Invest Clin 66(2): 191 - 204, 2025 https://doi.org/10.54817/IC.v66n2a06

The p300-NF-κB pathway induces the activation of the NLRP3 inflammasome and the pyroptosis of neurons in an *in vitro* model of Alzheimer's disease.

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Keywords: Alzheimer's disease; p300; pyroptosis; NLRP3 inflammasome.

Abstract. Inflammation-induced neuronal death is the primary cause of Alzheimer's disease (AD). p300 plays an important role in brain disorders. However, the role of p300 in AD remains unclear. This study aimed to investigate the potential of p300 in an *in vitro* model of AD. Protein expression was detected using western blotting. The mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. Cytokine release was detected using an enzyme-linked immunosorbent assay. Cellular function was determined using the cell counting kit-8, lactate dehydrogenase, and flow cytometry assays. Chromatin immunoprecipitation and luciferase assays verified the interaction between nuclear factor kappa B (NF-κB) and the NLR family pyrin domain containing 3 (NLRP3). E1A binding protein p300 (p300) was overexpressed in the $A\beta_{1-42}$ induced AD model in vitro. However, treatment with the p300 inhibitor (GNE-049) alleviated inflammation and $A\beta_{1-42}$-induced pyroptosis in the neurons. p300 activates NF-κB, which antagonizes the effects of GNE-049 and promotes neuronal pyroptosis. Moreover, NF-κB epigenetically activates the NLRP3 inflammasome. The p300/NF-κB pathway promotes neuronal pyroptosis in an *in vitro* AD model by activating the NLRP3 inflammasome. Therefore, the p300/NF-κB/NLRP3 signalling pathway may be a potential therapeutic target for AD.

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En el modelo in vitro de la enfermedad de Alzheimer, la vía p300 NF - Kappa B induce la activación del inflamasoma NLRP3 y la piroptosis neuronal en un modelo in vitro de la enfermedad de Alzheimer.

Invest Clin 2025; 66 (2): 191 – 204

Palabras clave: enfermedad de Alzheimer; P300; muerte por quemadura celular; cuerpo inflamatorio nlrp3.

Resumen. La muerte neuronal inducida por la inflamación es la principal causa de la enfermedad de Alzheimer (AD). El p300 juega un papel importante en las enfermedades cerebrales. Sin embargo, se desconoce el papel del p300 en la AD. El objetivo de este estudio es explorar el potencial del p300 en modelos in vitro de AD. Se utilizó Western blot para detectar la expresión de proteínas. Los niveles de ARNm se determinaron mediante la reacción cuantitativa en cadena de la polimerasa de transcripción inversa. Se utilizó la prueba de inmunoabsorción enzimática para detectar la liberación de citocinas. La función celular se determinó mediante el contador celular Kit - 8, la lactato deshidrogenasa y la medición con citometría de flujo. La interacción entre el factor nuclear Kappa b (nf - Kappa b) y el dominio Pirin 3 (nlrp3), que contiene la familia NLR, fue verificada por inmunoprecipitación de cromatina y detección de luciferasa. La proteína de unión a E1A p300 (p300) está sobreexpresada en un modelo de AD inducido por $A\beta_{1-42}$. Sin embargo, el tratamiento con un inhibidor del p300 (GNE - 049) redujo la inflamación y redujo la muerte por piroptosis neuronal inducida por $A\beta_{1-42}$. El p300 activa NF - Kappa b, que inhibe el efecto del GNE - 049 y promueve la muerte por piroptosis neuronal. Además, NF-κB epigeneticamentelly activa el NLRP3 inflamasoma Epigenética NF-Kappa B activa los cuerpos inflamatorios nlrp3. La vía p300 / NF - Kappa B promueve la muerte focal neuronal en modelos in vitro de AD activando el inflamasoma NLRP3. Por lo tanto, la transmisión de la señal p300/NF-Kappa B/NLRP3 puede ser un objetivo terapéutico potencial para la AD.

Received: 08-03-2025 Accepted: 10-05-2025

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disorder [1]. The symptoms include apraxia, agnosia, aphasia, and emotional disturbance [2]. AD is the leading cause of dementia and accounts for > 60% of all cases [3]. Currently, nearly 50,000,000 people suffer from AD worldwide [4]. In China, among the 15.07 million people (≥60 years old) with dementia, 9.83 million (65.2%) were diagnosed with AD [5]. Moreover, the incidence of AD is increasing with an aging population[6]. However, the pathogenesis of AD is complex, and there are no effective prognostic biomarkers [7]. Therefore, identifying the potential molecular mechanisms underlying AD may provide novel therapeutic strategies.

Pyroptosis is a form of programmed cell death characterized by inflammasomes[8].

The NLR family pyrin domain containing 3 (NLRP3) is the main inflammasome expressed in brain tissues [9]. Stimuli-induced activation of NLRP3 inflammasomes cleaves caspase1 [10]. The cleaved caspase-1 then cleaves gasdermin D (GSDMD) and induces the accumulation of the N-terminus of GSDMD (GSDMD-N), which drives GSDMD to move to the cell membranes [11]. The enrichment of GSDMD-N in the cell membranes contributes to pore formation and subsequent relapse, releasing interleukin (IL)-1β and IL-18 [12,13]. The activation of the NLRP3 inflammasome is frequently observed in patients with AD. The NLRP3 inflammasome mediates neuroinflammation, cell senescence, and loss of neurons, which are key causes of AD [14-16]. However, inhibition of the NLRP3 inflammasome restores neuronal function and alleviates AD development [17].

