Clin. Lab. 2021;67:XXX-XXX


## ORIGINAL ARTICLE

# Biglycan Expression Promotes β-Amyloid-Induced Microglial Activation via TLR2 in Mouse Cell Culture Model

Wenbiao Duan [1], Jinhui Zou [1], Xue Chen [1], Chungou Xiao [1], Wei Jiang [2]

[1] *Department of Anatomy, Zhaoqing Medical College, Zhaoqing City, Guangdong Province, China*
[2] *Department of Anatomy & Histology, Shenzhen University Health Science Centre, Shenzhen City, Guangdong Province, China*

## SUMMARY

***Background:*** **Alzheimer disease (AD) is one of the most frequent neurodegenerative disorders that results in the progressive loss of memory and severe impairments and death. The experimental results showed that the neuroinflammation involving microglia and cytokines, especially the neuritic plaques composed of aggregates of β-amyloid protein, also play a major risk in AD. Biglycan (BGN) is involved in the regulation of neuronal cell division that could induce the expression of proinflammatory factors. Furthermore, BGN also exerts effects on β-amyloid-induced microglial dysfunction and contributes to AD pathogenesis. However, the mechanisms underlying the regulatory role of BGN on β-amyloid-induced microglial activation remain unclear. This study intended to investigate whether BGN could promote β-amyloid induced microglial activation through TLRs in immortalized murine microglial (BV2) cells.**

***Methods:*** **The levels of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) were detected by enzyme-linked immunosorbent assay (ELISA). The Cell Counting kit-8 (CCK8) assay was performed to detect cell viability. The expression level of a microglia marker (CD11b) was detected by immunofluorescence. The mRNA and protein expression levels of BGN and Toll-like receptor 2 (TLR2) were determined by quantitative RT-PCR and western blotting.**

***Results:*** **BGN was upregulated in activated microglial cells. Knockdown of BGN efficiently attenuated β-amyloid-induced microglial activation and expressions of pro-inflammatory factors. Furthermore, the present findings provided evidence showing that BGN could regulate β-amyloid-induced microglial activation through TLR2 in BV2 cells.**

***Conclusions:*** **Our results suggested that TLR2 signaling may be involved in the regulatory role of BGN in β-amyloid-induced microglial activation.**

**(Clin. Lab. 2021;67:xx-xx. DOI: 10.7754/Clin.Lab.2020.200252)**

**Correspondence:**
Wei Jiang
Department of Anatomy & Histology
Shenzhen University Health Science Centre
Xueyuan Avenue No. 1066
Nanshan District
Shenzhen City, 518055, Guangdong Province
China
Phone: +86 755-26912819
Email: WeiJianglko@126.com

Manuscript accepted May 25, 2020

**KEY WORDS**

biglycan, Alzheimer disease, β-amyloid, TLR2, microglial activation

## INTRODUCTION

Alzheimer disease (AD) is one of the most frequent neurodegenerative disorders that result in the progressive loss of memory, late-life dementia and severe impairment and death [1-3]. The neuropathology of AD is complex and characterized by the accumulation of extracellular senile plaques and loss of synaptic connec-

tions within selective brain regions [4,5]. The neuro-inflammation also plays a major risk in the progression of AD, including microglia and cytokines [6,7], and especially the neuritic plaques composed of aggregates of β-amyloid protein [8,9]. Although the molecular mechanism underlying synapse dysfunction in AD is still elusive, over the past decade substantial evidence has indicated that β-amyloid may be implicated in the pathogenesis of AD [10]. The accumulation of β-amyloid can affect the activation of microglial cells and secrete pro-inflammatory factors, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) [11-13]. Consequently, β-amyloid accumulation leads to neural cell injury and neurological disorders.

