

Original Research

Muramyl dipeptide promotes A β 1-42 oligomer production via the NOD2/p-p38 MAPK/BACE1 signaling pathway in the SH-SY5Y cells

Yan-Jie Chen¹, Yuan-Jin Chan¹, Wen-Jing Chen², Ya-Ming Li^{1,*} and Chun-Yan Zhang^{3,*}¹Geriatrics Department of Chinese Medicine, Huadong Hospital, Fudan University, Shanghai, 200040, P. R. China²Geriatrics Department of Chinese Medicine, Huashan Hospital, Fudan University, Shanghai, 200040, P. R. China³Department of Chinese Medicine, East Hospital, Tongji University, Shanghai, 310000, P. R. China*Correspondence: doctorymli@163.com (Ya-Ming Li); cyzhang0810@163.com (Chun-Yan Zhang)DOI: [10.31083/j.jin.2020.03.112](https://doi.org/10.31083/j.jin.2020.03.112)This is an open access article under the CC BY 4.0 license (<https://creativecommons.org/licenses/by/4.0/>).

The relationship between chronic bacterial colonization in the brain and Alzheimer's disease is attracting extensive attention. Recent studies indicated that the components of bacterial biofilm drive the amyloid- β production. Muramyl dipeptide, the minimal bioactive peptidoglycan motif common to all bacteria, contributes to the development of many central inflammatory and neurodegenerative disorders. However, the involvement of Muramyl dipeptide in amyloid- β production is not completely defined. In our present study, wild type mice received an intracerebroventricular injection of normal saline or Muramyl dipeptide. Data showed that the production of A β 1-42 oligomers was significantly increased after Muramyl dipeptide injection in the wild type mice or incubation of the SH-SY5Y cells with Muramyl dipeptide. Moreover, the action of Muramyl dipeptide was dose- and time-dependent. The above results suggested a possibility that the Muramyl dipeptide-induced A β 1-42 oligomer production might be related to the NOD2/p-p38 MAPK/BACE1 pathway. To confirm this, the SH-SY5Y cells were transfected with siRNA NOD2. Data showed that the transfected SH-SY5Y cells exhibited decreased expression of A β 1-42 oligomer, NOD2, p-p38 MAPK, and BACE1 after treatment with Muramyl dipeptide. Finally, SH-SY5Y cells were pretreated with SB203580, an inhibitor of the p-38-MAPK pathway. The results indicated that these pretreated SH-SY5Y cells exhibited decreased expression of A β 1-42 oligomer, p-p38 MAPK, and BACE1 after treatment with Muramyl dipeptide. In conclusion, these results suggested that Muramyl dipeptide was the trigger factor for A β 1-42 oligomer production, which probably acts via the NOD2/p-p38 MAPK/BACE1 signaling pathway.

Keywords

Alzheimer's disease; Muramyl dipeptide; Nucleotide-binding oligomerization domain 2; β -site APP cleaving enzyme 1; A β 1-42 oligomer

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that typically manifests clinically as an isolated amnesic deficit and progresses to a characteristic dementia syndrome (Teipel et al., 2015). According to the classical amyloid hypothesis, the aggregation of amyloid- β (A β) into insoluble β -sheet fibrils initiates the pathological cascade of AD (De Strooper and Karran, 2016). Oligomers of the A β 1-42 protein are responsible for the development and progression of AD (Masters et al., 2015). Therefore, seeking and investigating the underlying cause that induces A β 1-42 oligomer production may be important in delaying the neurodegenerative progress of AD.

Recently, numerous studies indicated that bacteria could be a primary trigger point by inducing abundant cerebral production and deposition of A β . The study revealed that many bacteria exist in the brains of AD patients (Fulop et al., 2018): *Porphyromonas gingivalis* colonized in the brains of BALB/c mice could promote A β production (Dominy et al., 2019) and *Salmonella typhimurium* bacterial infections in the brains of 5XFAD mice resulted in accelerated A β deposition (Kumar et al., 2016). Studies have also demonstrated that the components of bacterial biofilms, such as the lipopolysaccharide (LPS) (Choi et al., 2017; Kim et al., 2017), drive the process of senile plaque formation (Allen and Morales, 2016; Miklosy, 2016). N-acetylmuramyl-L-alanyl-D-isoglutamine (Muramyl dipeptide, MDP) is the smallest essential bioactive peptidoglycan motif commonly present in all bacteria (Park et al., 2017). It is an inflammation-inducing molecule in the central nervous system (CNS) disorders (Cheng et al., 2018; Liu et al., 2015). Moreover, it exacerbates neuronal damage (Liu et al., 2015) and down-regulates cognition-related receptors (Grigoriev et al., 2008). Hence, MDP might play a pathological role in AD. The relationship between MDP and A β has not been addressed yet. Combining numerous evidence of bacterial components participating in A β production (Allen and Morales, 2016; Choi et al., 2017; Kim et al., 2017; Miklosy, 2016), we hypothesized that MDP might be involved in this process. Therefore, defining the involvement of MDP in A β 1-42 oligomer production

and seeking the potential molecular mechanisms might disclose the veil of bacteria promoting the development of AD.

