# Upregulation of microRNA-206 enhances lipopolysaccharide-induced inflammation and release of amyloid-β by targeting insulin-like growth factor 1 in microglia

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**Abstract.** Activated microglia are capable of facilitating amyloid-β (Aβ) accumulation via the release of inflammatory factors, thus resulting in the exacerbation of Alzheimer's disease (AD). MicroRNAs (miRs) participate in the activation of microglia, which is associated with AD. Insulin-like growth factor 1 (IGF1) is a neuroprotective, anti-inflammatory factor, which is able to accelerate clearance of Aβ peptides. The present study aimed to investigate the precise role of miR-206 and IGF1 in lipopolysaccharide (LPS)-induced microglial inflammation. The expression levels of miR-206 and IGF1 were detected in 60 peripheral blood samples from patients with AD and matched age subjects using quantitative polymerase chain reaction. A dual luciferase reporter gene assay was used to indicate the relationship between miR-206 and IGF1. In addition, the role of miR-206 was determined by gain and loss of function experiments in LPS-treated microglia. The results demonstrated that miR-206 upregulation enhanced LPS-induced inflammation and Aβ release in microglia by directly targeting the 3'-untranslated region of IGF1. These effects were attenuated following treatment with exogenous IGF1, thus indicating that the miR-206/IGF1 signaling pathway may be considered a novel therapeutic target for the treatment of AD-associated microglial inflammation.

## Introduction

Alzheimer's disease (AD), which is the most common neurodegenerative disorder in the elderly population, is characterized by deposition of amyloid-β (Aβ) and neuroinflammation (1). Aβ deposition has been detected in various areas of the brain, resulting in activation of microglia and neuronal death (2). Various mechanisms and pathways of Aβ generation and deposition have been established, including the inflammatory response (3). Microglia are the resident macrophages of the brain, which have a dual role in Aβ deposition. Microglia help eliminate Aβ deposition via phagocytosis; however, activated microglia are capable of facilitating Aβ accumulation via the release of inflammatory factors, including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, which are associated with the Aβ cascade during the development of AD (4). Therefore, the inflammatory response mediated by activated microglia has a key role in Aβ generation and deposition, and thus AD pathogenesis; however, the underlying molecular mechanisms remain to be elucidated.

Small noncoding RNA molecules, including microRNAs (miRNAs, miRs), have an important role in cell differentiation and activation. miRNA dysfunction may contribute to neuroinflammation under pathological conditions (5). Previous studies have demonstrated that miRNAs participate in the differentiation of progenitor cells into microglia, and in the activation process (6,7). Alterations to miRNA expression have been associated with AD. It was previously reported that miR-155 upregulation may contribute to a microglia-mediated neurotoxic response (8). In addition, Duan *et al* (9) demonstrated that miR-206 positively regulated the lipopolysaccharide (LPS)-induced inflammatory response in human astrocytes.

Insulin-like growth factor 1 (IGF1) promotes synaptogenesis, neurogenesis, neuroprotection, and exerts anti-inflammatory effects in the brain (10). Circulating serum IGF1 can accelerate the clearance of Aβ peptides (11), and loss of serum IGF-I input to the brain may be an early biomarker of disease onset in a murine model of AD (12). An IGF1 polymorphism has been reported as a key regulator for genetic susceptibility to late-onset AD in a Han Chinese population (13), thus indicating an essential role for IGF1 in the development of AD.

The role of miR-206 and IGF1 in LPS-induced microglial inflammation remains unknown; therefore, the present study aimed to investigate the precise role of miR-206 and IGF1 in the microglial inflammatory response induced by LPS. The expression levels of miR-206 and IGF1 were detected in 60 peripheral blood samples from patients with AD and

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matched age subjects using quantitative polymerase chain reaction (qPCR). A dual luciferase reporter gene assay was used to determine the association between miR-206 and IGF1. In addition, the role of miR-206 in LPS-treated microglia was determined by gain and loss of function experiments. The results demonstrated that miR-206 upregulation was able to enhance LPS-induced microglial inflammation, which was reversed following exogenous IGF1 treatment, thus indicating that miR-206/IGF1 signaling may be considered a novel therapeutic target for the treatment of microglial inflammation in AD.

