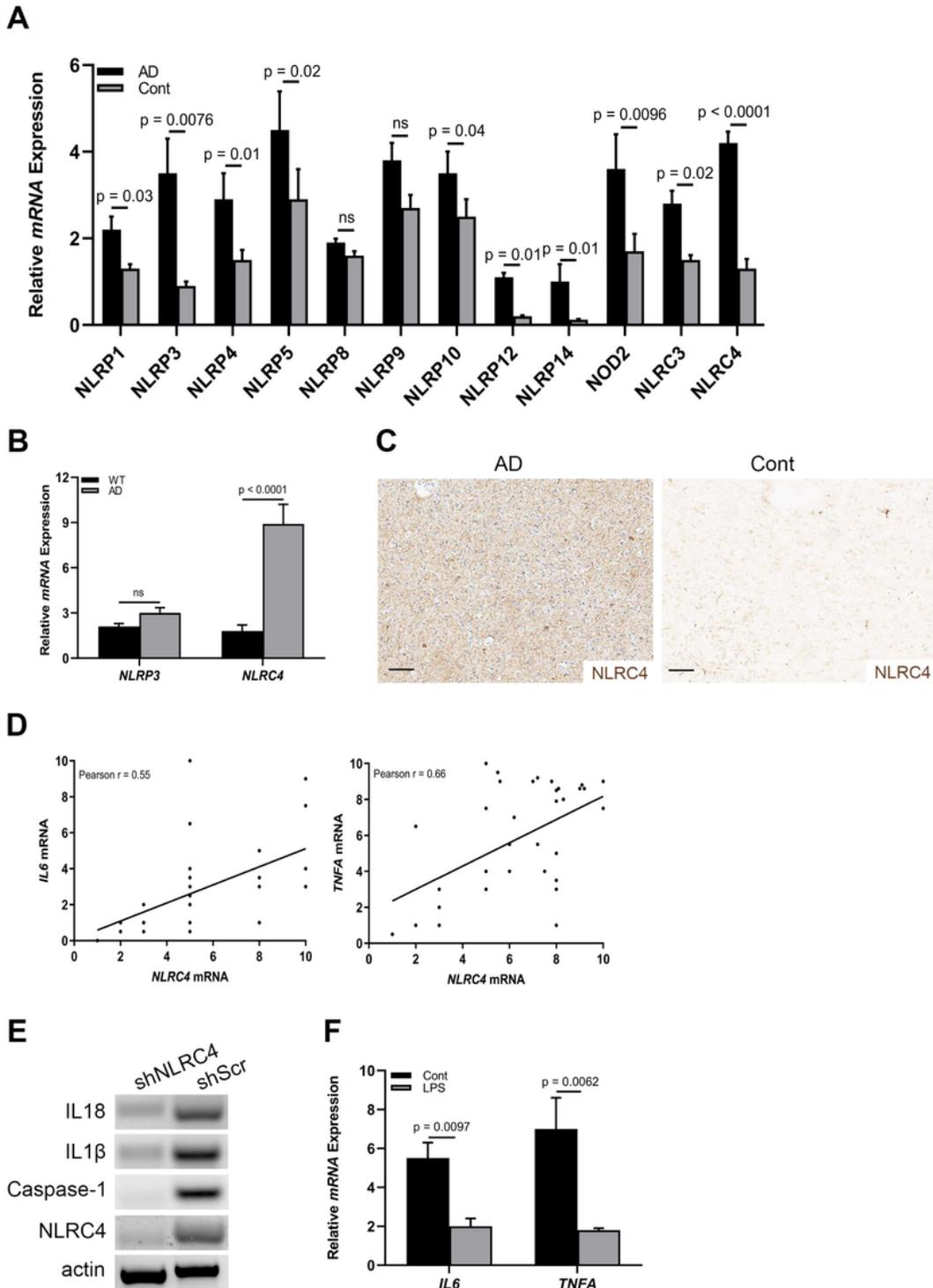


Figure 2

Figure 2

NLRC4 inflammasome is activated in patients with AD. A. RT-PCR analysis showing the inflammasome associated genes expression (NLRPs and NLRCs) in human health cerebra (n = 3) and AD cerebra (n = 3)

tissues. B. RT-PCR analysis showing NLRP3 and NLRC4 mRNA expression in microglia of AD and WT mice (n = 6) C. IHC staining of NLRC4 in health control cerebra (n = 3) and AD cerebra (n = 3, bar, 10 μ m). D. Pearson analysis showing the correlation between NLPC4 mRNA and IL6 or TNFA mRNA. E. Western blotting analysis showing the expression of NLRC4, Caspase-1, IL1 β and IL18 in BV2 cells. F. RT-PCR analysis showing IL6 and TNFA mRNA expression in BV2 cells infected with shNLRC4 after LPS stimulation (n = 6). All the experiments were performed at least three times.

