NLRC3 delays the progression of AD in APP/PS1 mice via inhibiting PI3K activation

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Abstract

Background/Aims: NLRC3 inhibits inflammatory responses. Epidemiological studies indicate that neuroinflammation induces and accelerates the onset of Alzheimer’s disease (AD). This study was designed to determine whether NLRC3 plays a role in neuroinflammation, Aβ accumulation and neuroprotection in AD mice.

Methods: Thirty 12-month-old APP/PS1 transgenic mice were randomized into three groups as model group, APP/PS1 +LVCON307 and APP/PS1 +LV-NLRC3 group. Ten 12-month-old wild-type C57 mice were chosen as control group. Mice in APP/PS1 +LVCON307 and APP/PS1 +LV-NLRC3 group were injected with LVCON307 or LV-NLRC3 through intracerebroventricular injection. Six months after LVCON307 or LV-NLRC3 injection, We carried out Morris water maze test on mice and harvested their brain tissues after the behavioral experiment. The deposition of amyloid protein and the changes of Nissle bodies were observed by ThS and Nissle staining. The expressions of NLRC3, 6E10, GFAP, Iba1, NeuN and PI3K were detected by immunohistochemistry or immunofluorescence. Western blot was used to analyze the expression of NLRC3, PI3K, GFAP and Iba1.

Results: The expression of NLRC3 is down-regulated in brain tissues of APP/PS1 mice. Mice in APP/PS1 group had a significant attenuation of learning and memory ability compared to the control group, the ability of learning and memory was improved in APP/PS1 +LV-NLRC3 mice. The expression of 6E10, GFAP, Iba1 and PI3K in brain and hippocampus slice of APP/PS1 and APP/PS1 + LVCON307 mice were significantly higher than those of the control group, while the expression of NLRC3 and NeuN was significantly lower than that of the control group. After overexpression of NLRC3, the expression of 6e10, GFAP, Iba1 and PI3K in APP/PS1 +
LV-NLRC3 group was significantly lower than that in APP/PS1 and APP/PS1 + LVCON307 group, while the expression of NLRC3 and NeuN was significantly higher than that in APP/PS1 and APP/PS1 + LVCON307 group. NLRC3 co-localized with NeuN. PI3K activation with 740YP increased the expression of GFAP and Iba-1 in hippocampus with exogenous NLRC3 protein.

Conclusion: NLRC3 may play an important role in the development and progression of AD. Down-regulation of NLRC3 can lead to the activation of PI3K, resulting in abnormal plaque deposition, glial cell activation and neuron loss during AD. NLRC3 delays the progression of AD in APP/PS1 mice via inhibiting PI3K activation.

Keywords: NLRC3 • inflammation • Aβ • neuron • PI3K • Alzheimer's disease

Introduction

Alzheimer's disease (AD), a deterioration of memory and cognitive functions that leads to death at last, is a progressive neurodegenerative disorder and the most common cause of age-related dementia. As the World Alzheimer Report (Prince, The Global Impact of Dementia: an Analysis of Prevalence, Incidence, Cost and Trends, 2015), there are more than 46.8 million people worldwide suffer from AD and the incidence is still increasing, this presents an enormous threat especially to the physical and mental health of the elderly population\(^1\).

The main neuropathological features of AD are formation of senile plaques comosited by amyloid beta (Aβ) peptide fibrils and dystrophic neuritis, neurofibrillary tangles (NFTs) formed of abnormal phosphorylated tau protein, neuronal loss and cerebrovascular amyloidosis. Currently, there is no particularly effective treatment and the pathogenesis of AD still remain unclear\(^2\).

Neuroinflammation mediated by gliocyte activation and neuron loss are identified as
two major hallmarks commonly seen in AD\textsuperscript{3–5}.

Microglia are resident innate immune cells which play important roles in regulating neurotoxicity caused by inflammatory response in the central nervous system (CNS). In adult human or mice, the density of microglia varies in different brain regions, and their concentration in hippocampus is the highest. Lipopolysaccharide (LPS) or A\textsubscript{β} can activate microglia, lead to over-production of inflammatory cytokines such as IL-6, IL-1\textbeta, reactive oxygen species (ROS) and so on. Overexpression of various inflammatory cytokines contributes to nerve injury, which leads to the occurrence and development of AD\textsuperscript{6}. Therefore, anti-neuroinflammatory via inhibition of microglial overactivation is regarded as a new promising strategy for preventing AD\textsuperscript{7,8}. Astrocyte is the important gliocyte, having supportive and protective functions to neurons in the brain. Astrocytes dynamically regulate the signal pathway and transmission in the brain, regulate the plasticity of neurons and synapses, and provide nutrition and metabolism support for neurons. In addition, astrocytes respond to the activity of neurons by regulating local blood flow. Local or chronic inflammation of the brain is the characteristic of AD, which is characterized by activated microglia and astrocytes around amyloid plaques, and neurofibrillary tangles\textsuperscript{9,10}.

There are two mechanisms related to the pathological process of AD: A\textsubscript{β} aggregation and activation of glial cells\textsuperscript{11,12}. However, the causal relationship between the two needs to be further confirmed. Studies have shown that the increase of astrocyte and microglia production in human brain has a linear relationship with the progress of disease. In patients with mild cognitive impairment and mild AD, the production of glial cells is considered to be an early phenomenon
of AD development\textsuperscript{11, 13–15}.

Nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs) which belong to a large family of cytoplasmic sensors, are pattern recognition receptors associated with immunity and inflammation in response to pathogen and damage associated molecular patterns. Dysregulation of their functional activities are found in several diseases including cancers, metabolic diseases, autoimmune disorders and inflammatory diseases\textsuperscript{16, 17}. NLRC3 (also known as CLR16.2 or NOD3), a newly discovered and incompletely characterized member of the NLR family, has a typical nucleotide binding domain-leucine-rich repeat configuration at its central and C-terminal domains and a caspase activation and recruitment domain (CARD) 3 at the amino terminal\textsuperscript{18}. Previous studies have reported that NLRC3 negatively regulates NLR-mediated inflammatory responses by competing to bind inflammasome components and inducing a reduction in caspase-1 activation and IL-1\textbeta{} release\textsuperscript{19}. Recently, it has been demonstrated that NLRC3 inhibition may trigger pro-inflammatory cytokine production by activating phosphoinositide 3-kinase (PI3K) and its downstream transcription factors\textsuperscript{20, 21}. Several studies have shown that sustained and disturbed activation of neuronal PI3K/AKT signaling is crucial for AD\textsuperscript{22, 23}.

However, the physiological role of NLRC3 in AD has remained largely unknown. Both in vitro and in vivo studies, little information is available on the neuroinflammatory and neuroprotective effects of NLRC3. Numerous in vivo clinical imaging and neuropathology studies suggest that gliocyte form the basis of the brains immune system and activated gliocyte play prominent roles in the pathogenesis of AD\textsuperscript{24}. Using a zebrafish model, Wang et al. first demonstrated that NLRC3 is important in
the development of microglia. In nlrc3-like−/− zebrafish, primitive macrophages initiate a systemic inflammation with increased proinflammatory cytokines and actively aggregated, highlighting an undefined potential function of NLRC3 in the development of AD.

In this project, we constructed lentiviral vectors containing NLRC3 through intracerebroventricular injection in 12-month-old APPswe/PS1-dE9 (amyloid precursor protein/presenilin protein 1 (APP/PS1)) double-transgenic model mice which represents mid-term of AD when animals develop Aβ formation, show pathological changes in brain tissue and structure, and exhibit learning and memory function obstacles. We wonder whether NLRC3 play a neuroprotective role through inhibiting the Aβ-induced gliocyte activation and release of inflammatory mediators via PI3K signal. Therefore, the aim of this study is to explore the neuroprotective effect of NLRC3 in Aβ-induced activated gliocyte and the early therapeutic effect of NLRC3 on AD mice.

MATERIALS AND METHODS

Animals and ethics statement

APP/PS1 double transgenic mice were bought from the Model Animal Research Center of Nanjing University [Animal license number: SCXK (su) 2012-0007, stock number: 0014406]. All time points referred to herein indicate the number of days after transferation of APP/PS1. The new transgenic AD APP/PS1 mouse model was obtained by breeding APP/PS1 AD mice, and the genotypes were confirmed by PCR of mouse tail genomic DNA using the 2*EasyTaq PCR SuperMix (Trans Technology, Lot:), according to the manufacturer’s instructions. All the experimental procedures were approved by the Animal Laboratory Administrative Center and the Institutional
Ethics Committee at Xiangya Medical University and also in accordance with the National Institutes of Health guidelines. Specific primers for APP gene contained within the Sangon Biotech (Shanghai) designed (forward Neo5’; 5’-GACTGACCACATCGACCAGTTCTG-3’ and reverse Neo3’; 5’-CTTGTAAGTTGGATTCTCATATCG-3’). Also, specific primers for PS1 gene contained within the Sangon biotech (Shanghai) designed (forward Neo5’; 5’-AATAGAGAACCGCGGAGCA-3’ and reverse Neo3’; 5’-GTGGATAACCCCTCCCCAGCTAGACC – 3’). After PCR reactions, the amplified products were separated in agarose gels and analyzed. C57/BL6 as wild type were purchased from the Experimental Animal Center of Jingda [Animal license number: SCXK (xiang) 2013–0004]. There were 4 groups including C57/BL6 as wild type (control) group, APP/PS1 (model) group, APP/PS1 + LVCON307 group and APP/PS1 + LV-NLRC3 group. Thirty 12-month-old APP/PS1 transgenic mice were randomized into three groups as model group, APP/PS1 + LVCON307 and APP/PS1 + LV-NLRC3 group. Ten 12-month-old wild-type C57 mice were chosen as control group. Mice in APP/PS1 + LVCON307 and APP/PS1 + LV-NLRC3 group were injected with LVCON307 or LV-NLRC3 through intracerebroventricular injection (4 µl, 10⁷ virus particles (VPs) /kg). Mice in control group and model group were administrated with the same volume of normal saline. All the animals were housed in barrier facilities (Experimental Animal Center, Xiangya Medical School of Central South University, temperature 22 °C, under a 12:12 h light-dark cycle light 7:00–19:00; humidity 40–60%), and food and water were freely available.