The p38 mitogen-activated protein kinase (p-38 MAPK) is a stress-activated enzyme that mediates various cellular activities, including inflammation and apoptosis (Kheiri et al., 2018). It is hyperactivated in the brains of AD patients and mice (Sun, 2003). Prolonged p-38 MAPK hyperfunction leads to accelerated generation and deposition of A β , while p-38 MAPK inhibitors reduce the A β plaque load (Colié et al., 2017). A β peptide is generated from the cleavage of A β PP primarily by the β -site APP cleaving enzyme 1 (BACE1) (Read and Suphioglu, 2019). Research has confirmed that p-38 MAPK promotes the expression of A β by increasing the level of BACE1 (Guo et al., 2011). In contrast, the blockage of p-38 MAPK is indicated to effectively ameliorate A β deposition by decreasing the expression of BACE1 (Schnöder et al., 2016). Hence, BACE1 is the crucial enzyme in the process of A β generation that is mediated by p-38 MAPK. Moreover, data from *in vitro* and *in vivo* experiments have verified that MDP could activate p-38 MAPK (Chen et al., 2018; Liu et al., 2015). The evidence above suggested that p-38 MAPK/BACE1 might be the missing link in MDP-induced A β production. However, the regulators involved in MDP-induced activation of p-38 MAPK are not confirmed.

Nod-like receptors (NLRs) mainly recognize exogenous pathogen-associated molecular patterns (PAMP) such as bacteria. NLRs participate in the pathological mechanism of multiple CNS disorders, including AD (Faustin and Reed, 2013; Ma et al., 2013). Nucleotide-binding oligomerization domain 2 (NOD2) is a member of the NLRs and functions as a specific intracellular recognition receptor for MDP (Al Nabhani et al., 2017). Physiologically, NOD2 has much lower expression in the brain, while its expression increases substantially after a bacterial infection (Nakamura et al., 2014). The higher expression of NOD2 destroys the neurovascular integrity, promotes inflammatory cell infiltration in the CNS, leads to neuronal damage and memory impairment, and eventually contributes to cerebral injury (Chauhan et al., 2009; Liu et al., 2015; Wang et al., 2018). Numerous studies demonstrated that p-38 MAPK mediates the proinflammatory effect of NOD2 in signaling transduction (Zhang et al., 2015). After binding with its ligands, NOD2 recruits the receptor-interacting protein 2 (RIP2). It activates the p-38 MAPK to stimulate a cascade of reactions such as the inflammatory response and oxidative stress (Negroni et al., 2018). NOD2, when agitated by MDP, directly contributes to the activation of p-38 MAPK. Therefore, it is considered the pivotal receptor that mediates MDP-induced A β production.

Based on the above evidence, we speculated the potential underlying mechanism: MDP promotes A β 1-42 oligomer production via the NOD2/p-38 MAPK/BACE1 pathway. To prove this, we carried out a preliminary exploration at the animal level. Wild type (WT) mice received an intracerebroventricular injection of normal saline (NS) or MDP. We detected the A β 1-42 oligomer and pathway-related proteins by the western blot. We further explored the mechanism at the cellular level. We incubated SH-SY5Y cells with MDP and demonstrated that MDP facilitates A β 1-42 oligomer production. Moreover, we pretreated SH-SY5Y cells with siRNA NOD2 or SB203580 to test the role of NOD2 and p-38 MAPK in A β 1-42 oligomer generation, respectively. Thus,

this research aims to examine the involvement of MDP in A β 1-42 oligomer production and elucidate the underlying mechanism.

2. Materials and methods

2.1 Animals

Animals were 28-week-old male wild type (WT) mice. They were purchased from GemPharmatech Company (Nanjing, P. R. China). The mice were pair-housed in standard cages with food and water. The colony room temperature was maintained at 25 ± 1 °C on a 12-hour light/dark cycle. After two weeks of acclimatization, the mice were randomly divided into two groups: (1) WT mice were injected stereotactically with 1.0 μ l of normal saline (NS) and (2) WT mice were injected stereotactically with 1.0 μ l of MDP (10 μ g/ μ l). The mice were exposed to NS or MDP for four weeks. They were then killed after anesthesia to samples. All experiments were conducted in accordance with the National Research Council's guide for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. The Animal Ethics Committee approved this study of the Department of Laboratory Animal Science of Fudan University (Approval No: 201902006S).

2.2 Reagents and antibodies

Muramyl dipeptide was purchased from InvivoGen (San Diego, CA, USA), while the rabbit anti-NOD2 antibody was purchased from Sigma (CA, USA). The mouse anti-phospho-p38 MAPK and anti- β -actin primary antibodies were purchased from Cell Signaling Technology (MA, USA). The rabbit anti-p-38 MAPK and anti-BACE1 antibodies were purchased from Proteintech (Chicago, USA). The rabbit anti-A β 1-42 antibody was purchased from Abcam (Cambridge, UK). The goat anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology (MA, USA). SB203580, an inhibitor of the p-38 MAPK pathway, was purchased from Cell Signaling Technology (MA, USA).