Nuclear phosphoprotein E1A binding protein p300 (p300) is an acetyl transferase [18]. p300 regulates numerous biological processes such as proliferation, autophagy, apoptosis, and pyroptosis [19,20]. Increasing evidence has suggested that p300 is abnormally expressed in patients with brain disorders. For instance, p300 is downregulated in ischemia/reperfusion injury, whereas overexpressed p300 enhances the anti-apoptotic effects of myocardin-related transcription factor A [21]. However, p300 deficiency inhibits neuroepithelial cell proliferation in diabetes-induced tube defects [22]. Moreover, high levels of p300 in human brains with AD contribute to neuronal loss [23]. However, the role of p300 in AD has not yet been completely elucidated. In the present study, we investigated the potential role of p300 in an *in vitro* model of AD. We hypothesized that p300-mediated neuronal pyroptosis exacerbates the progression of AD.

MATERIALS AND METHODS

Cell culture

The mouse neuronal cell lines HT22 and HEK293T were obtained from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37°C in a 5% CO_2.

HT22 cells were exposed to $A\beta_{1\text{-}42}$ (20 μM) and a p300 inhibitor (GNE-049, 500 nM) for 24 h. Cells in the control groups were cultured with FBS.

HT22 cells were transfected with small hairpin RNA shRNA) of nuclear factor kappa B (NF-κB) and overexpression plasmids or the control/vector using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA) assay

Cytokine levels were measured using ELISA kits (Abcam, Cambridge, USA), including IL-6, IL-1β, IL-18, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-10.

Lactate dehydrogenase (LDH) assay

The release of LDH was determined using the corresponding LDH kit (Abcam, Cambridge, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the HT22 cells. A HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China) was used to synthesize the cDNA. PCR was performed using the HiScript II One-Step RT-PCR Kit (Vazyme, China) on an ABI 7900 system. Glyceraldehyde-3-phosphate dehydrogenase served as the loading control. The mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

HT22 cells were harvested, and total protein was extracted. After centrifugation at 12000 × g, a BCA assay was performed to determine the protein concentration. Forty micrograms of protein were isolated using 10% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes. After sealing with 5% skim milk, the membranes

were incubated with primary antibodies, including anti-p300 (ab275378; 1: 1000, Abcam, UK), anti-p-p65 (ab32536; 1: 2000, Abcam, UK), and anti-GAPDH (ab9485; 1: 2500, Abcam, UK) and then with goat anti-rabbit IgG H&L (ab205718; 1: 10000, Abcam, UK). Subsequently, the bands were imaged using an enhanced chemiluminescence kit (6104-58-1; Sigma-Aldrich, Germany).

Luciferase assay

JASPAR (https://jaspar.elixir.no/) was used to predict the binding sites between NF-κB and the promoter of NLRP3. Binding was amplified and inserted into the pMIR-GLOTM luciferase vector (Promega). HEK293T cells were transfected with wild-type (WT)/mutant type (MUT) of NLRP3 and NF-κB shRNA/overexpression plasmids. After 48 h, luciferase activity was detected using a kit (Promega).

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was conducted on HT22 cells using a ChIP kit (Sigma-Aldrich). Briefly, cells were crosslinked with 1% formalin, afterwards, cells were lysed and sonicated. The sonicated chromatin was incubated with antibodies, including anti-NF-κB (ab32536; 1: 30, Abcam, UK) and anti-IgG (ab172730; 1: 50, Abcam, UK) using Protein G magnetic beads. Finally, the DNA fragments were analyzed by RT-qPCR.

Cell counting kit-8 (CCK-8) assay

The cells were seeded in a 6-well plate (4000 cells/well) and cultured for 0, 24, 48, and 72 h. The cells were then supplemented with CCK-8 reagent. Finally, cell viability was determined using a microplate reader at an absorbance of 450 nm.

Flow cytometry

Neuronal pyroptosis was detected using flow cytometry with propidium iodide (PI) and caspase-1 staining. Briefly, neurons were digested with ethylenediamine tetraacetic acid-free trypsin. Then, the cells were harvested by centrifugation at 1000 rpm for 5 min. Afterwards, cells were resuspended and washed with PBS twice. The cells were incubated in the dark with FAM FLICA™ Caspase-1 Kit (ICT098; Bio-Rad, USA) and PI (4 μL). The results were analyzed using a flow cytometer (Biosciences, USA).

Statistical analysis

Each independent experiment was performed in triplicate. Graphpad v.8. software was used to analyze the data. Data are presented as the mean±SD. Student's t-test and ANOVA were used to analyze differences. $p<0.05$ was considered statistically significant.