## Materials and methods

*Blood samples*. The peripheral blood samples were collected from patients with AD (30 cases; 16 male and 14 female; aged, 65-75-years-old; mean age, 68.6-years-old) and from normal age-matched individuals (30 cases; 18 males and 12 females; age, 65-75-years-old; mean age, 67.8-years-old) at the First Affiliated Hospital of Xinxiang Medical University (Weihui, China) between September 2013 and October 2014, according to the legislation and ethical boards of Xinxiang Medical University. The study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (Weihui, China). Individuals with significant illnesses, including diabetes, heart disease, stroke or cancer, were excluded from the present study. All individuals provided written informed consent. The blood samples were stored at -80˚C until further use.

*Cell culture and treatment*. Microglial BV-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA), and were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 95% air and 5% $CO_2$. BV-2 cells were plated in 6-well plates ($5x10^5$ cells/well) and were grown to 80% confluence. Ectopic miR-206 expression was introduced to the cells by transfection with miR-206 mimics, and miR-206 expression was inhibited by transfection with a miR-206 inhibitor (both Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Negative control mimics and negative control inhibitor were used as negative control (Shanghai GenePharma Co., Ltd., Shanghai, China). Following incubation of the BV-2 cells in 6-well plates for 24 h, the cell medium was removed, and the cells were treated with or without LPS (1 $\mu$g/ml; Sigma-Aldrich, St. Louis, MO, USA) for a further 24 h, or the cells were transfected with miR-206 mimics (50 nM) or a miR-206 inhibitor (100 mM), alongside IGF1 (5 $\mu$g/ml; Sigma-Aldrich) and/or LPS (1 $\mu$g/ml) for a further 12 h.

*RNA extraction and reverse transcription-qPCR*. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from the cells and blood samples. A total of 0.5 $\mu$g RNA was used to synthesize cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) at 42˚C for 60 min and 72˚C for 10 min. qPCR analysis was performed using a CFX96 Touch Real-Time PCR Detection system and SsoFast EvaGreen Supermix (both Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data were normalized to the expression levels of β-actin in each sample. β-actin was used as an endogenous control. The specific primers used were as follows: IGF1, forward 5'-AGAGCCTGCGCAATGGAATA-3', reverse 5'-ACCCTGTGGGCTTGTTGAAA-3'; β-actin, forward 5'-CATTAAGGAGAAGCTGTGCT-3' and reverse 5'-GTTGAAGGTAGTTTCGTGGA-3'; miR-206, forward 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCACACAC-3' and reverse 5'-ACACTCCAGCTGGGTGGAATGTAAGGAAGT-3; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'.

To quantify mature miR-206 expression, a MiScript SYBR-Green PCR kit (Guangzhou Ribobio, Co., Ltd., Guangzhou, China) was used. The specific primer sets for miRNA-206 and U6 were purchased from Genecopoeia (Rockville, MD, USA). The relative expression levels of miR-206 were normalized to U6. All PCR analyses were performed at the following conditions: 2 min at 55˚C and 7 min at 95˚C, followed by 40 cycles at 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 30 sec, and a final extension at 65-95˚C for 5 min. The $2^{-\Delta\Delta Cq}$ method was used to analyze the data (14).

*Western blot analysis*. Cells were collected and resuspended in cold radioimmunoprecipitation lysis buffer (Beijing CWBio Co., Ltd., Beijing, China) for 1 h on ice. The lysates were then centrifuged at 12,000 x g for 20 min at 4˚C. Protein samples (50 $\mu$g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto a nitrocellulose membrane (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The membrane was blocked in 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h, and was incubated with the primary antibody against anti-IGF1 (cat. no. 250710; 1:200; Abbiotec LLC, San Diego, CA, USA) and β-actin (cat. no. BA2305; 1:5,000; Wuhan Boster Biotechnology, Ltd.) overnight at 4˚C. The membrane was then washed three times in TBST (15 min/wash), and was incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (cat. no. BA1055; 1:3,000; Wuhan Boster Biological Technology, Ltd.) for 1 h at 37˚C. Immunoreactive proteins were detected using enhanced chemiluminescence (Wuhan Boster Biological Technology, Ltd., Wuhan, China). Bands were quantified using Image J software (NIH, Bethesda, MD, USA).

*Dual luciferase reporter assay*. The psiCheck-2 dual-luciferase reporter vector (Promega Corporation, Madison, WI, USA) harboring an IGF1 wild type (WT) or mutant (MUT) 3'-untranslated region (UTR; Sangon Biotech, Co., Ltd., Shanghai, China) was inserted into the *Bg*III and *Kpn*I restriction sites at the 3'-end of the *Renilla* luciferase gene. For the luciferase assay, $5x10^5$ cells were cultured to ~80% confluence in 6-well plates. Subsequently, the cells were co-transfected with the miR-206 mimics or inhibitor, and either the WT or MUT 3'-UTR of the IGF1 dual luciferase reporter vector. Following incubation for 6 h with the transfection reagent/DNA complex, the medium was replaced with

fresh complete medium containing 10% FBS. A total of 48 h post-transfection, a Dual Luciferase Reporter Gene Assay kit (antibodies-online Inc., Atlanta, GA, USA) was used to determine the luciferase activities in each group on a GloMax® 96 Microplate Luminometer (Promega Corporation). The activity of *Renilla* luciferase was normalized against that of firefly luciferase.