Microglia are the brain's tissue macrophages that are resident innate immune cells in the central nervous system [14,15]. Microglial cells are confined by the fully developed blood-brain barrier in mature brain and retain the ability to self-renew through proliferation in the brain to replace senescent cells [16,17]. Microglial cells possess a number of receptors such as Toll-like receptors (TLRs) to detect microbial and viral pathogens and tissue injury [18]. TLRs have an important role in the effects of microglia on regulating chronic inflammation, which lead to neurotoxicity in neurodegenerative disease [19,20]. The aggregates of β-amyloid can activate microglial cells and become a prominent source of pro-inflammatory mediators such as TNF-α and directly induce neuronal death [21,22]. Therefore, inhibition of β-amyloid to induce pro-inflammatory cytokine secretion and microglial activation has been considered a useful therapy for AD.

Recent studies have reported that BGN, a small leucine-rich repeat proteoglycan, which was detected mainly at aquaeductus cerebri and involved in the regulation of neuronal cell division [23]. In macrophages, BGN is directly involved in cell signaling through TLR4 and TLR2, which rapidly activates the p38, ERK, and NF-κB and induces the expression of pro-inflammatory factors [24,25]. Based on these studies, it raises the possibility that BGN can exert an effect on β-amyloid induced microglial dysfunction and thus contribute to the pathogenesis of AD. This is the first study showing the expression of BGN in activated microglial cells and investigating whether the underlying mechanism is involved with TLRs.

## MATERIALS AND METHODS

### Cell culture and cell transfection

Immortalized murine microglial cells (BV2) were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 mg/mL penicillin, and 0.1 mg/mL of streptomycin (Invitrogen) at 37°C and 5% $CO_2$. BV2 cells were grown to 70% - 80% confluence and treated with 1 or 5 μM of β-amyloid (Aβ1-42, Sigma) for 24 hours. The β-amyloid is soluble in DMSO at 0.5 mM and diluted into 10 mM sodium phosphate for use. The biglycan and TLR2-specific shRNA plasmid stably expressed a short hairpin RNA (shRNA) and lentiviral helper plasmid pHelper1.0 (gag/pol element); and Helper2.0 (VSVG element). The shRNA targeting BGN (5'-GCTATTGAGTTGGAGGA CCTA-3'), TLR2 (5'-GCGGAAGATAATGAACACC AA-3') and the negative control (NC) shRNA (5'-TTCTCCGA ACGTGTCACGT -3') were constructed by GeneChem Corporation (Shanghai, China). The shBGN, shTLR2 and shNC plasmid were transfected into cells using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions.

### Western blot analysis

BV2 cells were lysed and extracted in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), 1:10 complete protease inhibitors (Roche). The concentration of protein was determined using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). Protein (30 μg) was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were incubated in blocking solution (5% non-fat milk) for 1 hour. The membranes were incubated with 1:1,000 dilution primary antibody (anti-TLR2 (ab16894), anti-BGN (ab49701), anti-β-actin (ab8227), Abcam) overnight at 4°C. Then, the membranes were incubated with secondary antibody for 1 hour and visualized using electrochemiluminescence. Signals were detected by a chemiluminescence system and quantified by ImageJ.

### Cell viability assay

Cells were seeded in 96-well plates at a density of 1 x $10^4$ cells per well and were either untreated or treated with 1 μM or 5 μM β-amyloid for 24 hours prior to harvesting. Then, 10 μL of WST-8 solution (Cell Counting Kit-8; ab228554, Abcam) was added to each well and incubated at 37°C for 3 hours. The plate was measured on a microplate reader (PerkinElmer, Envision) at a wavelength of 460 nm.