Lentiviral vectors (LV)

In this experiment, the construction and titer determination of LV-NLRC3 and negative control lentivirus (LVCON307) were constructed and purified by GeneChem
Biomedical Co. Ltd (Shanghai, China). The titers were $5 \times 10^8$ and $6 \times 10^8$ transducing units (TU)/ml.

Intracerebroventricular injections

Mice were anesthetized by intraperitoneal injection of ketamine and intracerebroventricular injection with microinjector. The Bregma point was fully exposed by cutting an incision about 0.5 cm long along the median line of the head at the beginning of the operation. At 1.5 mm posterior to the point and 1 mm lateral to the right, the microinjector was used to penetrate the skull vertically at a depth of 3 mm and retained for 5–10 minutes. Then slowly inject LV-NLRC3 or LVCON307 at the speed of 1 ul/min, and then retain the needle for 5–10 minutes. Drew the needles and sutured skin. After 6 months, the mice were decapitated and executed. The brains were quickly removed on ice and sagittally cut along the median line.

Morris water maze and Behavior test

Morris water maze was used to evaluate spatial learning and memory (SLM) function of mice at age 18 month in each group, according to the protocol of van Praag et al. (1999) and Akers et al. (2014). Mice were housed and habituated for 2 h in behavioral testing rooms before tests. All tests were conducted on consecutive days in a dimly illuminated room with standard conditions of temperature and free from any stray noise. All behavioral apparatus were cleaned with 75% ethanol and dried between each animal. Behavioral outcome was recorded by 2 trained assistants who were blinded to the information of each mouse. The apparatus consisted of a circular tub (120 cm in diameter, 50 cm in height) with a black inner wall and of transparent platform (10 cm diameter) submerged 1 cm below the water surface, which was painted with distinct geometric cues. The water maintained at the range of $(24 \pm 1 \degree C)$. Forty mice underwent four trials per day in the learning stage. Each
mouse was placed into the water and randomly started from each of four different locations facing the pool wall in the trial. The trial was terminated and the latency was recorded when the mouse found the platform within 90 s. Otherwise, the trial was terminated and the mouse was gently guided to the platform. On day 5, a probe trial was conducted to estimate the memory function of each mouse. After the platform removed, mouse was put into the pool as before to evaluate its memory ability. The swim paths were recorded with an overhead video camera and tracked with automated software (San Diego Instruments, San Diego, CA, USA). The time to reach the platform during water-maze training, number of the times the target area (former platform) was crossed, and time spent in each quadrant were recorded during the probe trial.

Histology
After the Morris water maze test, 5 mice from each group were deeply anesthetized (300 mg/kg, i.p. 10% chloral hydrate in DDW) and were perfused with ice-cold normal saline (50 ml) to remove blood from the vasculature, and then with 4% paraformaldehyde in phosphate buffered saline(50 ml). Their brains were removed and incubated overnight in 4% paraformaldehyde, and then half of each brain was dehydrated in 30% sucrose in PBS for frozen sections, while half was done routine fixation, dehydration, paraffin embedding for paraffin sections. Another 5 mice from each group were deeply anesthetized and perfused with ice-cold normal saline (50 ml), and then their brain samples were harvested and stored at -80 °C for Real time PCR and western blot analysis.

Organotypic hippocampal slice culture
Prepare surgical equipments: brushes, scissors (big and small), plastic pipette(thin, middle and short), blades, plastic plates and curved dissecting forceps and plastic
knifes (long). Make sure to keep everything sterilized. Sterilize the surface of the
super-clean workbench with alcohol, turn the ultraviolet light on, sterilizing for two
hours. Use a container of ice to place the cutting medium. The composition of the
cutting medium are (PH = 7.2): Earle’s MEM (cat.61100-06, Lot.74k4064 Gibco),
Hepes 5.95 g, Tri-base 1.21 g, Glucose 1.8 g, MgCl2 6 ml (all reagents from Gibco or
Sigma). Get packs of cell well dishes and place inside each of the wells an insert of
Millipore. Add to the outside edge of the well 1 mL of tissue medium. Put dishes into
the incubator.

Add 1 ml tissue culture medium to each well of 6-well plate, inserts of Millipore
were then placed into the wells where the tissue culture medium had been added.
The composition of the culture medium are (PH = 7.25, in 1L): BME (B 9638),
EBSS(E7510), NaCl(S5886) 1.167 g, NaHCO3(S 5761) 0.42 g, Ascorbica cid (A
4544), Glucose(G 7021), Hepes (free acid H3375) 6.36 g, CaCl2·2H2O(C 7903)
0.0293 g, MgSO4(M 2643) 0.203 g, Glutamine(G 7029), 0.39 g, Insulin (2 mg/ml
stock I4011) 0.667 ml, Penicillin(P 3032) 24.8 mg, 20% Horse serum (all reagents
from Gibco or Sigma). Put dishes into the incubator. Prepare the chopper (LEICA
VT1200S, VIBRATOME LINE), cut head of pups (postnatal 6-9 days) quickly and
remove skull, membrane till you get to brain. Put brain in cutting medium with
paper and slice into two pieces and get hippocampus. Use a brush to gently move
the separated pieces of hippocampus with cutting medium onto chopping board. Cut
the hippocampus into 400 micron slices, and gently place these sections in cutting
medium. Get the spare 6-well plates containing the culture medium from the
incubator. Place 2-5 pieces of dorsal hippocampal at approximalcoronal section in
each pup on the Millipore inserts of each well. Excess cutting medium should be
sucked. Place plate in incubator and label (name, date). After 2-3 hours, use sucker
to remove old medium of the bottom in each well and add new culture medium. Feed slice culture tissue each other days about 10 days. Treatment slices culture tissue about 6-10 days. NLRC3 protein was used for 48 hours, Aβ was used 36 hours before tissue harvested.