RESULTS

p300 is upregulated in an in vitro model of AD

p300 is frequently upregulated in patients. Therefore, we determined the p300 expression in an AD model *in vitro*. We found that p300 mRNA expression in HT-22 cells exposed to $A\beta_{1\text{-}42}$ was markedly increased compared with that in the control group (Fig. 1A). This finding was consistent with the Western blot results. $A\beta_{1\text{-}42}$ treatment markedly increased the protein expression of p300 (Fig. 1B).

p300 deficiency inhibits neuroinflammation

p300 is a key regulator of the inflammatory response and mediates cerebral injury by activating inflammation-related signalling. Therefore, we hypothesized that p300 promotes AD pathogenesis by inducing neuroinflammation. As shown in Fig. 2A-E, the release of proinflammatory cytokines, such as IL-6, IL-1β, IL-18, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, was significantly increased after $A\beta_{1\text{-}42}$ exposure, whereas IL-10 was markedly decreased (Fig. 2F). However, GNE-049 treatment significantly alleviated the effects of $A\beta_{1\text{-}42}$, inhibited the release of IL-6, IL-1β, IL-18, TNF-α, and IFN-γ, and increased the release of IL-

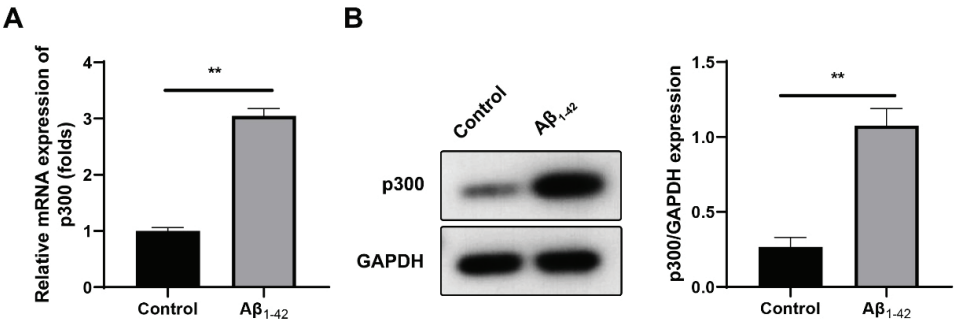

Fig. 1. The expression of p300 in in vitro model of AD.
(A) RT-qPCR was conducted to detect p300 mRNA expression in HT-22 cells exposed to $A\beta_{1\text{-}42}$. (B) Western blot was conducted to detect p300 protein expression in HT-22 cells exposed to $A\beta_{1\text{-}42}$. The difference in comparison was analyzed using the Student t-test. AD: Alzheimer's disease; p300: E1A binding protein p300; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. $^{**}p<0.01$.