2.3 Cell culture and treatments

The SH-SY5Y cells were kindly donated by the State Key Laboratory of Medical Neurobiology, Fudan University (Shanghai, P. R. China). The SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) and were supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μ M streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were subcultured every 2-3 days. For experiments, the cells were grown to 70-80% confluence, and the media were replaced with Opti-MEM (Invitrogen).

2.4 Preparation of MDP

MDP was prepared as described. Briefly, the lyophilized powder of MDP was dissolved in endotoxin-free water at a concentration of 10 mg/ml and stored at -20 °C. Before use, this MDP solution was diluted to concentrations of 5 μ g/ml, 10 μ g/ml, and 20 μ g/ml in cold Opti-MEM media.

2.5 Immunofluorescence staining

For immunofluorescence staining, 30-50% confluent SH-SY5Y cells were seeded on coverslips before various treatments were applied, as described below. Cells were washed with PBS and underwent fixation with 4% paraformaldehyde for 20 minutes

at room temperature. After that, blocking was performed with the Blocking Buffer for Immunol Staining (Beyotime Biotechnology, Shanghai, P. R. China) for 1 h. The cells were then incubated overnight with the primary antibody anti-A β 1-42 (1: 100) at 4 °C and goat anti-rabbit IgG 488-conjugated secondary antibody for 1 h. The nuclei were stained with DAPI for 5 minutes. All samples were assessed using the Laser Scanning Confocal Microscopy (LEICA TCS SP8, Germany).

2.6 Knockdown of NOD2 with siRNA

NOD2 small interfering RNAs (siRNA: sense 5'-GCCUGAUGUUGGUCAAGAATT-3' and antisense 5'-UUCUUGACCAACAUCAGGCCA-3') were synthesized by Hippo Biotechnology. Cells were grown to 30-40% confluency and then transfected with Lipofectamine RNAiMAX (Invitrogen) and siRNA according to the manufacturer's instructions. RT-PCR was utilized to verify the silencing efficiency after 48 h of siRNA transfection.

2.7 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured SH-SY5Y cells by using the TRIzol reagent, and a ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan) was used to obtain cDNA. Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green RT-PCR Master Mix kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol and then amplified with the real-time PCR detection system (Eppendorf AG, Hamburg, Germany). Amplification conditions were set as 40-cycles program (95 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s). The mRNA level of the target gene described below was normalized to that of β -actin, and the results were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Sequences of the upstream and downstream PCR primers to detect NOD2 mRNA used in qRT-PCR were 5'-TGT GCG GAC TCT ACT CTT-3' and 5'-GTG AAC CTG AAC TTG AAC TC-3', respectively. Sequences of the upstream and downstream PCR primers to detect BACE1 mRNA were 5'-TCT GTC GGA GGG AGC ATG AT-3' and 5'-CCA CGG AAA CTT TGT AAT GA -3', respectively. Sequences of the upstream and downstream PCR primers to detect β -actin were 5'-GTG GAC ATC CGC AAA GAC-3' and 5'-TAG AAA GGG TGT AAC GCA ACT A-3', respectively.

2.8 Western blot analysis

The SH-SY5Y cells and the hippocampus tissues of the WT mice were collected and lysed in the loading buffer. The protein suspensions were collected, and a BCA kit determined the protein concentration. The lysates were then evaluated for protein expression using the western blot. After denaturation, equal amounts of proteins were loaded on a 10% SDS-polyacrylamide gel. Proteins were separated by gel electrophoresis under a constant voltage of 100 V before being transferred to the nitrocellulose membrane (Millipore, USA). After getting blocked at room temperature, proteins on the membrane were incubated with primary antibodies. After washing, the membranes were further incubated with secondary antibodies for 1 h. Western blot bands were captured using the Gel Imaging System (BIO-RAD, USA).

2.9 Statistics

All results are expressed as the Mean \pm SD. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software

Inc., San Diego, CA, USA). All experiments were repeated independently three times. Statistical significance of the difference among different groups was analyzed by one-way ANOVA or the Student's *t*-test. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1 MDP upregulated the expressions of A β 1-42 oligomer, NOD2, p-p38 MAPK, and BACE1 in the WT mice

As mentioned earlier, the NOD2/p-38 MAPK/BACE1 pathway was related to the MDP-induced A β 1-42 oligomer production. Hence, the pathway-related proteins were detected (Fig. 1A). As seen in Fig. 1A and 1B, the WT mice, subjected to a 10 μ g/ μ l injection of MDP, exhibited a higher expression of the hippocampal A β 1-42 oligomer. Further, the WT mice injected with MDP also exhibited higher expressions of NOD2, p-p38 MAPK, and BACE1 (Fig. 1B). Thus, these results suggested that MDP upregulated the expressions of A β 1-42 oligomer, NOD2, p-p38 MAPK, and BACE1 in the WT mice.