*Enzyme-linked immunosorbent assay (ELISA) determination of IL-1β, TNF-α and Aβ*. The culture supernatants were collected from the treated BV-2 cells, in order to measure the levels of TNF-α and IL-1β. To measure Aβ peptide 1-42 (Aβ1-42) levels, cell lysates (the same preparation of lysates as used for western blotting) were used. Mouse IL-1β ELISA kit, mouse TNF-α ELISA kit and mouse Aβ1-42 ELISA kit were purchased from Nanjing SenBeijia Biotechnology Co., Ltd. (Nanjing, China) and were used to measure the levels of IL-1β, TNF-α and Aβ, according to the manufacturer's protocols. The optical density was detected at 450 nm using a microplate absorbance reader (Synergy™ Mx; BioTek, Winooski, VT, USA).

*β-secretase activity assay*. β-secretase activity in the BV-2 cells was determined using a β-Secretase Fluorometric Assay kit (Biovision Inc., Milpitas, CA, USA). Ice-cold extraction buffer (Beyotime Institute of Biotechnology, Wuhan, China) was used to extract protein from the treated cells. Following a 20 min incubation on ice, cell lysates were centrifuged at 10,000 x g for 5 min. The supernatant was collected and maintained on ice. A 50 $\mu$l sample (total protein, 150 $\mu$g) was added to each well in a 96-well plate, followed by 50 $\mu$l 2X reaction buffer and 2 $\mu$l β-secretase substrate, and the plate was incubated in the dark at 37˚C for 1 h. Fluorescence was detected at excitation and emission wavelengths of 335 and 495 nm respectively, using a GloMax® 96 Microplate Luminometer (Promega Corporation). β-secretase activity is expressed as relative fluorescence units per $\mu$g of protein sample.

*Statistical analysis*. All experiments were repeated at least three times with similar results, and the data are expressed as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were compared using a one-way analysis of variance with Dunnett test as a post-hoc comparison, or with the Student's t-test. Correlation was analyzed using Pearson's correlation. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*miR-206 expression levels are negatively correlated with the mRNA expression levels of IGF1 in the blood of patients with AD*. The expression levels of miR-206 and IGF1 were detected using SYBR green qPCR analysis. Blood samples from 30 patients with AD and 30 normal individuals were tested, the results indicated that miR-206 was significantly increased in the peripheral blood samples from patients with AD, as compared with in the paired normal blood samples (P=0.0004; Fig. 1A). However, as shown in Fig. 1B, the mRNA expression levels of IGF1 were significantly reduced in the peripheral blood from patients with AD, as compared with in the normal control individuals (P=0.0005). In addition, the expression levels of miR-206 were negatively correlated with the mRNA expression levels of IGF1 in the peripheral blood samples from patients with AD (Fig. 1C).

*IGF1 is a molecular target of miR-206*. The negative correlation detected between miR-206 and IGF1 in patients with AD promoted us to elucidate whether miR-206 directly targeted IGF1. Several miRNA public target-prediction algorithms [Pictar (http://pictar.mdc-berlin.de), TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do)] were used to assess this hypothesis. As shown in Fig. 2A, the binding site between miR-206 and IGF1 was conserved. The present study generated a dual luciferase reporter containing a putative binding site alongside a mutant construct (Fig. 2A). Luciferase activity was significantly reduced in cells co-transfected with miR-206 mimics and WT-IGF1, as the concentration of miR-206 mimics increased (P=0.0006, 0.008, 0.0002 and 0.0001 for 5, 10, 25 and 50 nM, respectively); however, decreased luciferase levels were not detected following co-transfection with the MUT-IGF1 vector. Furthermore, luciferase activity exhibited no significant alterations in the cells that were co-transfected with the miR-206 inhibitor and WT-IGF1 or MUT-IGF1. These data indicate that miR-206 directly targets the 3'-UTR of IGF1. Furthermore, as shown in Fig. 2D and E, ectopic expression of miR-206 resulted in a significant reduction in IGF1 mRNA (P=0.007) and protein (P=0.006) expression levels, whereas transfection with the miR-206 inhibitor restored the expression of IGF1 (P=0.0004 and 0.0002 for mRNA and protein, respectively). These results suggest that miR-206 may regulate the expression of IGF1 at the transcriptional level by directly targeting its 3'-UTR.