Immunohistochemistry, Immunofluorescent and confocal microscopy

Immunohistochemistry was performed to investigate the expression of Aβ, NLRC3 and Glial fibrillary acidic protein (GFAP) in the mice brains. Briefly, brains sections were dewaxed and rehydrated in decreasing concentrations ethanol. Then, endogenous peroxidase of the sections was blocked with 3% H2O2 under room temperature for 10 minutes. Slides were washed with PBS for several times and blocked in 2% BSA with 0.3% Triton X-100 for 40 min at 37 ºC. Thereafter, the sections were incubated with primary antibodies (GFAP 1:200 Millipore, Billerica, MA, USA; 6E10 1:1000 Thermo Fisher Scientific; NLRC3 1:100 abnova, Taiwan, China) overnight at 4 ºC. Then, rinsed in PBS and incubated with biotinylated secondary antibody (Vector, 1:200) and streptavidin-horseradish peroxidase (Jackson, Immunoreaserch, West Grove, PA, USA) for 1 h at 37 ºC, and then rinsed for another 3 min × 3 with PBS before reaction with diamino-benzidine (DAB) (Vector) solution. The sections were observed under a microscope. Nissl staining was performed to quantify neuronal density in sections.

For immunofluorescent staining, the sections were boiled in citric acid buffer (pH 6.0) for 20 min in a bain marie oven. After the sections were cooled, they were treated with 0.3% Triton X-100 and 2% BSA for 1 h at room temperature. The sections were then incubated overnight at 4 ºC with a primary antibody (neuron-specific nuclear protein (NeuN) 1:200 CST, USA; NLRC3 1:100 abnova, Taiwan, China), and then with a secondary antibody (1:300 Alexa-Fluor-488-conjugated goat
anti-mouse IgG2a antibody, Life Technologies, catalog number A21131; 1:300 Alexa-Fluor-555-conjugated goat anti-rabbit IgG1 antibody, Life Technologies, catalog number A31572) in PBS containing 1% BSA at room temperature for 2 h. The sections were mounted onto slides, embedded with SlowFade® Gold (Invitrogen), and covered with a coverslip.

Aβ plaques in brains were visualized using Thioflavin-S fluorescence (ThS) staining. ThS was dissolved in 50% of ethanol at 500 mM and brain sections were stained for 7 min. As a differentiation step to remove a nonspecific binding of the dye, a slide was soaked into 100, 95 and 90% ethanol solutions for 10sec each and then moved into PBS.

Quantitative real-time PCR

The level of NLRC3 mRNA was detected using Real-time qPCR kits by technicians who were blinded to the experimental groups. Total RNA was extracted from frontal cortex and hippocampus using the Trizol Plus RNA Purification System (Ambion, Invitrogen) according to the manufacturer’s instructions. RNA was quantified using the BioSpec Nano spectrophotometer (Shimadzu) and cDNA was reverse transcribed using the cDNA Synthesis kit (Thermo scientific, Lot 00285583) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the UltraSYBR Mixture (With ROX1) (CEBIO, CW2601) with the following cycling parameters: 95 °C for 10 minutes followed by 38 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 32 seconds, followed by amplicon dissociation (95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 15 seconds, 60 °C for 15 seconds, increasing at 0.5 °C/ cycle until 95 °C was reached). All gene expression data were normalized to β-actin expression. All reactions were performed in triplicate. Gene expression results were calculated using the delta delta cycle
threshold (two delta delta CT) method (Livak and Schmittgen, 2001). The two delta delta CT method was used to determine mean fold changes in gene expression between the control and target genes.

Western blot

Brain tissues were lysed in RIPA buffer containing phosphatase inhibitor and complete protease inhibitor cocktail. Aliquots of brain lysates were separated on SDS-PAGE, transferred to membranes and immunoblotted with primary antibodies, then horseradish peroxidase-conjugated secondary antibody (1:1000). Bands were revealed by use of an enhanced chemiluminescence (ECL) system (Thermo Scientific, Rockford IL) and density was quantified by use of Imagequant 5.2 (Healthcare Bio-Sciences, Philadelphia, PA). The primary Abs were used as following: Iba-1 and NeuN (1:1000 dilution, CST, USA), GFAP (1:1000 dilution; Millipore, USA), NLRC3 (1:1000 dilution; abnova), PI3K (1:500 dilution, CST, USA), β-actin and GAPDH (Proteintech, Chicago, USA). Secondary horseradish peroxidase-conjugated Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (1:1000 dilution).