3.2 MDP upregulated the expressions of A β 1-42 oligomer, NOD2, p-p38 MAPK, and BACE1 in the SH-SY5Y cells

The pathway was further explored at the cellular level by employing the SH-SY5Y cell line. The concentration gradient and time gradient were set up for MDP. Results from the western blot (Fig. 2A) showed that MDP upregulated the expression of A β 1-42 oligomer in a dose-dependent manner after incubation with the SH-SY5Y cells for 24 h (Fig. 2B). It was noticed that when SH-SY5Y cells were treated with 5 μ g/ml MDP, the expression level of A β 1-42 oligomer was upregulated, but this increase was not statistically significant (Fig. 2B). Meanwhile, the incubation of the SH-SY5Y cells with 10 μ g/ml MDP (Fig. 2C) resulted in an increase in the expression of the A β 1-42 oligomer in a time-dependent manner (Fig. 2D). These results suggested that MDP promoted A β 1-42 oligomer production in the SH-SY5Y cells.

The pathway-related proteins were then detected. Results from the western blot (Fig. 2A and 2C) showed that MDP upregulated the expressions of NOD2, p-p38 MAPK, and BACE1 in a dose- and time-dependent manner (Fig. 2B and 2D). Moreover, results from qRT-PCR showed that MDP also upregulated the mRNA level of NOD2 and BACE1 in a dose- and time-dependent manner (Fig. 2E and 2F).

These data from *in vivo* and *in vitro* experiments suggested that the MDP-induced A β 1-42 oligomer production might be related to the NOD2/p-38 MAPK/BACE1 pathway. In the next step, SB203580 and siRNA NOD2 were employed to test the role of p-38 MAPK and NOD2 in the MDP-induced A β 1-42 oligomer production, respectively.

3.3 Inhibition of P-38 MAPK prevented MDP-induced upregulation of BACE1 and A β 1-42 oligomer in the SH-SY5Y cells

SB203580, an inhibitor of the p-38 MAPK pathway, was employed to determine the engagement of p-38 MAPK in the MDP-induced upregulation of BACE1 and A β 1-42 oligomer. As seen in Fig. 3A and 3B, after MDP treatment, the ratio of p-p38 MAPK/p-38 MAPK in the SH-SY5Y cells pretreated with SB203580 was lower than that in the cells not pretreated with SB203580. Detection of the level of BACE1 (Fig. 3A) was the next step. It was

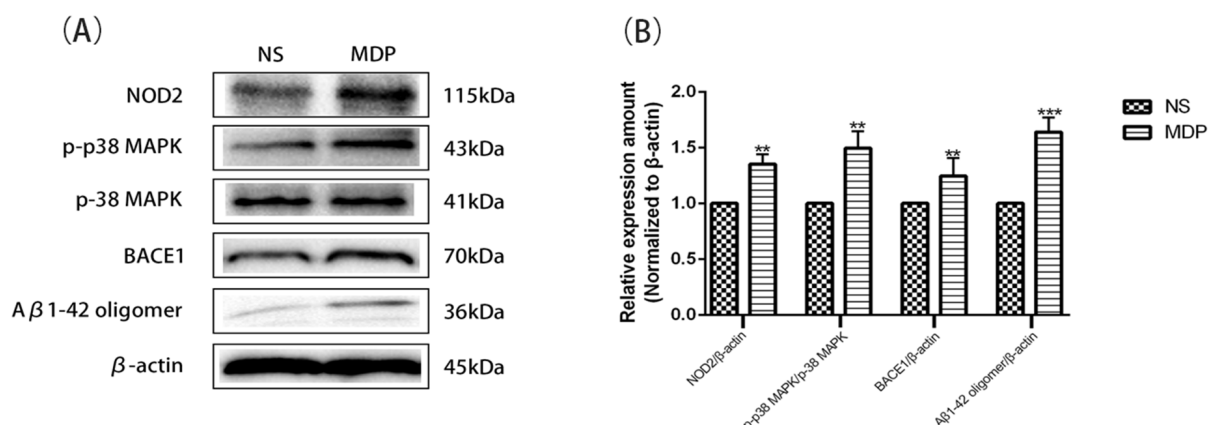


Fig. 1. MDP upregulates the expressions of Aβ1-42 oligomer, NOD2, p-p38 MAPK, and BACE1 in WT mice. (A) The effect of MDP (10 μg/μl) on the upregulation of hippocampal NOD2, p-p38 MAPK, BACE1, and Aβ1-42 oligomer. (B) The corresponding bar graphs showing the quantification of the respective molecules in (A). Results are expressed as the mean ± SEM from at least three separate experiments. Note. **versus NS: $P < 0.01$, ***versus NS: $P < 0.001$.