*miR-206 and IGF1 expression are upregulated and downregulated respectively, in LPS-treated microglial BV-2 cells*. LPS induces the expression of several miRNAs, which are involved in LPS-mediated inflammation (15). To determine whether miR-206 and IGF1 are involved in LPS-mediated immune responses in microglial cells, the expression levels of miR-206 and IGF1 were determined in LPS-stimulated BV-2 cells (microglial cells of BV-2 mice). The BV-2 cells were stimulated with various concentrations of LPS for a range of durations. The expression levels of miR-206 were significantly increased in a time-dependent (P=0.008, 0.0007, <0.0001 and <0.0001 for 4, 8, 12 and 24 h, respectively; Fig. 3A) and dose-dependent manner (P=0.038, 0.008 and <0.0001 for 100, 500 and 1,000 ng/ml, respectively; Fig. 3B), whereas the mRNA expression levels of IGF1 were markedly decreased in a time-dependent (P=0.009, 0.0005, <0.0001 and <0.0001 for 4, 8, 12 and 24 h, respectively; Fig. 3C) and dose-dependent manner (P=0.029, 0.0007 and <0.0001 for 100, 500 and 1,000 ng/ml, respectively; Fig. 3D), particularly when the cells were treated with 1 $\mu$g/ml LPS for 12 h. These results suggest a possible involvement for the miR-206/IGF1 pathway in LPS-mediated immune responses in brain microglia.

*miR-206 modulates the production of inflammatory cytokines and amyloidogenesis induced by LPS*. To investigate the

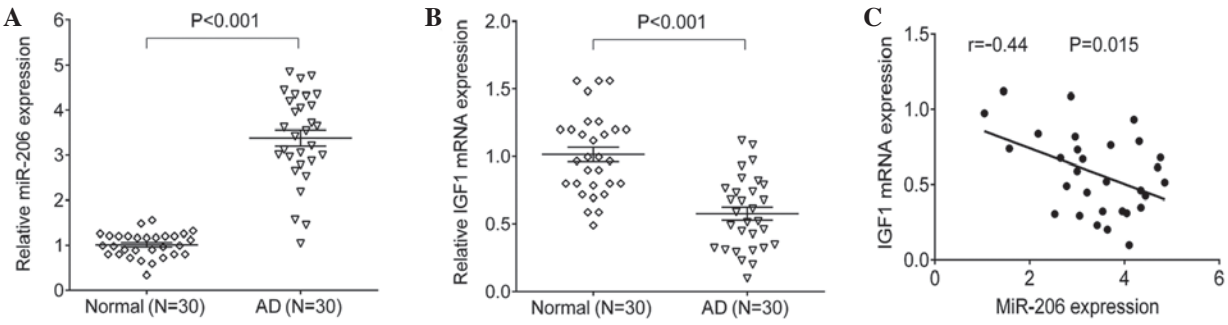


Figure 1. Correlation between miR-206 and IGF1 in patients with AD. (A) Relative expression levels of miR-206 in the blood samples of normal control individuals and patients with AD. (B) Relative mRNA expression levels of IGF1 in the blood samples of normal control individuals and patients with AD. (C) Correlation analysis between miR-206 and IGF1 expression. Data are presented as the mean ± standard deviation. AD, Alzheimer's disease; IGF1, insulin-like growth factor 1; miR, microRNA.