Data and Statistical Analyses

The 3D image overlays were visualized with the Leica Application Suite (LAS) Advanced Fluorescence Lite software (LAS AF Lite, 2.4.1 build 6384, Leica). The ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the immunohistochemistry results. Analyses of plaque distributions were transcribed manually into the computer-acceptable format by keeping research colleagues blind. In the case of single mean comparison, Student t test were performed to analyzed the data. In case of multiple mean comparisons, ANOVA and further Newman–Keuls posttest, or two-way repeated-measures ANOVA, followed by
Bonferroni tests was used. Data are expressed as means ± standard deviations of the means (SD). P value < 0.05 was considered statistically significant (Prism version 6.0 software (GraphPad), USA).

Results

The expression of NLRC3 is down-regulated in brain tissues of APP/PS1 mice.

We did gene identification for all the mice. We further investigated the expression of NLRC3 in AD. q-PCR showed that the mRNA of NLRC3 was detected in the hippocampus of both control and APP/PS1 mice. Compared with the control group, the expression of NLRC3 mRNA in hippocampus of APP/PS1 mice decreased significantly. The change trend of protein expression is consistent with mRNA of NLRC3. The expression of NLRC3 protein was detected in the hippocampus of both control and APP/PS1 mice, and the expression of NLRC3 protein was significantly decreased in the hippocampus of APP/PS1 group (Fig. 1A-B). Total NLRC3 mRNA and protein levels were significantly decreased in the brains of APP/PS1 mice compared to controls.

Overexpression of NLRC3 improved the learning and memory ability of APP/PS1 mice.

As the expression of NLRC3 were decreased in the brains of APP/PS1 mice, we hypothesized that over-expression of NLRC3 may alleviate the progression of AD. To answer this question, in the current study mice were divided into four groups with different treatments (control, APP/PS1 groups, APP/PS1 + LVCON307 and APP/PS1 + LV-NLRC3 groups). The spatial learning and memory ability of mice were assessed with the Morris water maze. Mice in APP/PS1 group had a significant attenuation of learning and memory ability compared to the control group (Fig. 2A-C). There was
no significant difference in the time taken to reach the platform between the four groups on day 1 (P > 0.05). However, the time to reach the platform was significantly shorter in the APP/PS1 + LV-NLRC3 group than APP/PS1 group on day 2 (41.60 ± 0.49 vs. 53.40 ± 0.40 s, respectively, P < 0.001), day 3 (28.30 ± 2.07 vs. 49.60 ± 1.27 s, respectively; P < 0.001), day 4 (21.10 ± 1.41 vs. 46.00 ± 1.16 s, respectively, P < 0.001) and day 5 (16.60 ± 0.97 vs. 47.80 ± 1.74 s, respectively, P < 0.001) (Fig. 2A). There was no significant difference between APP/PS1 and APP/PS1 + LVCON307 group. We then evaluated the memory ability at the same time and found that the retention time in the target area were significantly increased in the APP/PS1 + LV-NLRC3 group (20.80 ± 2.34) than APP/PS1 group (9.00 ± 1.07; t = 4.58, P < 0.001) in the probe trial (Fig. 2B). Our data suggest that APP/PS1 mice displayed AD-like symptoms with impaired learning and memory ability, and this defect can be partially rescued in consistent with the overexpression of NLRC3 in APP/PS1 + LV-NLRC3 group (Fig. 2C).

Overexpression of NLRC3 inhibited the deposition of Aβ, decreased the activation of glial cells, and reversed the degeneration of neurons in APP/PS1 mice. NLRC3 co-localized with NeuN.

The APP/PS1 model is known to produce elevated levels of Aβ and develop AD-like phenotypes by expressing mutant APP and PS1. Aβ-dependent inflammation is considered to reflect the extent of injury and toxicity in AD. With 6E10 staining, we found that there was little deposition of Aβ in control group. In APP/PS1 and APP/PS1 + LVCON307 group, scattered or agglomerated deposition of Aβ could be seen. After overexpression of NLRC3, deposition of Aβ in APP/PS1 + LV-NLRC3 group decreased significantly compared with that in APP/PS1 and APP/PS1 + LVCON307 group (Fig. 3A).
Uncontrolled and over-activated microglia and astrocytes are prominent source of pro-inflammatory factors such as IL-1β, IL-6 and TNF-a, which are neuro-toxic\textsuperscript{26}. GFAP is a kind of intermediate filament protein, which mainly exists in astrocytes and is a specific marker of astrocyte activation in the nervous system. Iba1 plays an important role in regulating some immune and pathophysiological functions of microglia, and can be used as a specific marker of microglia activation. To reveal the changes of astrocytes and microglial, we performed immunostaining using GFAP and Iba1 antibody. In brain tissues of control group, there were low expression of GFAP and Iba1. In contrast, there were increased amount of GFAP and IBA1 detected in brain tissues of APP/PS1 group and APP/PS1 + LVCON307 group. However, the expression of GFAP and Iba1 in brain tissues of APP/PS1 + LV-NLRC3 group was significantly reduced compared with that in APP/PS1 group and APP/PS1 + LVCON307 group (Fig. 3B, C).