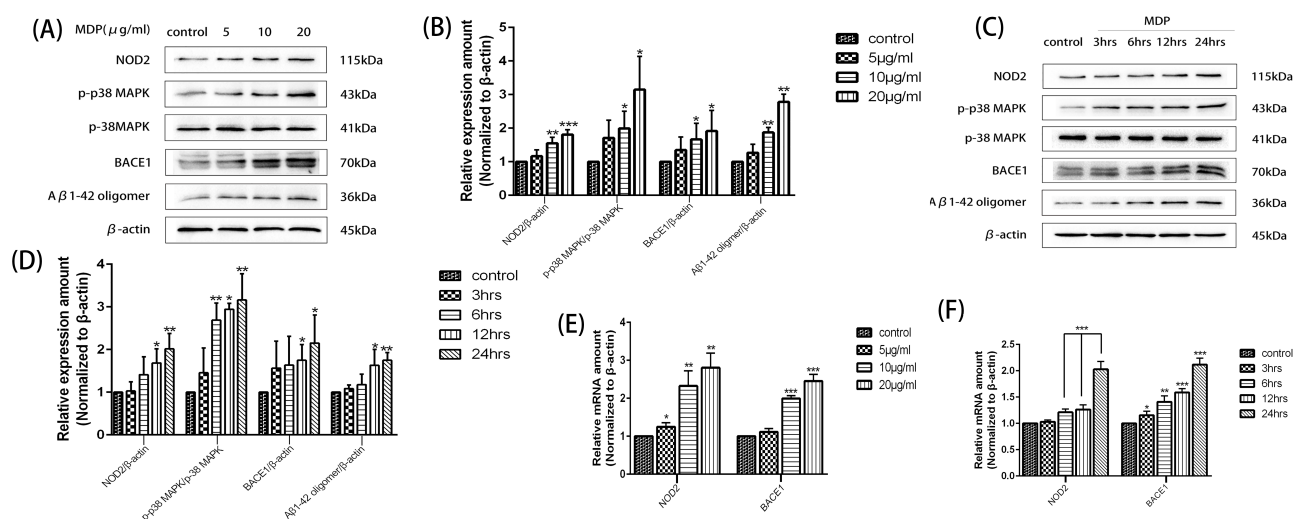


Fig. 2. MDP upregulates the expressions of NOD2, p-p38 MAPK, BACE1, and Aβ1-42 oligomer in the SH-SY5Y cells at different concentrations (5 μg/ml, 10 μg/ml, 20 μg/ml) and at different times (3 h, 6 h, 12 h, and 24 h). (A) The western blot determines the effect of different concentrations of MDP on the upregulation of NOD2, p-p38 MAPK, BACE1, and Aβ1-42 oligomer in the SH-SY5Y cells after incubation for 24 h. (C) The western blot detects the effect of 10 μg/ml MDP on the upregulation of NOD2, p-p38 MAPK, BACE1, and Aβ1-42 oligomer in the SH-SY5Y cells at different times. (B and D) are the corresponding bar graphs showing quantification of (A) and (C), respectively. The mRNA levels of NOD2 and BACE1 are shown in (E) and (F), respectively. Results are expressed as the Mean ± SEM from three separate ($n = 3$) experiments performed independently. Note. *versus control: $P < 0.05$, **versus control: $P < 0.01$, *** versus control: $P < 0.001$.

found that the SH-SY5Y cells pre-treated with SB203580 exhibited a decrease in the BACE1 levels at both the gene expression level (Fig. 3C) and the mRNA level (Fig. 3H). Moreover, the level of Aβ1-42 oligomer was also analyzed. The anti-Aβ1-42 antibody from Abcam (ab201060) could detect two forms of Aβ1-42, including the Aβ1-42 monomer and the Aβ1-42 oligomer. Results from the Laser Scanning Confocal Microscopy (Fig. 3F) showed that the fluorescence intensity of Aβ1-42 was enhanced by MDP and was repressed by SB203580 in comparison with the intensity of the control cells (Fig. 3G). We were considering the results from western blot (Fig. 3A) [that the SH-SY5Y cells pre-treated with SB203580 exhibited decreased expression of the Aβ1-42 oligomer

after treatment with MDP, but without any change in the expression of the Aβ1-42 monomer (Fig. 3D-3E)], we concluded that the Aβ1-42 oligomers mainly caused the change in the fluorescence intensity. These results suggested that MDP induced the upregulation of BACE1 and Aβ1-42 oligomer via a p-38 MAPK-dependent pathway in the SH-SY5Y cells.

3.4 siRNA NOD2 reversed the MDP-induced upregulation of p-p38 MAPK, BACE1, and Aβ1-42 oligomer in the SH-SY5Y cells

The SH-SY5Y cells were transfected with siRNA NOD2. It was seen from the results that the silencing efficiency was higher than 70% (Fig. 4I), and the expression of NOD2 was suppressed by