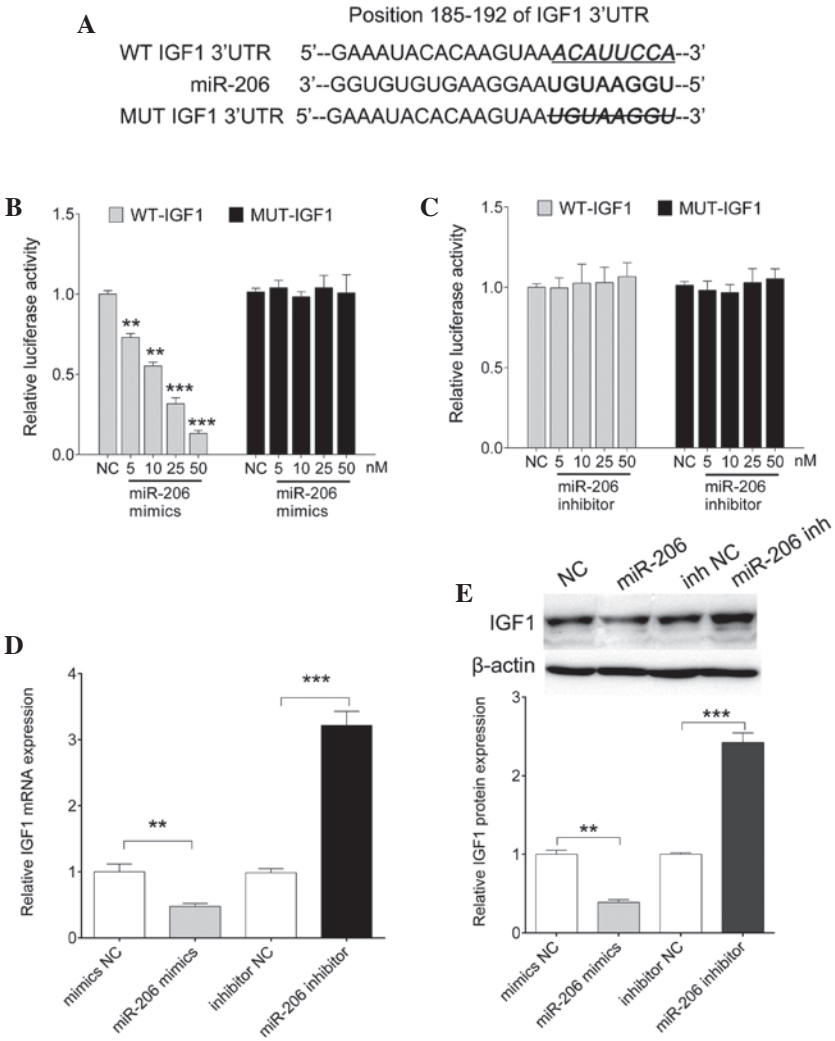


Figure 2. miR-206 directly targets the 3'-UTR of IGF1. (A) Predicted position of IGF1 3'-UTR targeted by miR-206, obtained from TargetScan. A MUT 3'-UTR was constructed (indicated by italics and strikethrough showing replacement with these bases). (B) Luciferase activity of the IGF1 3'-UTR was significantly suppressed following transfection with increasing concentrations of miR-206 mimics. MUT-IGF1 3'-UTR abrogated the miR-206-mediated suppression of luciferase activity. (C) Transfection with a miR-206 inhibitor did not alter the luciferase activity of IGF1 WT or MUT 3'-UTR. (D) Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression levels of IGF1 following transfection with miR-206 mimics or a miR-206 inhibitor. (E) Western blotting was used to detect the expression of IGF1 protein following transfection with miR-206 mimics or a miR-206 inhibitor. Data are presented as the mean ± standard deviation. $^{**}$P<0.01 and $^{***}$P<0.001, vs. NC. IGF1, insulin-like growth factor 1; miR, microRNA; UTR, untranslated region; WT, wild type; MUT, mutant; NC, negative control; inh, inhibitor.

involvement of miR-206 in inflammatory cytokine production, miR-206 was upregulated or downregulated in BV-2 cells by transfection with miR-206 mimics or an inhibitor for 24 h. Subsequently, the cells were treated with LPS (1 $\mu$g/ml)