One pathological feature of the AD is neuronal loss. Many studies have suggested that the pronounced decline of neurons in the brain contribute to the deterioration of cognitive function. In this experiment, NeuN was analyzed to evaluate neuron damage. NLRC3 and NeuN were labeled by Red fluorescence (Cy3) and green fluorescent proteins, respectively. Two fluorescent densities were analyzed separately, or merged with photoshop software to identify co-location of NLRC3 and NeuN. NLRC3 localized at neurons. Both the red and green fluorescence were strong in brain tissues of control group indicating high levels of NLRC3 and NeuN. The expressions of NLRC3 and NeuN in the brains of APP/PS1 and APP/PS1 + LVCON307 group were significantly down regulation compared to that in control group. Compared with the APP/PS1 group and APP/PS1 + LVCON307 group, the red and green fluorescence of APP/PS1 + LV-NLRC3 group was significantly stronger, but still
weaker than the control group suggesting that expression of NLRC3 were negatively associated with degree of neuron-degeneration progression. NLRC3 was co-localization with NeuN (Fig. 3D, E).

Overexpression of NLRC3 attenuates the activation of PI3K in the brains of APP/PS1 mice.

Studies have confirmed that PI3K and its downstream signaling pathway play an important role in the occurrence and development of AD. Studies have also shown that NLRC3 can regulate the expression of PI3K in different diseases and NLRC3 can interact with PI3K. Then the next question is NLRC3 related to PI3K in AD? We found that contrary to the trend of NLRC3, PI3K expression was significantly up-regulated in the hippocampus of APP/PS1 mice by WB. There was no significant difference in PI3K expression between APP/PS1 and APP/PS1 + LVCON307 group. After overexpression of NLRC3, the activation of PI3K was significantly inhibited in APP/PS1 + LV-NLRC3 group (P < 0.05; Fig. 4A). Immunohistochemistry also indicated that the expression of PI3K localized in neurons was significantly higher in APP/PS1 group than that in control group. The activation of PI3K was inhibited after overexpression of NLRC3 in APP/PS1 + LV-NLRC3 group (Fig. 4B).

NLRC3 reverses senile plaque deposition, neuronal loss and PI3K activation induced by Aβ in hippocampal slices.

ThS-negative diffuse senile plaques are characterized as a pathological sign of AD. ThS staining was applied to analysis the number and area of the senile plaques in mice hippocampal slices. No senile plaques deposition was observed in control group. In Aβ and Aβ + PBS group, the senile plaques were distributed in intracellular and extracellular spaces in spherical or irregular shapes with different sizes. Hippocampal slices in Aβ + NLRC3 group exhibited less senile plaques burden
compared with Aβ and Aβ + PBS group (Fig. 5A).

In this experiment, Nissl bodies were analyzed to evaluate neuron damage. With Nissl staining, we detected that the neurons were in distinct and regular structure, and neuronal density were clearly increased in control group. In contrast, hippocampal slices in Aβ and Aβ + PBS group revealed injuries including remarkable neuron loss, karyopycnosis and disappearance of Nissl bodies. After administration of exogenous NLRC3 protein, neuronal density were increased while the vacuolization of endochylema and karyopycnosis were decreased significantly in Aβ + NLRC3 group compared to Aβ and Aβ + PBS group (Fig. 5B).

We further examined the expression of PI3K in hippocampal slices of each group. The results showed that the trend of PI3K detected by WB in hippocampal slices were consistent with that in vivo. In Aβ group, PI3K was significantly up-regulated compared with that in control group. After administration of exogenous NLRC3 protein, the activation of PI3K was significantly inhibited in Aβ + NLRC3 group compared to Aβ and Aβ + PBS group (Fig. 5C).

PI3K agitation partially reverses the protective effect of NLRC3 on hippocampus of mice exposed to Aβ.

The deposition of senile plaques, dysfunction of glial cells and loss of neurons in AD are related to the activation of PI3K. Results above suggested that NLRC3 overexpression could reduce plaque deposition and neuron loss in the hippocampus of AD model, decrease the abnormal activation of glial cells, and inhibit the activation of PI3K. Next, we explored whether activation of PI3K reverse the protective effect of NLRC3 on hippocampus of mice exposed to Aβ?

In this experiment, elevations of GFAP and Iba-1 in response to Aβ were observed in Aβ group. In Aβ + NLRC3 group, NLRC3 could inhibit the elevation of GFAP and Iba-1
induced by Aβ. In Aβ + NLRC3 + 740YP group, PI3K activation with 740YP increased the expression of GFAP and Iba-1 in hippocampus with exogenous NLRC3 protein. That was to say, PI3K activation with 740YP could antagonize the effect of NLRC3 to some extent (P < 0.05, Fig. 6A).

To explore the effect of PI3K activation on neurons after NLRC3 administration in hippocampal slice, we further investigated the information of neuron loss under different interventions with immunofluorescence. The results showed that the expression of NeuN (red fluorescent) in Aβ group was significantly lower than that in the control group. After exogenous NLRC3 administration, the expression of NeuN in Aβ + NLRC3 group was significantly higher than that in the Aβ group, but still lower than that in the control group. However, after administration of 740YP (PI3K agonist), the expression of NeuN in Aβ + NLRC3 + 740YP group was higher than that in Aβ + NLRC3 group. There was a certain degree of reversal effect (Fig. 6B).