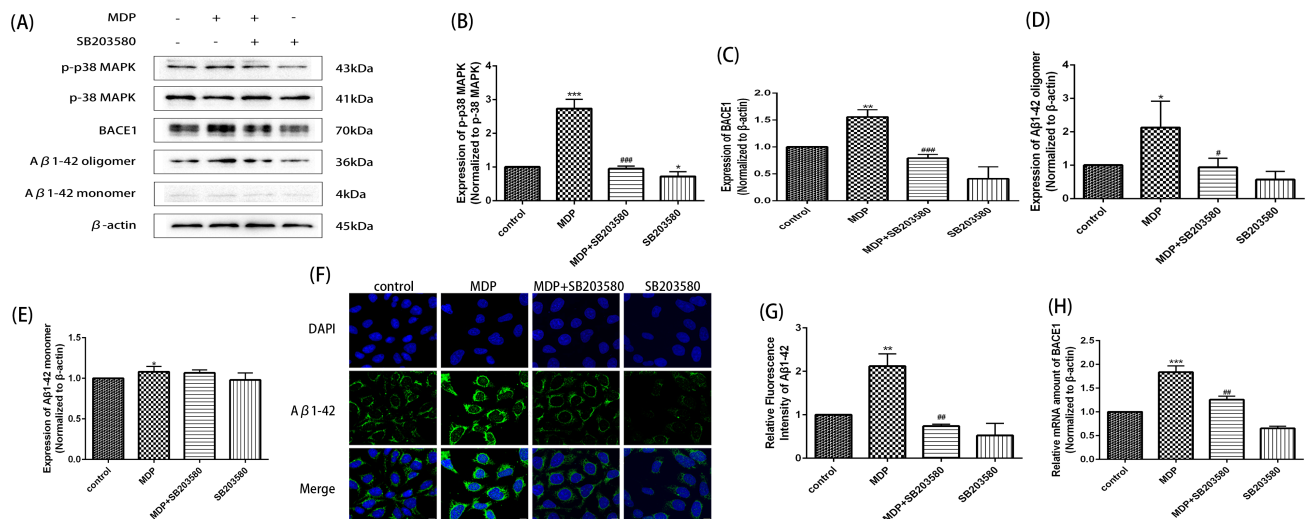


Fig. 3. SB203580 suppresses the MDP-induced upregulation of BACE1 and Aβ1-42 oligomer in the SH-SY5Y cells. (A) The western blot determines the expressions of p-p38 MAPK, p-38 MAPK, BACE1, Aβ1-42 monomer, and Aβ1-42 oligomer. The mRNA level of BACE1 is shown in (H). (B-E, G) are the corresponding bar graphs showing quantification of parameters in (A) and (F), respectively. (F) Aβ1-42 location within the cytoplasm of SH-SY5Y cells is induced by MDP (10 μg/ml) and is decreased by SB203580 (10 μM). Aβ1-42 is shown in green and DAPI in blue color. Bar = 10 μm. Results are expressed as the mean ± SEM from at least three separate experiments.

Note. *versus control: $P < 0.05$, **versus control: $P < 0.01$, ***versus control: $P < 0.001$, #versus MDP group: $P < 0.05$, ##versus MDP group: $P < 0.01$, ###versus MDP group: $P < 0.001$.

siRNA (Fig. 4A-4B). The next step was the detection of the level of the Aβ1-42 oligomer. From the LSCM results (Fig. 4G), it was observed that the fluorescence intensity of Aβ1-42 was enhanced by MDP and was repressed by siRNA (Fig. 4H). Combining with results from the western blot (Fig. 4A) that the SH-SY5Y cells transfected with siRNA exhibited decreased expression of Aβ1-42 oligomer after treatment of MDP, but without any change in the expression of the Aβ1-42 monomer (Fig. 4E-4F). It was concluded that the Aβ1-42 oligomers mainly caused the change in the fluorescence intensity. Thus, NOD2 was a fatal receptor that mediated the MDP-induced Aβ1-42 oligomer production.

Finally, the effect of siRNA NOD2 on the MDP-induced activation of p-38 MAPK and BACE1 was assessed (Fig. 4A). As seen in Fig. 4C-4D, the SH-SY5Y cells transfected with siRNA, exhibited decreased expression of p-p38 MAPK and BACE1 after treatment with MDP. Results from qRT-PCR (Fig. 4J) showed that siRNA NOD2 repressed the mRNA level of BACE1 that was induced by MDP. Therefore, it was suggested that NOD2 mediated the MDP-induced Aβ1-42 oligomer production via the p-p38 MAPK/BACE1 pathway in the SH-SY5Y cells.

4. Discussion

The etiology of AD is highly complex and emphasizes the primacy of the Aβ1-42 oligomer (De Strooper and Karran, 2016). Recent observations demonstrated that bacteria could invade the central nervous system (CNS) and drive the formation of senile plaques (SPs) that are considered as the underlying cause of AD (Dominy et al., 2019; Kumar et al., 2016). The amyloid beta-precursor protein (AβPP) and Aβ might occur in bacterial biofilms. Meanwhile, SPs were reported to contain elements of biofilm constituents, revealing that the co-localization of biofilms with Aβ is a signature finding in AD (Fulop et al., 2018). Some

scholars suggested that the production and deposition of Aβ may be related to certain critical components in the biofilms (Allen and Morales, 2016; Miklossy, 2016). LPS, derived from the gram-negative bacterial biofilm, could have the expression of Aβ in the brains of ICR mice (Choi et al., 2017; Kim et al., 2017). We expected to seek an extensively representative component to elucidate the molecular mechanism of bacteria in promoting Aβ production. MDP is an immunoreactive derivative of peptidoglycan that is commonly found in all bacteria (Park et al., 2017); it plays a vital role in the amplification of inflammation in neurodegenerative diseases (Cheng et al., 2018). Therefore, MDP emerged as a suitable candidate to mimic the bacteria-induced infected microenvironment in the brain.