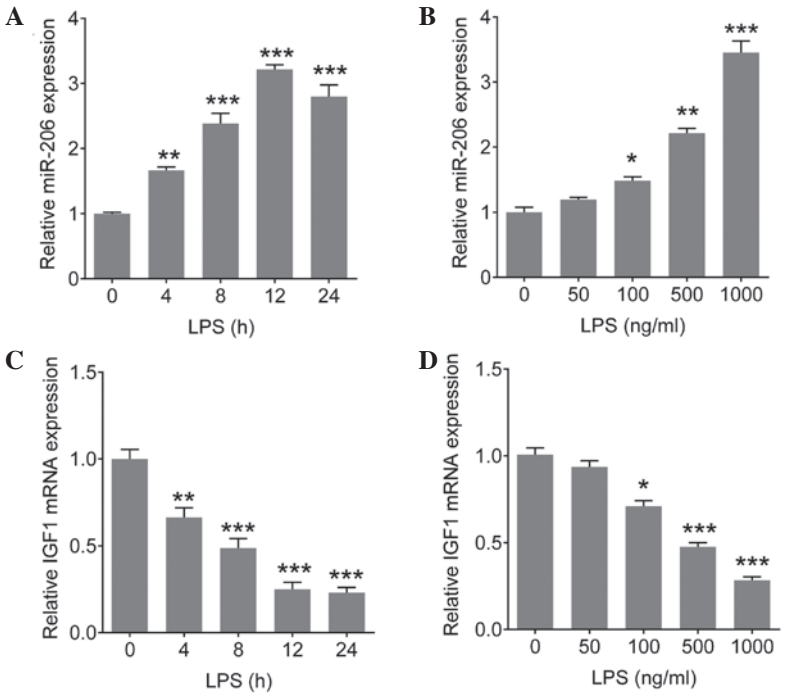


Figure 3. (A and B) Upregulation of miR-206 and (C and D) downregulation of IGF1 was observed in LPS-treated microglia. (A and C) BV-2 cells were exposed to 1 $\mu$g/ml LPS for 0, 4, 8, 12 or 24 h. (B and D) BV-2 cells were exposed to LPS at 0, 50, 100, 500 or 1,000 ng/ml for 12 h. Total RNA was extracted, and the miR-206 expression levels and IGF1 mRNA expression levels were detected using quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001, vs. 0 h or 0 ng/ml group. miR, microRNA; LPS, lipopolysaccharide; IGF1, insulin-like growth factor 1.

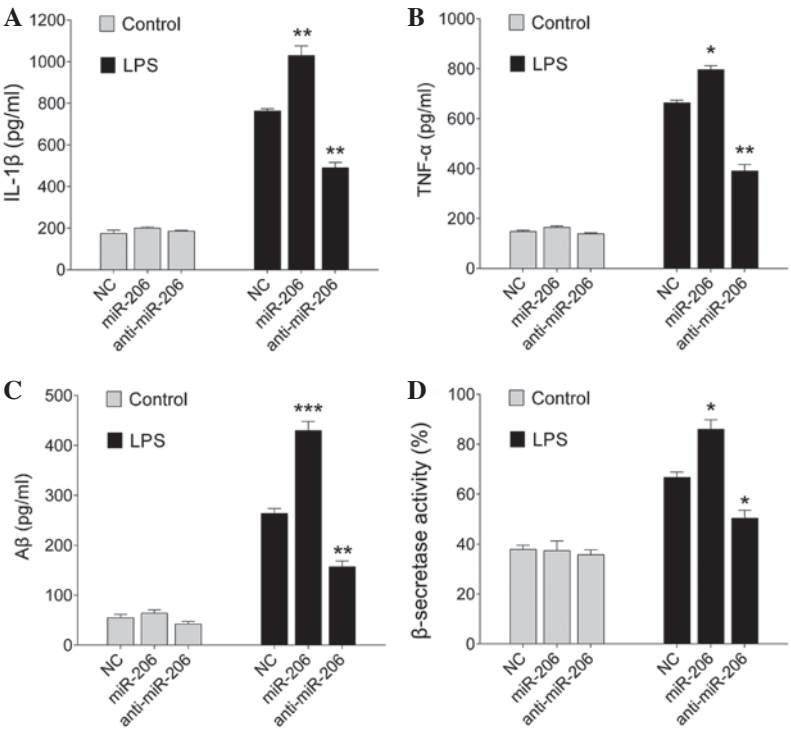


Figure 4. miR-206 enhanced LPS-induced inflammatory cytokine production and Aβ generation. BV-2 cells were transfected with miR-206 mimics, a miR-206 inhibitor or the NC for 24 h, and were then exposed to LPS (1 $\mu$g/ml) for 12 h. Levels of (A) IL-1β, (B) TNF-α, (C) Aβ and (D) β-secretase were detected by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. NC. miR, microRNA; LPS, lipopolysaccharide; Aβ, amyloid-β; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, negative control.

for an additional 12 h. Upregulation of miR-206 significantly increased the production of IL-1β and TNF-α, whereas downregulation of miR-206 markedly reduced their expression (P=0.006 for miR-206 and P=0.005 for anti-miR-206, an P=0.036 for miR-206 and P=0.006 for anti-miR-206; Fig. 4A and B, respectively), as determined by ELISA.