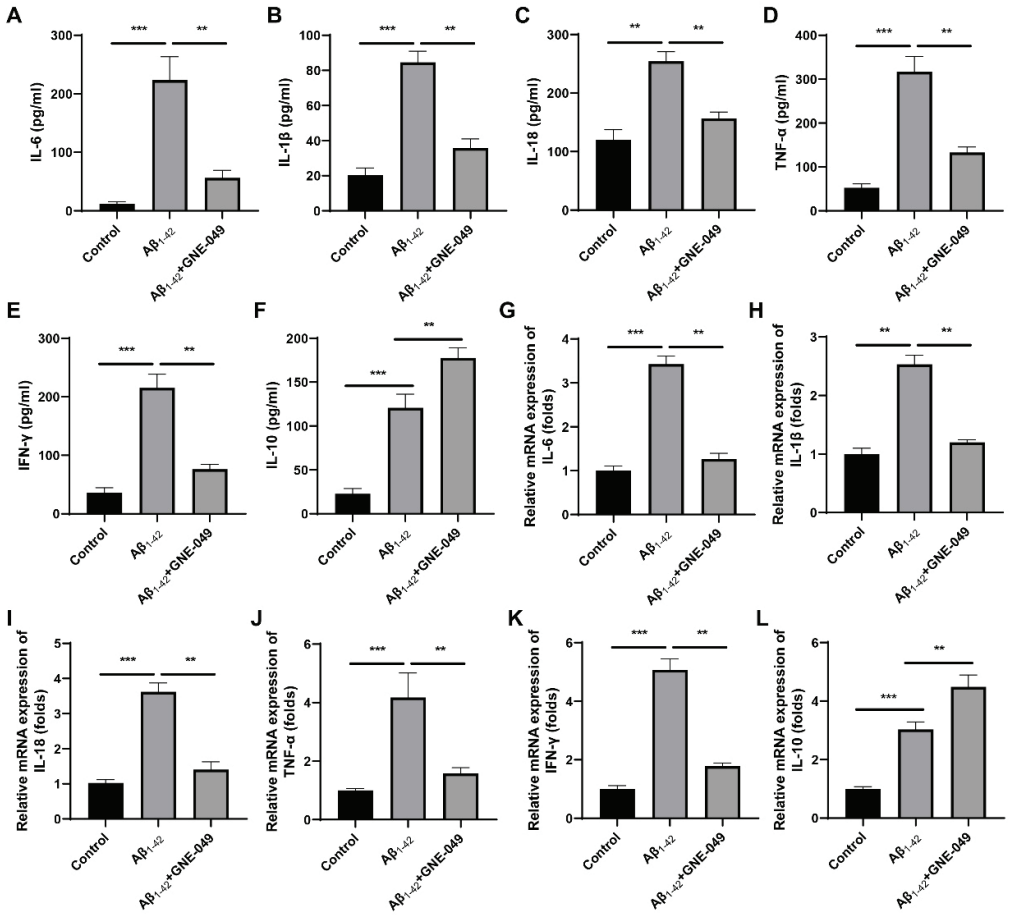

Fig. 2. p300 deficiency inhibits neuroinflammation.
(A-F) ELISA was used to detect the release of cytokines in HT-22 cells. (G-L) RT-qPCR was conducted to detect cytokine mRNA expression in HT-22 cells. Comparison difference was analyzed using one-way ANOVA. p300: E1A binding protein p300; IL-6: interleukin 6; IL-1β: interleukin 1β; IL-18: interleukin 18, TNF-α: tumor necrosis factor α; IFN-γ: interferon γ; IL-10: interleukin 10. ELISA: enzyme-linked immunosorbent assay; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. $^{**}p<0.01$, $^{***}p<0.001$.

10. This finding is consistent with the RT-qPCR results. GNE-049 treatment suppressed the mRNA expression of IL-6, IL-1β, IL-18, TNF-α, and IFN-γ (Fig. 2 G-K), while increasing IL-10 mRNA expression (Fig. 2 L). These findings suggest that the inhibition of p300 expression suppresses neuroinflammation.

p300 deficiency inhibits the pyroptosis of neurons

Inflammation-induced pyroptosis, which is characterized by the activation of the inflammasome and an increase in cytotoxicity and death, is a key cause of AD. p300 protein expression was markedly reduced by GNE-049 treatment (Fig. 3A). $A\beta_{1-42}$ exposure markedly increased LDH release (Fig. 3B), which was antagonized by GNE-049 treatment. Moreover, GNE-049 treatment promoted neuronal viability (Fig. 3C). GNE-049 treatment markedly alleviated the pyroptosis in the neurons induced by $A\beta_{1-42}$ exposure (Fig. 3D). Additionally, GNE-049 treatment suppressed the mRNA expression of NLRP3, PYD and CARD domain containing (ASC), and caspase-1 (Fig. 3E). These findings suggested that p300 inhibition alleviates neuronal pyroptosis in AD.

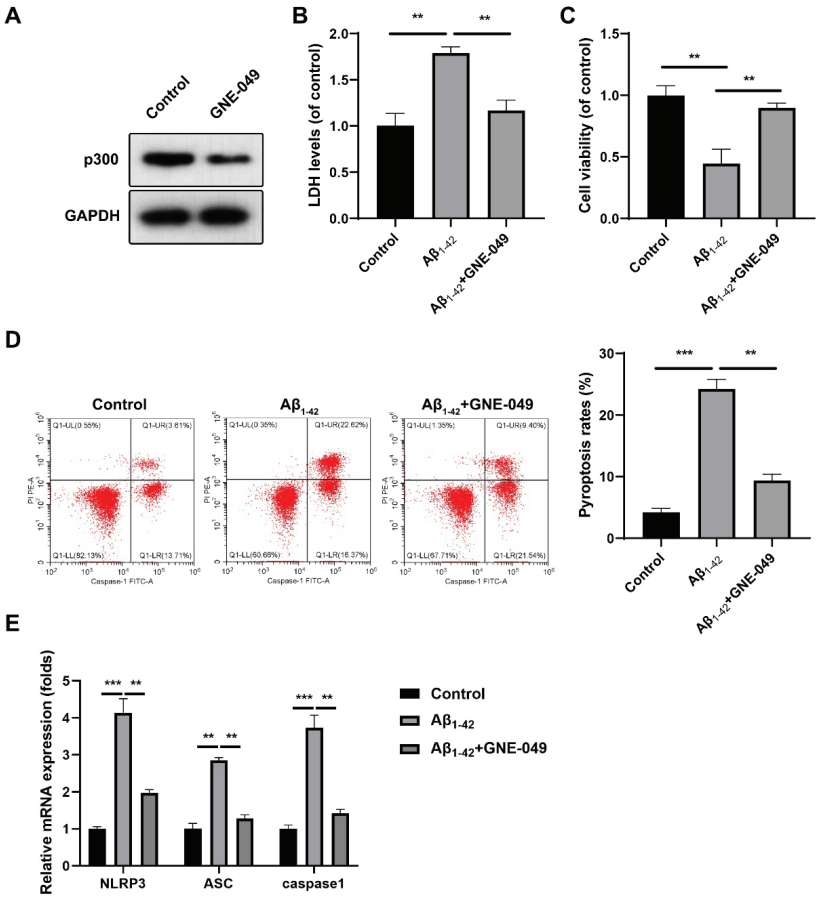

Fig. 3. p300 deficiency inhibits the pyroptosis of neurons.
(A) p300 protein expression was detected using Western blot. (B) LDH assay was conducted to detect cytotoxicity of HT-22 cells. (C) CCK-8 was performed to determine the cell viability of HT-22 cells. (D) Flow cytometry was used to detect the pyroptosis of HT-22 cells. (E) RT-qPCR was conducted to detect mRNA expression in HT-22 cells. Comparison difference was analyzed using one-way ANOVA. p300: E1A binding protein p300; NLRP3: NLR family pyrin domain containing 3; ASC: PYD and CARD domain containing; LDH: lactate dehydrogenase; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. $^{**}p<0.01$, $^{***}p<0.001$.