To test the above hypothesis, the WT mice were first injected with MDP. Results from the western blot confirmed the role of MDP in promoting the production of the cerebral Aβ1-42 oligomer (Fig. 1A). To investigate the developing effects of MDP on Aβ1-42 oligomer production at the cellular level, the SH-SY5Y cells were further incubated with MDP. Results from the western blot showed that MDP up-regulated the expression of Aβ1-42 oligomer in a dose- and time-dependent manner (Fig. 2A and 2C). It was initially determined that bacterial MDP was the possible trigger factor that promoted Aβ1-42 oligomer production under *in vivo* and *in vitro* conditions. Nevertheless, the precise mechanism remained unclear.

BACE1 is thought to initiate the amyloidogenic pathway that cleaves AβPP to form the N-terminus of the Aβ peptides (Read and Suphioglu, 2019). Extensive research has mentioned that cerebral BACE1 is hyperactivated in AD patients and mice (Fukumoto et al., 2004). However, sustained BACE1 inhibition could reverse the amyloid deposition, indicating that BACE1 is closely related to the process of Aβ production (Hu et al., 2018). Bacteria and

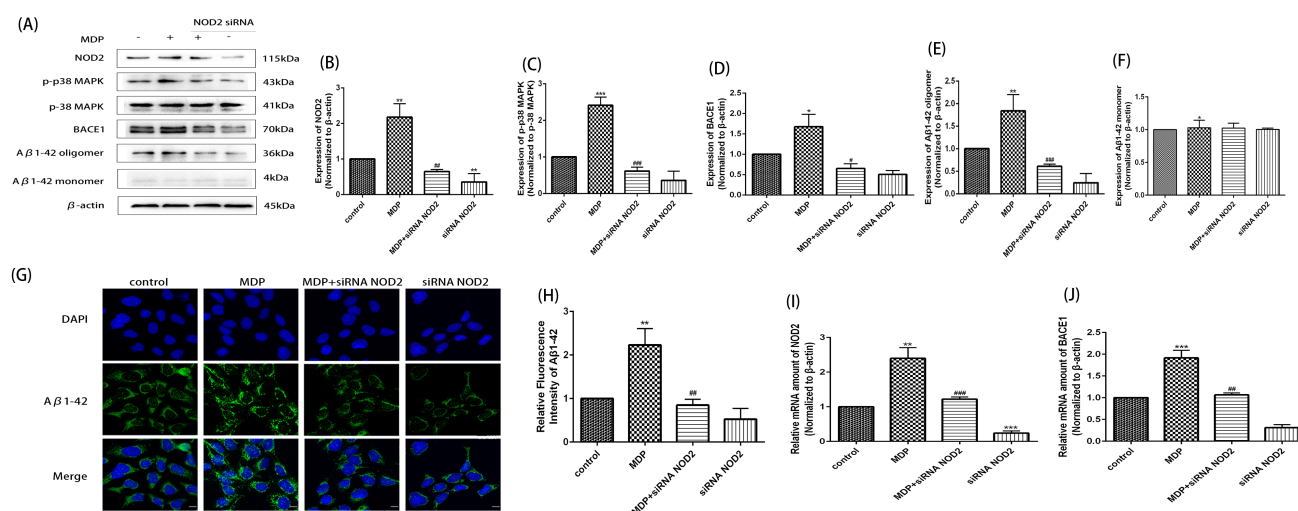


Fig. 4. siRNA NOD2 inhibited the MDP-induced upregulation of p-p38 MAPK, BACE1, and Aβ1-42 oligomer in the SH-SY5Y cells. (A) The expressions of NOD2, p-p38 MAPK, p-38 MAPK, BACE1, Aβ1-42 monomer, and Aβ1-42 oligomer were determined by western blot. The mRNA level of NOD2 and BACE1 were shown in (I) and (J). (G) Aβ1-42 location within the cytoplasm of the SH-SY5Y cells was induced by MDP (10 μg/ml) and was repressed by siRNA NOD2 (50 nM). Aβ1-42 is shown in green and DAPI in blue color. Bar = 10 μm. (B-F, H) are the corresponding bar graphs showing quantification of (A) and (G), respectively. Results are expressed as the mean ± SEM from at least three separate experiments.

Note. *versus control: $P < 0.05$, **versus control: $P < 0.01$, ***versus control: $P < 0.001$, #versus MDP group: $P < 0.05$, ##versus MDP group: $P < 0.01$, ###versus MDP group: $P < 0.001$.

their active components have been reported to upregulate the expression of BACE1 (Choi et al., 2017; Kim et al., 2017). Despite this, the relationship between MDP and BACE1 is undefined.