### Quantitative real-time PCR

BV2 cells were harvested and total RNA was isolated using a total RNA kit. BGN and TLR2 mRNA expression levels were measured by qRT-PCR using PrimeScript RT reagent Kit and SYBR Green Master Mix kits. The cycling conditions comprised 10 minutes polymerase activation at 94°C and 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. RNA expression data was analyzed by cycle threshold. The BGN and TLR2 primers were as follows:

BGN:

Forward: 5'-TCTATCTGCACTCCAACAACAT-3'

Reverse: 5'-GATGCCATTATAGTAGGCCCTC-3'

TLR2:

Forward: 5'-TTGTCCCGTGCAAACTTGCCGGGGA

GGA-3'
Reverse: 5'-AAGTCCCGTTATTACTTGCCGGTTAG GA-3'

**Enzyme-linked immunosorbent assay (ELISA)**
The proinflammatory factors concentrations were assessed from cultured BV2 cells, using Human TNF alpha and IL-1 beta ELISA Kit (ab181421 & ab46052, Abcam) according to the manufacturer's instructions. The plates were measured at the absorbance of 450 nm.

**Immunofluorescence**
The BV2 cells were grown to 5 x $10^5$ cells/well and fixed with 4% paraformaldehyde for 30 minutes. The cells were blocked with 3% BSA and incubated with 0.1% Triton X-100 for 1 hour. Then incubated at 4°C with the primary antibodies anti-CD11b (ab62817, Abcam, 1:100) followed by the secondary antibodies Alexa Fluor® 594 (Thermo Scientific, USA, 1:1,000). The cell's nuclei were detected by 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI, Thermo Scientific, USA). The images were acquired with the fluorescence microscope (Olympus, BX51) and analysis with ImageJ software.

**Statistics**
All data were performed using SPSS 17.0 statistical analysis software and presented as mean ± SD from 3 independent experiments. Statistical analysis was performed using Student's *t*-test for comparison between two groups. The data were analyzed using one-way ANOVA. Differences between means were considered significant at $p < 0.05$.

## RESULTS

**Biglycan expression is upregulated in activated microglial cells.**
To investigate the effects of β-amyloid on microglial cells (BV2), we examined the expression levels of pro-inflammatory cytokines, TNF-α and IL-1β, after treatment with 1 or 5 μM of β-amyloid. As demonstrated in Figure 1A, the treatment of β-amyloid induced the intracellular β-amyloid concentration directly. Compared with the control group, treatment with β-amyloid dose-dependently induced the expression levels of TNF-α and IL-1β (Figure 1A). As shown in Figure 1B, CCK8 assay showed that β-amyloid treatment significantly increased the microglial viability rate. To evaluate whether β-amyloid could induce microglial activation, a microglial marker CD11b was detected by immunofluorescence. Figure 1C showed that CD11b expression was enhanced significantly by β-amyloid treatment in a dose-dependent manner. These results suggested that β-amyloid increased the activation of microglia.
Next, we investigated whether β-amyloid could regulate BGN expression in activated microglial cells. The mRNA and protein expression levels of BGN were determined by quantitative RT-PCR and western blotting. As shown in Figure 1D and 1E, β-amyloid treatment caused higher levels of BGN expression than in the control group. These results indicated that BGN was upregulated in activated microglial cells.

**Biglycan knockdown inhibits β-amyloid-induced expressions of pro-inflammatory factors in microglial cells.**
To demonstrate the effect of BGN on β-amyloid-induced expressions of pro-inflammatory factors, BGN was efficiently knocked down in BV2 cells and co-treated with 5 μM of β-Amyloid for 24 hours. The mRNA and protein expression levels of BGN in BV2 cells were assessed (Figure 2A and 2B). As expected, BGN were significantly knocked-down in sh-BGN group compared to the control group (shNC group). ELISA results showed that compared with shNC group, the pro-inflammatory factors, TNF-α and IL-1β, were significantly decreased (Figure 2C). Therefore, BGN knockdown might protect BV2 cells against β-amyloid-induced expressions of pro-inflammatory factors.

**Knockdown of biglycan attenuates β-amyloid-induced activation of microglia.**
To assess whether knockdown of BGN also inhibited β-amyloid-induced microglial activation, the CD11b level was measured by using immunofluorescence in BV2 cells with BGN knockdown. As detected by fluorescence microscopy, the numbers of CD11b positive cells were significantly lower in the shBGN group compared with the shNC group after β-amyloid treatment (Figure 3A). Next, we detected microglial viability by CCK8 assay after BGN was knocked down. Consistent with immunofluorescence results, the viability rate of microglial cells was significantly decreased in the shBGN group compared with the shNC group after β-amyloid treatment (Figure 3B). These results suggested that knockdown of BGN efficiently attenuated β-amyloid-induced microglial activation.