Collectively, this study confirms that the down-regulation of NLRC3 can lead to the activation of PI3K, resulting in abnormal plaque deposition, glial cell activation and neuron loss during AD. NLRC3 over-expression inhibited the activation of PI3K, on the contrary, this effect of NLRC3 could be partially reversed by 740Y-P (PI3K agonist) (Fig. 7). That is to say, NLRC3 delays the progression of AD in APP/PS1 mice via inhibiting PI3K activation.

**DISCUSSION**

Our study demonstrated the novel role of the NLRC3 in a mouse model of AD and thus its potential involvement in AD pathogenesis. The expression of NLRC3 decreased in APP/PS1 mice as compared with control mice. As well, function augmentation of the NLRC3 could effectively improve spatial learning and memory
function and NeuN expression, inhibit plaque deposition, neuronal loss and PI3K activation in APP/PS1 mice.

AD is the most common cause of dementia in the elderly and has insidious onset, chronic progression and long duration. Although, to date, several independent hypotheses have been proposed to explain the disease, pharmacological treatments of AD are limited. Thus, further studies are needed to target mechanism of AD and develop specific preventive drugs. In the present study, spatial learning and memory function were significantly decreased, neurons were injured seriously, plaque deposition were increased and PI3K signals were activated in APP/PS1 mice, which is almost consistent with the results of zhou-jun et al\textsuperscript{27}.

The activation of PI3K pathway plays an important role in the occurrence and development of AD. Many studies have found that PI3K activation can lead to abnormal activation and functional changes of glial cells. In AD, the activation of glial cells leads to a series of adverse reactions, such as neuroinflammation, oxidative stress, etc. Many signal factors, including PI3K and AKT, play an important role in glial cell-activation and neuritis\textsuperscript{28}. In bacterial LPS - induced neuritis, P13K-Akt can be activated by binding to its specific receptor and interact with multiple upstream molecules to regulate glial cell-induced neurodegeneration\textsuperscript{29}.

Increasing evidence suggests that inflammation is a prominent feature of AD and correlates to its pathogenesis. Microglia and astrocytes are main mediators of inflammation in brain. In a healthy brain, these cells release molecules that keep the brain running smoothly. In Alzheimer disease, they become dysregulated, causing detrimental effects such as neuroinflammation that can promote the development and progression of neurodegeneration\textsuperscript{30}. Activation of microglia and
astrocytes initiates the inflammatory cascade that leads to the release of potentially neurotoxic cytokines are suggested to play a decisive role in the pathogenesis of AD. Our examination demonstrated that in the brain tissues of APP/PS1 mice, the activation of glial cells, as assessed by Iba1 and GFAP immunoreactivity, were increased accompany with the activation of PI3K. On the other hand, the expression of NLRC3 and NeuN and the number of Nissl bodies decreased distinctly in the brain tissues of APP/PS1 mice compared with control mice. Overexpression of NLRC3 significantly inhibited the activation of PI3K and reversed the increasing of GFAP and Iba1, also improved the contents of NeuN and Nissl bodies. These data suggested that NLRC3 could alleviate neurodegeneration in APP/PS1 mice.

In AD brain, Aβ plaques surrounding astrocytes and microglia can secrete inflammatory mediators to regulate neuroinflammation. IL-1β, IL-6, and TNFα are involved in the initiation and progression of AD by deregulating Aβ-mediated inflammation and APP metabolism. We performed LV-NLRC3 studies to identify the contribution of NLRC3 in APP/PS mice during Aβ formation. As expected, APP/PS mice which received LV-NLRC3 had significantly decreased Aβ burden compared with APP/PS mice that did not received LV-NLRC3. However, APP/PS mice which received LV-NLRC3 still had significantly increased Aβ burden compared with control mice. These results supported the notion that disruption of NLRC3 may increase Aβ generation. In a AOM-DSS inflammation-induced colorectal cancer model, defective inflammasome activation leads to loss of epithelial integrity, massive leukocyte infiltration and increased chemokines expression in the NLRC3−/− mice, NLRC3 seems important to suppress inflammation. On the other hand, Shiau CE, et al.
found that NLRC3-like can alleviate unwarranted inflammation in microglia precursor cells and exert neuroprotective effect through decreasing proinflammatory factors such as IL-1β and IL-8 during systemic inflammation\textsuperscript{33}. In line with these findings, our data demonstrated lower levels of GFAP and Iba1 in APP/PS1 + LV-NLRC3 mice than that in APP/PS1 mice. These results might further confirm the hypothesis that neuronal protection effect of NLRC3 in APP/PS1 mice, as it can regulate the functions of glial cells and alleviate the neuron loss by inhibiting the activation of PI3K signaling pathway.

However, research into NLRC3 and its role in AD pathogenesis is still in its infancy. PI3K activity of astrocytes is highly associated with neurodegenerative diseases. Increased PI3K were found in the immediate vicinity of extracellular Aβ deposition in patients with dementia and in an AD mouse model. In astrocytes, Aβ elicits the production of ROS and nitric oxide (NO), which can activate PI3K and its downstream signals, thereby leading to AD-related events in the brain including neurodegeneration and inflammation\textsuperscript{34}. Moreover, functional inhibition of PI3K abrogated the production of Aβ plaques, IL-1β, IL-6, and TNF. We proposed that Aβ attenuated the expression of NLRC3 and subsequently increased PI3K signals activity, thereby leading to production of pro-inflammatory cytokines from astrocytes and microglia. In our study, expression of PI3K in APP/PS1 mice significantly increased compared with that in control mice, while NLRC3 decreased significantly by contrast. Treatment with NLRC3 significantly reversed the increase of PI3K in APP/PS1 mice and Aβ hippocampal slices. NLRC3 may lessen PI3K activity its downstream signaling pathways, which are involved in inflammation and AD. The results indicated that NLRC3 may exert an important role through PI3K signaling.
pathways in AD progression. However, the detailed molecular mechanism by which NLRC3 regulates AD progression needs further investigation.