It was found that the WT mice injected with MDP exhibited a higher expression of BACE1 (Fig. 1A). The above *in vivo* published result was consistent with our *in vitro* result that indicated that MDP increased both the gene expression and the mRNA level of BACE1 in a dose- and time-dependent manner in the SH-SY5Y cells (Fig. 2A, 2C, 2E, and 2F). It was suggested that the MDP-dependent upregulation of the expression of Aβ1-42 oligomer might be related to BACE1. Hence, it was feasible to seek the mechanism of MDP-induced Aβ1-42 oligomer production based on BACE1. Accumulating research has proved that the p-38 MAPK plays a critical role in the regulation of BACE1 and Aβ in AD pathogenesis (Schnöder et al., 2016). MDP has been reported to activate the p-38 MAPK (Chen et al., 2018; Liu et al., 2015). As expected, the WT mice and the SH-SY5Y cells exhibited a higher expression of p-p38 MAPK after treatment with MDP (Fig. 1A, 2A, and 2C). However, little information is available regarding the crosstalk between the p-38 MAPK and MDP-induced upregulation of BACE1 and Aβ1-42 oligomer. Therefore, the p-38 MAPK inhibitor, SB203580, was utilized to observe the role of MDP in this process. It was noticed that the SH-SY5Y cells pretreated with SB203580 exhibited a lower ratio of p-p38 MAPK/p-38 MAPK after treatment with MDP in comparison with the ratio observed in the control cells (Fig. 3A-B). Meanwhile, SH-SY5Y cells pretreated with SB203580 exhibited decreased BACE1 levels at both the gene expression (Fig. 3A) and the mRNA levels (Fig. 3H). The next step was the detection of the Aβ1-42 oligomer. Results from LSCM and western blot confirmed that SB203580 repressed the increased expression of Aβ1-42 oligomer that was induced by MDP (Fig. 3A and 3F). It was revealed that MDP in-

duced the upregulation of BACE1 and Aβ1-42 oligomer via a p-38 MAPK-dependent pathway.

Multiple studies indicate that NOD2 is a risk factor for neurodegenerative disorders like Parkinson's disease (PD) (Cheng et al., 2018; Ma et al., 2013) and multiple sclerosis (MS) (White et al., 2014). Previous research has revealed that NOD2 induces neuronal damage and cognitive dysfunction, thus implying a pathological role of NOD2 in AD as well (Chauhan et al., 2009; Liu et al., 2015). However, less information is available about the relationship between NOD2 and Aβ1-42 oligomer. To investigate this aspect, the SH-SY5Y cells were transfected with siRNA NOD2. It was shown that the MDP-induced increase in the Aβ1-42 oligomer level was repressed by siRNA NOD2 (Fig. 4A and 4H). This suggested that MDP might promote Aβ1-42 oligomer production in a NOD2-dependent manner.

Numerous studies have proved that NOD2 regulates the p-38 MAPK to amplify an inflammatory response (Negroni et al., 2018; Zhang et al., 2015). However, the involvement of NOD2 in MDP-induced upregulation of p-p38 MAPK remains uncertain. It was further detected that SH-SY5Y cells transfected with siRNA NOD2 exhibited a lower expression of p-p38 MAPK after treatment with MDP (Fig. 4A). Moreover, siRNA NOD2 inhibited the MDP-induced upregulation of BACE1 at both the gene expression and the mRNA levels (Fig. 4A and 4J). Thus, it was demonstrated that NOD2 mediated the MDP-induced Aβ1-42 oligomer production via the p-p38 MAPK/BACE1-dependent pathway in the SH-SY5Y cells.

It was initially demonstrated that MDP promoted Aβ1-42 oligomer production via the NOD2/p-38 MAPK/BACE1 pathway in the *in vivo* (mice) and the *in vitro* SH-SY5Y models. These results might provide a possible underlying mechanism to investigate bacteria-induced Aβ1-42 oligomer production or the amy-

loidogenesis in Alzheimer's disease. However, there are some limitations. (1) LPS has been reported to be associated with A β aggregation and neuronal toxicity (Martins, 2018). For example, the distribution of LPS on the cell membrane and its interaction with apolipoprotein E disorders the peripheral A β metabolism and further affects the A β generation in neurodegeneration and AD (Martins, 2015). Besides, MDP synergistically enhances the LPS-mediated biological activities (Kitauro et al., 2018). Therefore, the role of LPS needs to be considered in the investigation of bacteria-induced A β generation in SH-SY5Y cells. Moreover, we need to explore whether MDP plays a role in A β generation independent of LPS or synergistically with LPS in SH-SY5Y cells. (2) It is well-known that the pathological process of A β aggregation involving excessive A β production and perturbed A β clearance. However, our research focused on exploring the mechanism of A β production induced by MDP. More experiments need to determine whether MDP-induced A β precipitation is related to the perturbed A β clearance. (3) Neuroinflammation and A β aggregation are the primary pathogenic mechanisms of AD. NOD2 is one of the well-studied members of the NLRs. It has been reported that MDP activates the human NLRP1 inflammasome and induces interleukin-1 beta processing by NOD2 (Cui et al., 2014; Hsu et al., 2008; Pan et al., 2007). Therefore, it is necessary to explore if MDP mediates neuroinflammation in AD by NOD2. Thus, in our following study, we would like to further investigate the role of NOD2 in A β clearance induced by MDP to provide a precise mechanism for the bacterial promotion of the deposition of A β .

Author contributions

Ya-Ming Li and Chun-Yan Zhang designed the research study. Yan-Jie Chen performed the research. Yuan-Jin Chan provided help and advice on the western blot experiments. Yan-Jie Chen analyzed the data. Yan-Jie Chen and Wen-Jing Chen wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Animal Ethics Committee approved this study of the Department of Laboratory Animal Science of Fudan University (Approval No: 201902006S).

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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