**Biglycan regulates β-amyloid-induced microglial activation via TLR2**
To subsequently investigate whether TLR2 was involved in the regulatory effects of BGN on β-amyloid-induced microglial activation, TLR2 was efficiently knocked down in BV2 cells and co-treated with 5 μM of β-amyloid for 24 hours. The TLR2 mRNA and protein expression levels were determined by quantitative RT-PCR (Figure 4A) and western blotting (Figure 4B). As expected, the TLR2 expression levels were significantly knocked down in the shTLR2 group compared to the shNC group. The following analysis investigated whether BGN could regulate β-amyloid-induced expressions of pro-inflammatory factors and microglial activation through TLR2 in BV2 cells. TLR2 knockdown decreased the expression of pro-inflammatory factors, TNF-α and IL-1β (Figure 4C). Immunofluorescence assay showed that, compared with the control group,

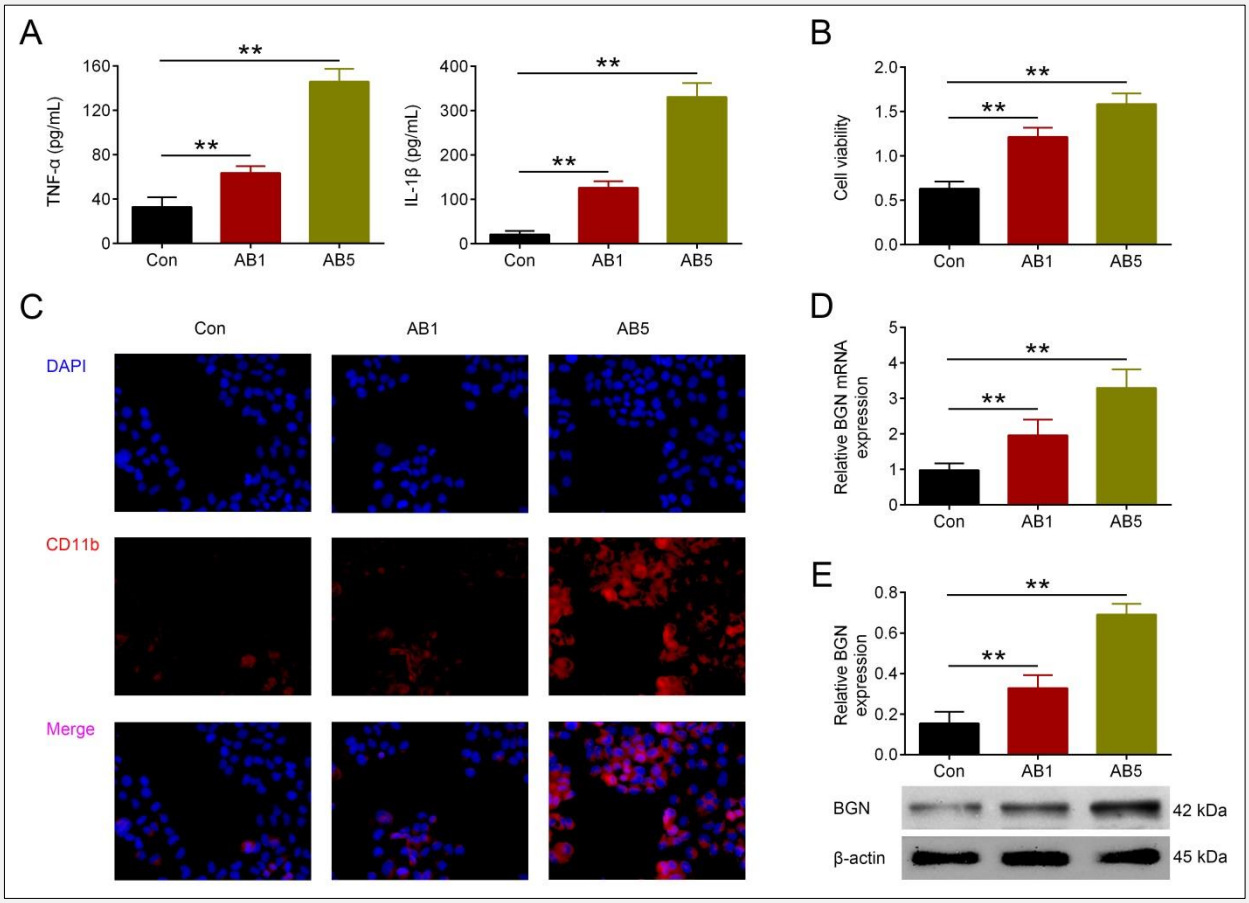


**Figure 1. Expression of BGN in activated microglial cells.**

**BV2 cells were exposed to different concentrations of β-amyloid for 24 hours. (A) The β-amyloid induced expression levels of internalized β-amyloid (Aβ1-42), TNF-α, and IL-1β. (B) β-Amyloid increased the viability rate in BV2 cells. (C) CD11b expression was enhanced significantly after treatment of β-amyloid. (D) β-Amyloid treatment groups showed high levels of BGN mRNA expression. (E) BGN expression was significantly increased in β-amyloid treatment group. (Data was shown as means ± SD, ** - $p < 0.01$).**

shTLR2 reversed β-amyloid-induced overexpression of CD11b (Figure 4D). Besides, significantly decreased cell viability was found in shTLR2 group than control group (Figure 4E). These findings provided evidences showing that BGN could regulate β-amyloid-induced microglial activation through TLR2 in BV2 cells.

## DISCUSSION

AD is a common disease worldwide, and nearly 50 million people have AD or related dementia [26]. The disease is characterized by an irreversible brain neurodegenerative disorder, neurofibrillary tangles, and β-amyloid aggregation that slowly destroy memory and cognitive function. Overexpression of β-amyloid protein leads to senile plaque formation and correlative memory impairment, which eventually cause pathologically abnormal and neurodegenerative diseases [27,28]. Several studies have reported that high levels of β-amyloid could activate microglia and further induce an inflammatory response [29-31]. Recently, the small leucine-rich proteoglycan family member BGN, which is present in the extracellular matrix of central nervous system, was demonstrated to exert protective effects on inflammatory responses or neurodegenerative disorders. However, the mechanisms underlying the effects of BGN on β-amyloid-induced microglial activation remain unclear.

In the present study, we investigated the role of BGN in β-amyloid-induced microglial activation. These results found that β-amyloid enhanced the activation of mi-