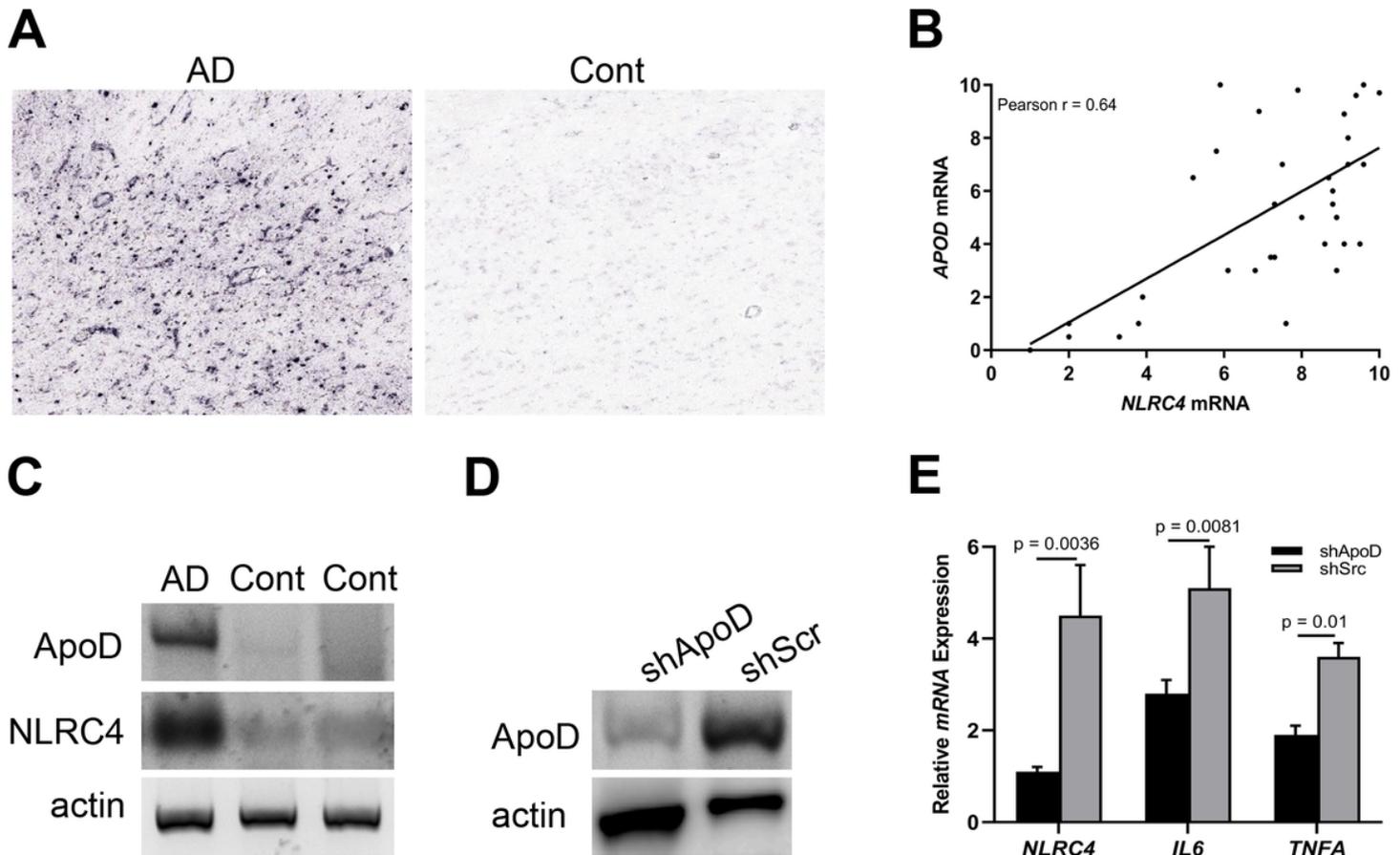


Figure 3

Figure 3

ApoD is required for microglial NLRC4 inflammasome activation. A. ISH staining of ApoD in health control cerebra (n = 3) and AD cerebra (n = 3, bar, 10 μ m). B. Pearson analysis showing the correlation between APOD and NLRC4 mRNA. C. Immunoblotting assay showing the expression of ApoD and NLRC4 expression in brain tissues of AD patients and controls. The β -actin protein was chosen as the internal control. D. Immunoblotting assay showing the expression of ApoD in the BV2 cells infected with shApoD and shSrc. E. RT-PCR analysis showing NLRC4, IL6 and TNF1 mRNA expression in BV2 cells infected with shApoD after LPS stimulation (n = 6). All the experiments were performed at least three times.