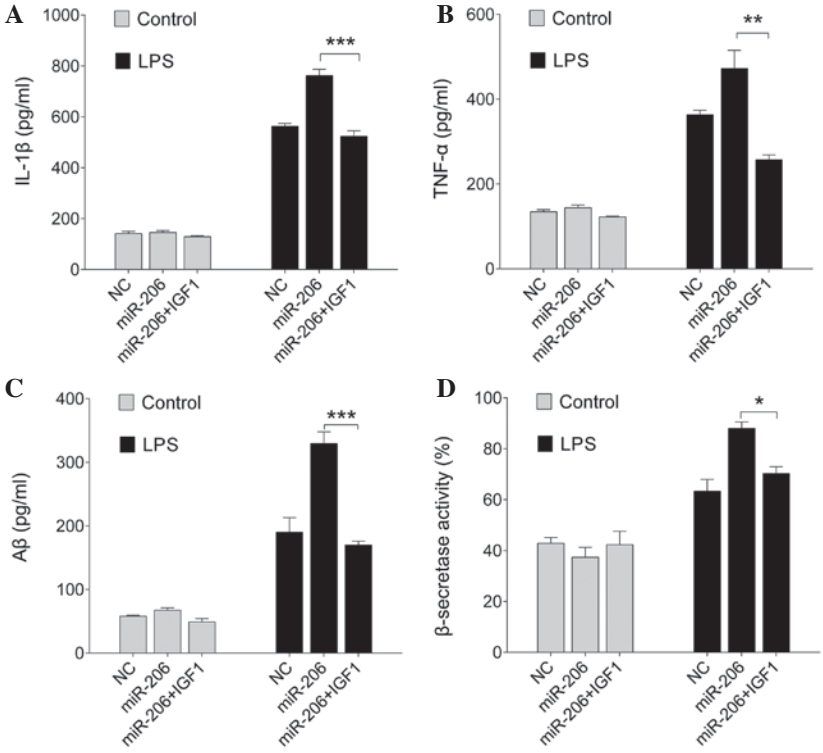


Figure 5. Exogenous IGF1 abrogates the miR-206-modulated LPS-induced proinflammatory response and Aβ generation. BV-2 cells were transfected with miR-206 mimics or the NC, and were then treated with exogenous IGF1 and exposed to 1 $\mu$g/ml LPS for a further 12 h. The concentration of (A) IL-1β, (B) TNF-α and (C) Aβ, and (D) the activity of β-secretases was measured by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001. miR, microRNA; LPS, lipopolysaccharide; Aβ, amyloid-β; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, negative control.

Microglia are a major source of neuroinflammation, which induces amyloid generation (16). To assess the involvement of miR-206 in modulating amyloidogenesis, BV-2 cells were transfected with miR-206 mimics or an inhibitor, and were stimulated with LPS for 12 h. As shown in Fig. 4C and D, when unstimulated, the cells expressed low levels of Aβ1-42 and β-secretase, whereas the expression levels of Aβ1-42 and β-secretase were increased in response to LPS (1 $\mu$g/ml) after 12 h. In addition, miR-206 overexpression increased LPS-induced Aβ1-42 (P<0.0001 and P=0.008 for miR-206 and anti-miR-206, respectively; Fig. 4C), and promoted the activation of β-secretases, the rate-limiting enzymes in Aβ generation (P=0.022 and P=0.037 for miR-206 and anti-miR-206, respectively; Fig. 4D). Notably, downregulation of miR-206 decreased LPS-induced Aβ1-42 and the activation of β-secretases (Fig. 4C and D). These results indicate that overexpression of miR-206 may have a pro-inflammatory and pro-amyloidogenesis effect.

*IGF1 abrogates the miR-206-modulated LPS-induced inflammatory response and amyloidogenesis.* IGF1 has an important anti-inflammatory role in activated microglial cells (17). To further investigate the link between miR-206-mediated regulation of IGF1 and inflammation, BV-2 cells were transiently transfected with miR-206 mimics for 24 h, and were then treated with exogenous IGF1 (5 $\mu$g/ml) and exposed to 1 $\mu$g/ml LPS for an additional 12 h. ELISA was used to quantify the levels of IL-1β, TNF-α, Aβ1-42 and β-secretases. As expected, overexpression of miR-206, together with IGF1, abrogated the miR-206-modulated LPS-induced proinflammatory response and amyloidogenesis (P=0.0007, 0.006 and <0.0001; Fig. 5A-D, respectively). These results indicate that the miR-206/IGF1 pathway is involved in the process by which LPS induces the production of proinflammatory cytokines and amyloidogenesis.