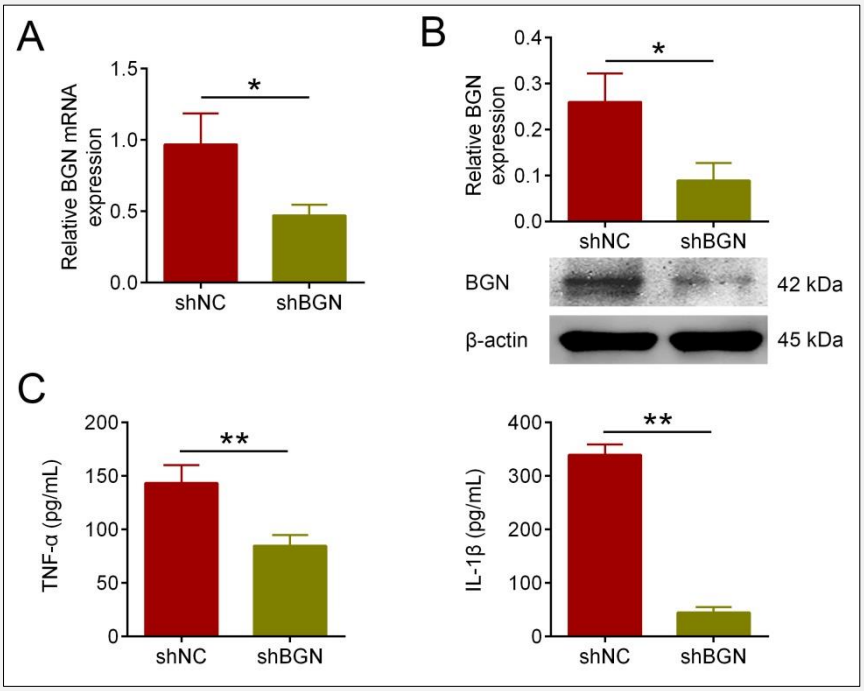


**Figure 2. Effect of BGN on β-amyloid-induced expressions of pro-inflammatory factors in microglial cells.**

**BGN expression was knocked down in BV2 cells and exposed to 5 μM of β-amyloid for 24 hours. (A) BGN mRNA expression level was significantly knocked down in the shBGN group. (B) BGN expression level was significantly decreased in the shBGN group. (C) TNF-α and IL-1β expressions were decreased after β-amyloid treatment. (Data was shown as means ± SD, * - p < 0.05, ** - p < 0.01).**

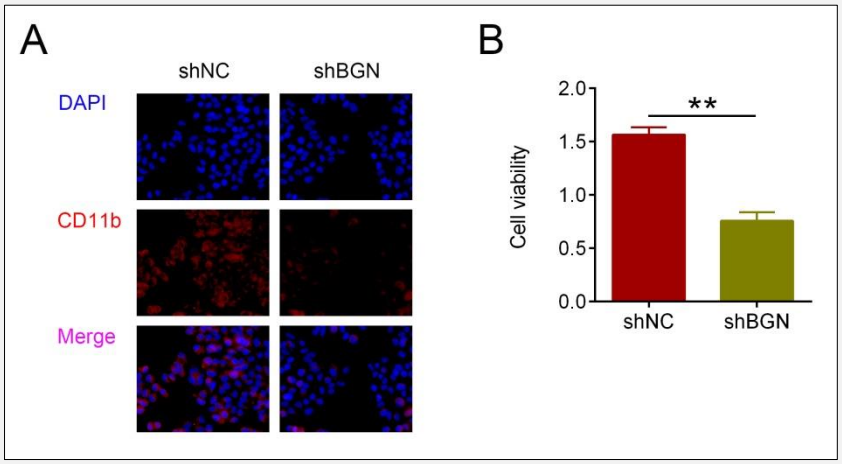


**Figure 3. Effect of BGN on β-amyloid-induced microglial activation.**

**BGN expression was knocked down in BV2 cells and exposed to 5 μM of β-amyloid for 24 hours. (A)The level of CD11b was significantly lower in the shBGN group compared to the control group. (B) The cell viability rate was decreased in the shBGN group compared to the control group. (Data was shown as means ± SD, ** - p < 0.01).**