p300 activates NF-κB signaling

p300 participates in the inflammatory response by activating inflammatory signalling. Therefore, we hypothesized that p300 mediates neuroinflammation by activating NF-κB signaling. $A\beta_{1\text{-}42}$ exposure significantly increased the protein expression of p-p65 (Fig. 4), which GNE-049 alleviated.

p300 induces neuroinflammation via activating NF-κB signaling

To verify the role of NF-κB in AD, neurons were transfected with an NF-κB-overexpression plasmid. We found that overexpression of NF-κB alleviated the effects of GNE-049 and promoted the release of IL-6, IL-1β, IL-18, TNF-α, and IFN-γ, as well as decreased IL-10 (Fig.5A-F). Moreover, overexpression of NF-κB markedly increased the mRNA expression of IL-6, IL-1β, IL-18, TNF-α, and IFN-γ (Fig. 5G-K), but decreased IL-10 mRNA expression (Fig. 5L).

p300 induces pyroptosis via activating NF-κB signaling

A rescue assay was conducted to confirm further the role of p300/NF-κB signaling in AD. We found that overexpression of NF-κB markedly alleviated the effects of GNE-049 and promoted neuronal cytotoxicity (Fig. 6A). However, NF-κB overexpression suppressed neuronal viability (Fig. 6B). Overexpression of NF-κB alleviated the effects of GNE-049 and promoted pyroptosis in neurons (Fig. 6C). Moreover, overexpression of NF-κB markedly increased the mRNA expression of NLRP3, ASC, and caspase-1 (Fig. 6D). These findings suggest that p300 regulates neuronal pyroptosis by activating NF-κB signaling.

p300-dependent activation of NF-κB epigenetically activates NLRP3.

NF-κB, a key transcription factor in inflammatory signalling, participates in biological processes via its downstream regulation. We found that NLRP3 mRNA expression was markedly increased by p300 overexpression and returned to normal levels after transfection with NF-κB shRNA (Fig. 7A). NF-κB regulates its downstream activity by binding to the promoters of its target genes. Therefore, we hypothesized that NF-κB binds to the NLRP3 promoter (Fig. 7 B). Fig. 7C shows the binding motif for NF-κB. Four binding sites were identified in the promoter of NLRP3 (Fig. 7c). Overexpression of p300 and NF-κB markedly enhanced the transcription of NLRP3 (Fig. 7D). To identify the site that binds NF-κB, the 3'-UTR of the binding

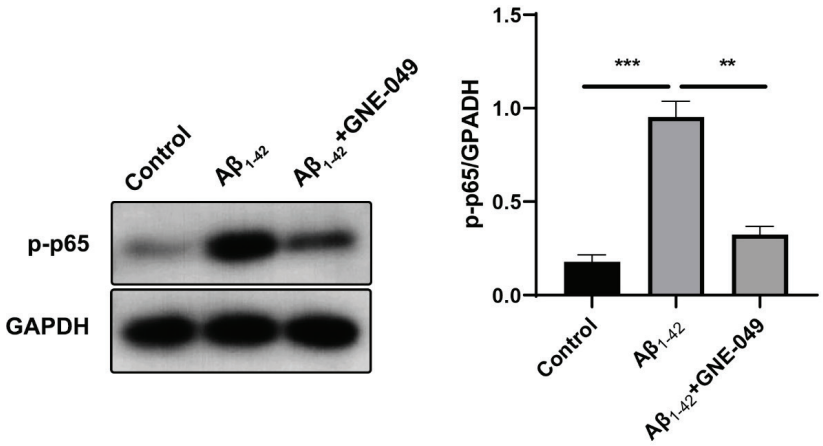

Fig. 4. p300 activates NF-κB signaling.
Western blot was performed to detect the p-p65 protein expression in HT-22 cells. Comparison difference was analyzed using one-way ANOVA. p300: E1A binding protein p300; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. $^{**}p<0.01$, $^{***}p<0.001$.