Finally, behavioral analysis demonstrated that function of NLRC3 impeded AD progression and improved neuropsychiatric signs, positively affecting cognition and spatial learning and memory.

Collectively, our data suggest that NLRC3 is involved in the progression of AD, hence may provide a novel therapeutic strategy and biomarker for AD. The drawbacks of this study are as follows. This research only uses the hippocampal slice culture in vitro to study the mechanism. No specific studies have been conducted on cell lines of nervous system. Therefore, we cannot explore the mechanisms in a deeper level. Moreover, we have not studied brain slices of other parts except hippocampal slices. Whether they have the same changes in brain slices of other parts remains to be verified. Finally, we only established NLRC3 overexpression mice model without knockout group.

Our preliminary study found that NLRC3 and neurons have co-localization phenomenon. Next, we plan to culture neurons and explore the protective effect of NLRC3 on neurons through cell experiments. We will establish the AD model that overexpress or knock out NLRC3, and directly intervene the PI3K pathway to support our conclusions, the next level down.

Conclusion

In conclusion, we provide experimental evidence to support the critical role of NLRC3 in the progression of AD. The molecular mechanisms we established may provide important information for depicting the pathogenesis of AD and aid in the development of therapeutic interventions for AD.
ABBREVIATIONS

Alzheimer's disease (AD); amyloid beta (Aβ); neurofibrillary tangles (NFTs); central nervous system (CNS); Lipopolysaccharide (LPS); Nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs); nucleotide-oligomerization domain (NOD)-like receptor subfamily C3 (NLRC3); phosphoinositide 3-kinase (PI3K); reactive oxygen species (ROS); APPswe/PS1-dE9 (amyloid precursor protein/presenilin protein 1 (APP/PS1)); lentivirus NLRC3 (LV-NLRC3); negative control lentivirus (LVCON307); transducing units (TU); patial learning and memory (SLM); diamino-benzidine (DAB); Glial fibrillary acidic protein (GFAP); neuron-specific nuclear protein (NeuN); Thioflavin-S fluorescence (ThS); nitric oxide (NO); Real-time quantitative polymerase chain reaction (RT-qPCR).

DECLARATIONS

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Availability of data and materials

All raw data used in this manuscript are available on request.

Authors’ contributions

LHZ and JZ conceived and performed the research, analyzed the data, and wrote the manuscript. LHZ and ZXY contributed equally to this work, JZ designed the research. SHG, LZ and WG helped in funding the project. LHZ and LZ performed the research. All authors read and approved the final manuscript.
Ethics approval and consent to participate

All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All of the experimental protocols with rats have been approved by the institutional ethics committee of Xiangya Hospital, Central South University.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

NLRC3 is down-regulated in brain tissues of APP/PS1 mice. Expression of NLRC3 (A) mRNA and (B) protein was measured by qRT-PCR and Western blotting, respectively. The expression levels were normalized to GAPDH and β-actin. (A) The data are represented as the mean ± standard error of the mean (n = 8). An unpaired t-test was used for this analysis. **P < 0.01.

Figure 2

NLRC3 improves the learning and memory ability of APP/PS1 mice. (A) Learning latency were obtained and recorded. (B) The data are represented as the mean ± standard error of the mean (n = 10). An unpaired t-test was used for this analysis. **P < 0.01.

Figure 3

NLRC3 inhibits the deposition of Aβ, decreases the activation of glial cells, and reverses the degeneration of neurons in APP/PS1 mice. NLRC3 (A) inhibitor, (B) membrane potential, and (C) immunofluorescence were examined. The data are represented as the mean ± standard error of the mean (n = 6). ANOVA and Student–Newman–Keuls test were used for these analysis. **P < 0.01.

Figure 4

NLRC3 attenuates the activation of PI3K in the brains of APP/PS1 mice. (A) Expression of PI3K protein was measured by Western blotting. The data are represented as the mean ± standard error of the mean (n = 6). An unpaired t-test was used for this analysis. **p < 0.01.
Figure 5

NLRC3 reverses senile plaque deposition, neuronal loss and PI3K activation induced by Aβ.

Figure 6

PI3K agitation reverses the protective effect of NLRC3 on hippocampus of mice exposed to Aβ.
Proposed pathway of NLRC3 mediation contributes to AD. Aβ and APP/PS1 downregulate expression of NLRC3, and consequently increase PI3K expression and activation. PI3K-related functional proteins contribute to Aβ and APP/PS1-induced plaque deposition, glial cell activation, and neuron loss in AD. NLRC3 overexpression inhibits PI3K activation, whereas the effect of NLRC3 could be partially reversed by 740Y-P (PI3K agonist).

Supplementary Files

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