## Discussion

Activated microglia are associated with the progression of aging-associated neurodegenerative diseases, including AD, via the regulation of inflammation through the generation of IL-1β, TNF-α and other cytokines (18). Aβ deposition activates the complement system, which, in turn, stimulates microglia to release neurotoxic materials, including inflammatory factors (19). This positive feedback loop between Aβ deposition and microglial activation exacerbates the progression of AD, and the inflammatory response bridges this loop. Previous studies have indicated that aberrantly expressed miRNAs are increasingly being implicated in AD by regulation of Aβ, phosphorylation of tau protein and inflammation, which are the predominant pathomechanisms of AD (20,21). In addition, alterations in AD may be associated with the regulation of various miRNAs in blood and cerebral spinal fluid (CSF), particularly the brain-specific miRNAs secreted into blood and CSF, which could be considered as potential AD biomarkers (21,22). The present study demonstrated that miR-206 was significantly upregulated in blood samples from patients with AD compared with in age-matched

normal controls. In animal models of AD, previous studies have reported an increased expression of miR-206 in brain tissue, CSF and plasma of embryonic amyloid precursor protein (APP)/presenilin 1 transgenic mice (23) and Tg2576 mice (24). Furthermore, upregulation of miR-206 has been detected in serum from patients with mild cognitive impairment (25), and in the temporal cortex of human AD brains (24). These results indicated that upregulation of miR-206 in the peripheral circulation truly reflects the alterations in the AD brain.

Tian *et al* (23) and Lee *et al* (24) demonstrated that brain-derived neurotrophic factor (BDNF), a neuroprotective factor, was a target of miR-206. Similar to BDNF, IGFs, including IGF1 and IGF2, are the key regulators of memory, cognition and inflammation in the central nervous system (26,27). IGF2 was previously shown to reduce the number of hippocampal Aβ40- and Aβ42-positive amyloid plaques in APP mice (28,29). Furthermore, IGF2 may increase the protein levels of hippocampal BDNF and IGF1 (28). Delivery of IGF1 into the hippocampus of the APP mouse model Tg2576 was able to rescue behavioral deficits, promote dendritic spine formation and restore normal hippocampal excitatory synaptic transmission (29). In addition, microglia-specific deletion of the gene encoding the prostaglandin E2 receptor, a proinflammatory factor implicated in preclinical AD development, restored microglial chemotaxis and Aβ clearance, increased cytoprotective IGF1 expression, and was able to prevent memory deficits (30). The present study demonstrated that IGF1 was markedly reduced in blood samples from patients with AD. Notably, IGF1 was negatively correlated with miR-206 in human AD blood samples. As determined by dual luciferase reporter gene assay, miR-206 directly targeted the 3'-UTR of IGF1. In the present study, microglia were exposed to various concentrations of LPS for a range of durations; the results demonstrated that LPS induced miR-206 upregulation and IGF1 downregulation in a time- and dose- dependent manner. Lee *et al* (24) reported that miR-206 inhibition prevented the detrimental effects of Aβ42 in Tg2576 neurons *in vitro*, and third ventricle or intranasal administration of a miR-206 inhibitor into the cerebral ventricles of AD mice improved their memory function, and enhanced hippocampal synaptic density and neurogenesis. These results indicated that miR-206/IGF1 signaling may have a key role in microglia-mediated inflammation in AD.

In the present study, miR-206 expression was upregulated by transfecting microglial BV-2 cells with miR-206 mimics. The results revealed that following LPS treatment, increased miR-206 expression enhanced the release of proinflammatory cytokines, including IL-1β and TNF-α, and also increased the activity of β-secretase and the secretion of Aβ from microglia. Conversely, downregulation of miR-206 reduced the release of IL-1β and TNF-α, attenuated the activity of β-secretase and decreased the secretion of Aβ. Notably, exogenous IGF1 treatment abolished the effects of miR-206 on inflammation and Aβ generation in LPS-exposed microglia. Besides microglia, IGF1 treatment has been reported to reverse the Aβ-induced neurotoxic effects on survival of septal neurons (26). In addition, overexpression of miR-206 in astrocytes led to increased expression of inflammatory cytokines, including IL-6, IL-1β and chemokine (C-C motif) ligand 5 upon exposure to LPS, whereas knockdown of miR-206 had the opposite effects (9). A previous study suggested that loss of serum IGFI input to the brain may be an early biomarker of disease onset in AD mice (12).

In conclusion, the present study suggested that miR-206/IGF1 signaling may regulate microglial inflammation and amyloidogenesis, which are critical processes for the development of AD. In future experiments, we aim to determine the possible anti-AD effects of miR-206/IGF1 signaling in an animal model of AD. Taken together, these data indicated that targeting the small molecule miR-206, and treatment with exogenous IGF1 may be considered a novel therapeutic strategy for the treatment of inflammatory neurodegenerative diseases such as AD.

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