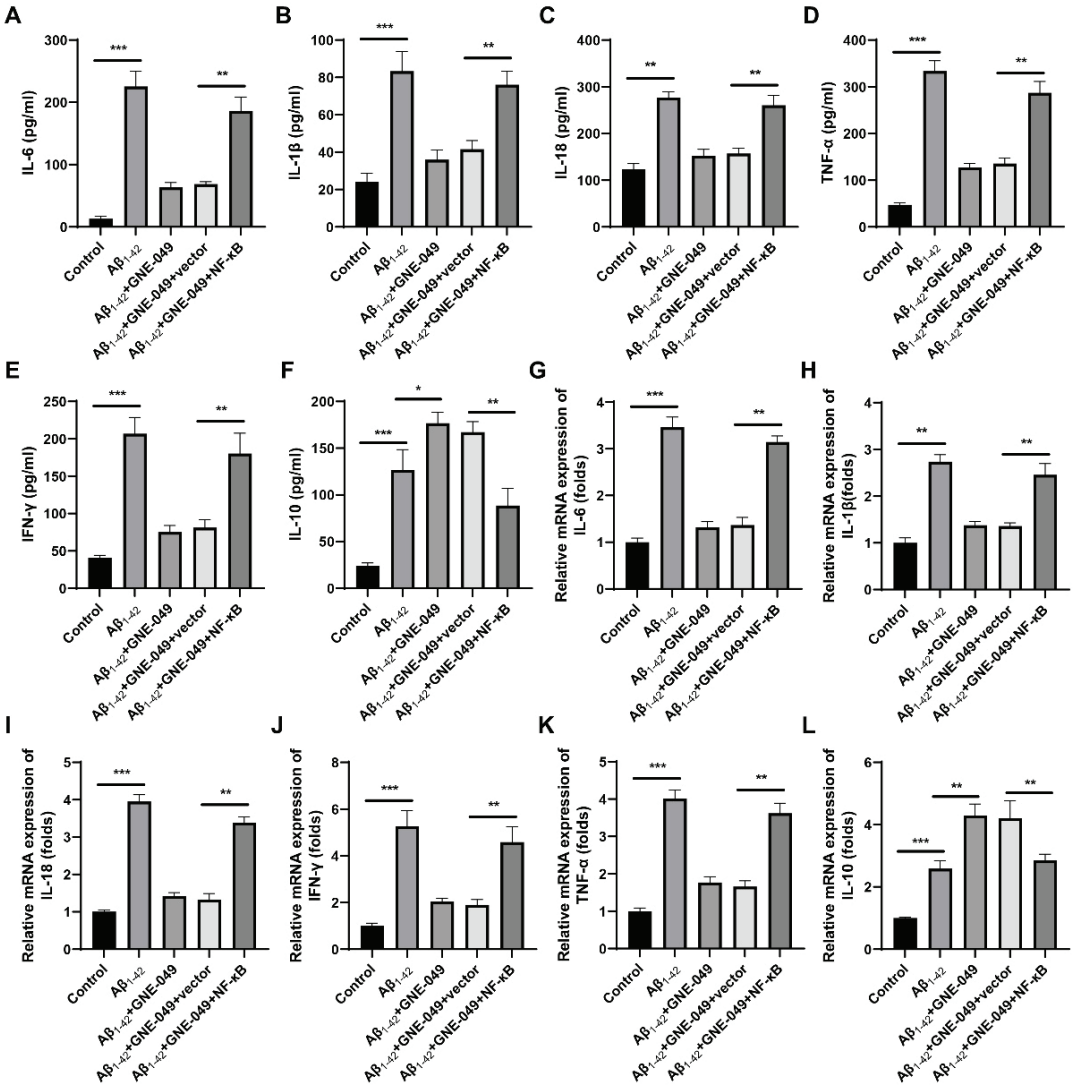

Fig. 5. p300 induces neuroinflammation via activating NF-κB signalling.
(A-F) ELISA was used to detect the release of cytokines in HT-22 cells. (G-L) RT-qPCR was performed to detect the p300 mRNA expression in HT-22 cells. Comparison difference was analyzed using one-way ANOVA. p300: E1A binding protein p300; IL-6: interleukin 6; IL-1β: interleukin 1β; IL-18: interleukin 18, TNF-α: tumor necrosis factor α; IFN-γ: interferon γ; IL-10: interleukin 10. ELISA: enzyme-linked immunosorbent assay; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

sites was mutated and inserted into luciferase reporters. Co-transfection with p300 and NF-κB significantly increased luciferase activity (Fig. 7E). Moreover, luciferase activity was markedly increased in MUT1/3/4 cells after $A\beta_{1\text{-}42}$ exposure, which was antagonized by GNE-049 treatment (Fig. 7F), whereas there was no significant alteration in MUT2. Additionally, p300 deficiency markedly suppressed the co-occupancies of site2 in HEK293T cells (Fig. 7G-H). These findings suggest that the p300-mediated activation of NF-κB epigenetically upregulates NLRP3 expression.