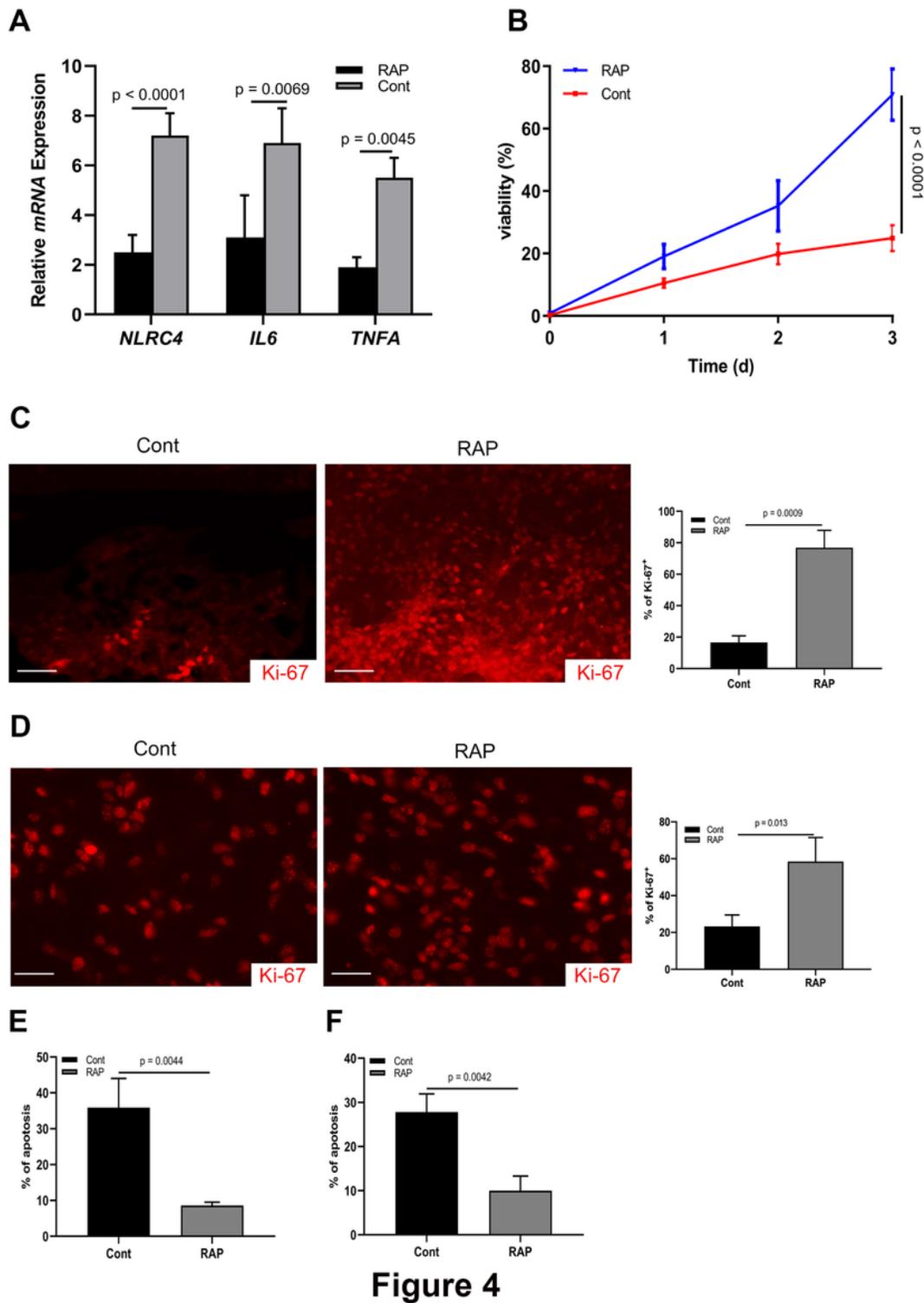


Figure 4

Figure 4

Blockage of ApoD promotes the NSCs self-proliferation. A. RT-PCR analysis showing NLPC4, IL6 and TNFA mRNA expression in BV2 cells treated with RAP or PBS after LPS stimulation (n = 3). B. MTT assay showing the viability of NSCs cultured in the supernatant of LPS-stimulated BV2 cells with or without RAP treatment (n = 3). C. Immunostaining for Ki-67 in the NSCs co-cultured with activated BV2 cells with RAP or PBS treatment (n = 3). D. Immunostaining for Ki-67 in the NSCs co-cultured with activated primary

microglia with RAP or PBS treatment (n = 3). E. FCM analysis showing the apoptosis of neurons cultured in the supernatant of LPS-stimulated BV2 cells with or without RAP treatment (n = 3). F. FCM analysis showing the apoptosis of neurons cultured in the supernatant of microglia of murine AD model with or without RAP treatment (n = 3). All the experiments were performed at least three times.

Supplementary Files

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