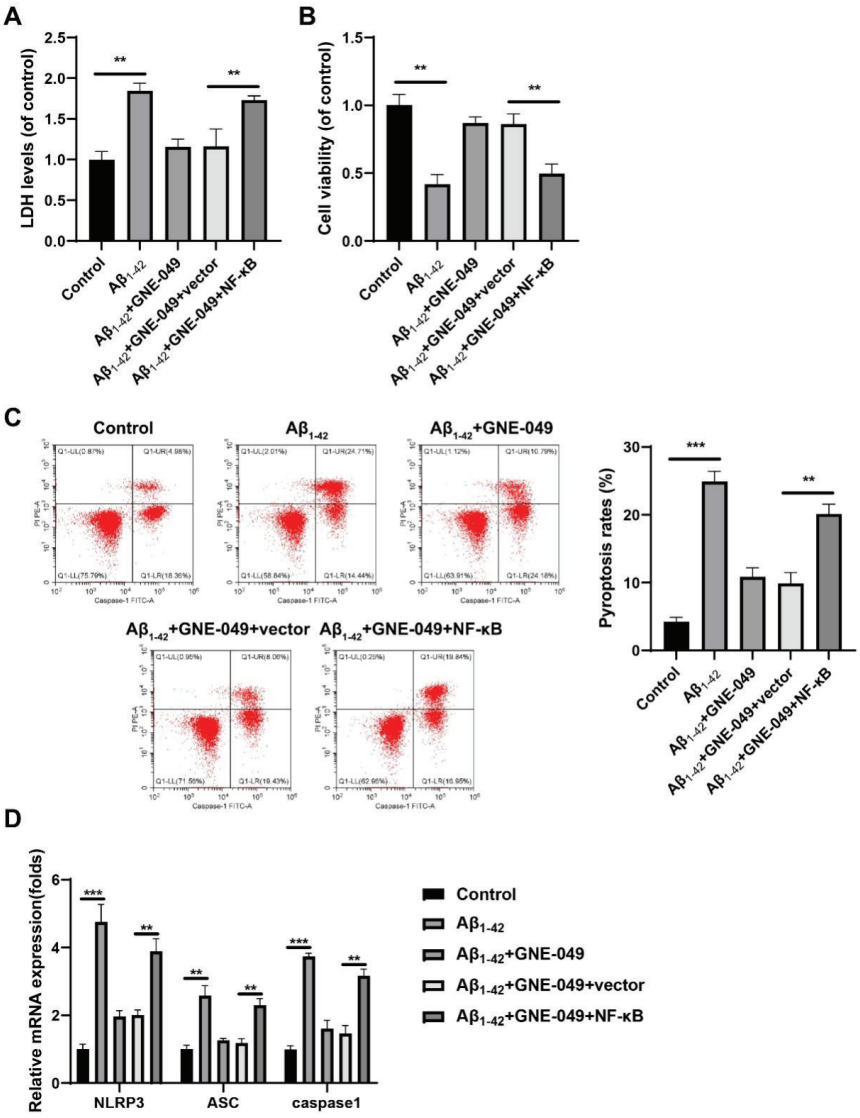

Fig. 6. p300 induces pyroptosis via activating NF-κB signaling.
(A) The LDH assay detected cytotoxicity in HT-22 cells. (B) The CCK-8 assay was performed to determine the viability of HT-22 cells. (C) Flow cytometry was used to detect pyroptosis in the HT-22 cells. (D) RT-qPCR was performed to detect the mRNA expression in HT-22 cells. Comparison difference was analyzed using one-way ANOVA. p300: E1A binding protein p300; NLRP3: NLR family pyrin domain containing 3; ASC: PYD and CARD domain containing; LDH: lactate dehydrogenase; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. $^{**}p<0.01$, $^{***}p<0.001$.

DISCUSSION

In this study, p300 was upregulated in *an in vitro* model of AD. Interestingly, p300 deficiency inhibits neuroinflammation and suppresses pyroptosis in the neurons. Moreover, p300 activates NF-κB, and its overexpression promotes pyroptosis in neurons. Additionally, the p300-mediated activation of NF-κB epigenetically upregulates NLRP3, which induces pyroptosis in neurons. Therefore, the p300/NF-κB/NLRP3 pathway may be a potential target in AD.

p300 is aberrantly expressed in several brain disorders. Chatterjee et al.[24] revealed that CBP/p300 activation enhances neuro-

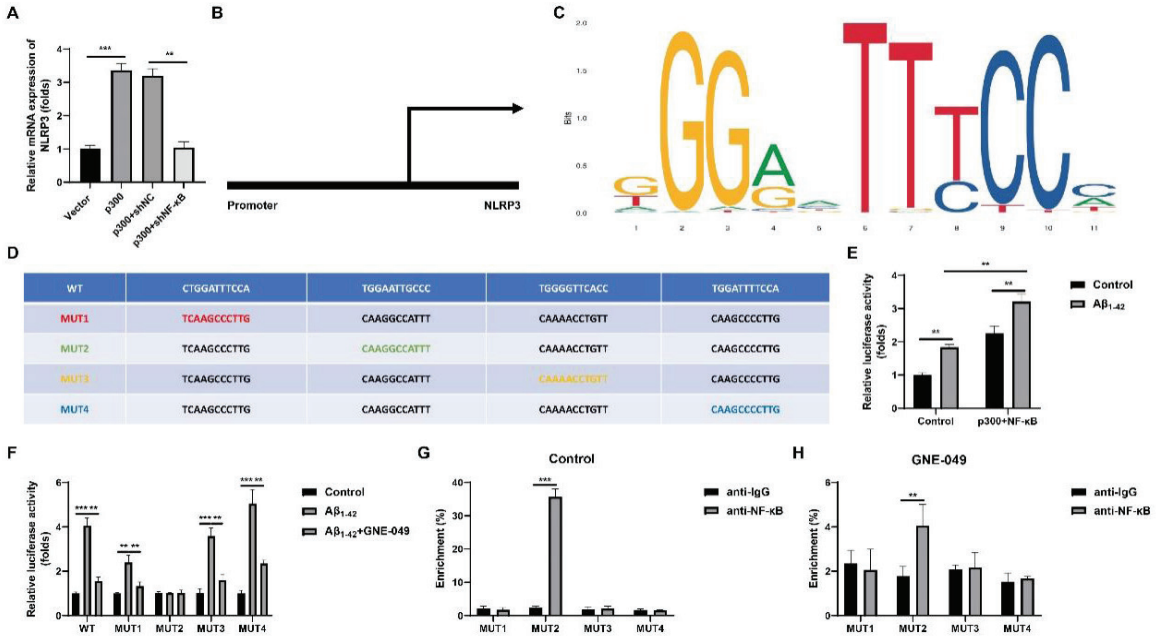

Fig. 7. p300-depedent activation of NF-κB epigenetically activates NLRP3.
(A) RT-qPCR was conducted to detect mRNA expression in HT-22 cells. (B) A hypothesis of NF-κB/NLRP3 signalling. (C) JASPAR was used to analyze the binding motif of NF-κB. (D) JASPAR was used to analyze the binding sites between NF-κB and the promoter of NLRP3. (E) Luciferase assay was conducted to confirm that the p300/NF-κB promoted the transcription of NLRP3 in HEK293T cells. (F) Luciferase assay was performed to verify the exact binding site between NF-κB and the promoter of NLRP3 in HEK293T cells. (G-H) ChIP assay was performed to verify the binding sites in HEK293T cells. The difference in comparison was analyzed using one-way or two-way ANOVA. p300: E1A binding protein p300; NLRP3: NLR family pyrin domain containing 3; WT: wild type; MUT: mutant type; RT-qPCR: reverse transcription-quantitative polymerase chain reaction. ChIP: chromatin immunoprecipitation. $^{**}p<0.01$, $^{***}p<0.001$.

genesis and prolongs memory duration, and maturation, and differentiation of adult neuronal progenitors. However, hyperactivation of p300 contributes to tauopathy pathogenesis [25]. Therefore, p300 may play protective and passive roles in brain disorders. This may be because the roles of p300 vary with disease subtype and signalling. Therefore, it is crucial to identify the role of p300 in AD, which is very important. CBP/p300 activation-mediated acetylation of tau exacerbates traumatic brain injury, which is the most significant non-genetic, non-aging-related risk factor for AD [26]. Moreover, p300-mediated autophagy promoted neuronal damage and inflammation in AD [27]. Therefore, p300 may promote the pathogenesis of AD. In this study, p300 was upregulated in an in vitro AD model. Targeting p300 may be an effective strategy for alleviating AD [25,26]. In this study, p300 deficiency induced by a specific inhibitor suppressed neuroinflammation and pyroptosis in the neurons. These findings suggest that p300 inhibition may alleviate neuronal loss during AD pathogenesis, consistent with previous studies.

p300, an acetyl transferase, has no DNA-binding domain [28]. p300 regulates gene expression by interacting with transcription factors. For instance, Sox8 induces the activation of the LIF interleukin 6 family cytokine downstream transcription factor signal transducer and activator of transcription 3 via p300 to promote astrocytic differentia-

tion [29]. Moreover, REST recruits CBP/p300 to the EAAT2 promoter to alleviate manganese-induced excitotoxicity [30]. Geong et al. [31] reported that p300/NF-κB promotes microglial activation and neuroinflammation. In the present study, p300 activated NF-κB. NF-κB is a key regulator of inflammatory signalling. NF-κB is a key mediator of brain inflammation in AD [32]. Activating NF-κB signaling stimulates the innate immune system and induces neurodegeneration and neuronal loss [33-35]. In this study, overexpression of NF-κB antagonized the effects of GNE-049 and promoted neuroinflammation and neuronal death.

Pyroptosis is a type of inflammation-related cell death process. Inflammation also induces necroptosis [36]. Although pyroptosis and necroptosis share some properties, such as lytic and inflammatory types of programmed cell death and releasing damage-associated molecular patterns, pyroptosis is differentiated from necroptosis, a backup cell death defense mechanism. In contrast, pyroptosis is a primary cellular response after sensing potentially damaging insults[37]. Necroptosis is characterized by the activation of receptor-interacting serine/threonine kinase 3/mixed lineage kinase domain-like pseudokinase signalling, whereas pyroptosis is characterized by the activation of inflammasomes and executed by GSDMD[38]. In this study, p300-dependent upregulation of NF-κB activated the NLRP3 inflammasome and increased the release of IL-1β and IL-18 after $A\beta_{1-42}$ exposure. Therefore, the p300/NF-κB/NLRP3 pathway-mediated neuronal death occurred via pyroptosis.

In conclusion, p300 was upregulated in an *in vitro* model of AD. p300-mediated upregulation of NF-κB epigenetically activates the NLRP3 inflammasome and pyroptosis in neurons. However, p300 inhibition alleviated neuroinflammation and neuronal pyroptosis. Therefore, targeting p300/NF-κB/NLRP3 may be a promising strategy for alleviating AD.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Third People's Hospital of Yunnan Province. All experimental animal procedures followed the Guidelines for the Care and Use of Laboratory Animals formulated by China's Ministry of Science and Technology.

Funding

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

Not applicable.

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- Wei Huang (WH): 0009-0008-9840-1633.

Author contributions

FQS performed data analysis and drafted the manuscript. FQS and WH conceived and supervised the study and reviewed the manuscript. FQS and WH ran the software and modified the code. FQS and WH were involved in the study design and contributed to the data collection procedure and interpretation.

ACKNOWLEDGEMENTS

Not applicable.

Conflicts of interest

The authors declared that they have no conflicts of interest regarding this work.

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