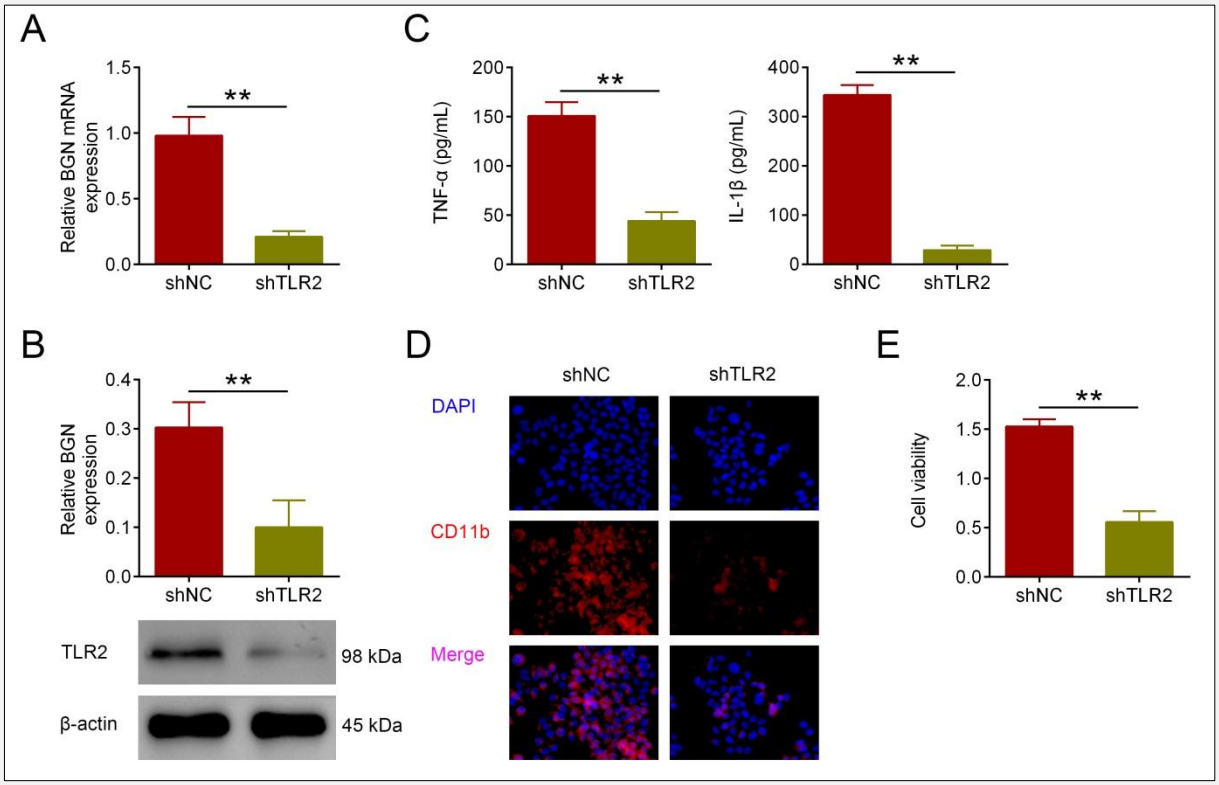


**Figure 4. Involvement of TLR2 in the effects of BGN on β-amyloid-induced microglial activation.**

**BV2 cells were exposed to 5 μM of β-amyloid for 24 hours. (A) The mRNA expression of TLR2 in BV2 cells. (B) The TLR2 expression level was significantly knocked down in the shTLR2 group compared to the control group. (C) TLR2 knockdown decreased the expression levels of TNF-α and IL-1β. (D) Immunofluorescence assay was used to determine CD11b level. (E) Cell viability was examined by CCK8 assays. (Data was shown as means ± SD, ** - $p < 0.01$).**

croglial cells. Furthermore, BGN expression was increased in activated microglial cells, evidenced by quantitative RT-PCR and western blot. Consistently, recent results show that BGN is a secretory product of macrophages that leads to the rapid activation of NF-Kb and exerts pro-inflammatory function [32,33]. BGN plays a critical role in regulating brain plasticity and peripheral metabolism. This study demonstrated that knockdown of BGN by shRNA in BV2 cells suppressed β-amyloid-induced neuron cell injury by attenuating the expressions of pro-inflammatory factors and reducing microglial activation. Pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 are reported to cause acute and chronic neuronal diseases. Our results indicated that BGN knockdown contributed to the protective effect of neuro-inflammatory responses in an AD cell model. Consistent with our findings, BGN was considered as a crucial pro-inflammatory factor of lipopolysaccharide-induced sepsis in BGN-null mice, due to a mitigated inflammatory response that results in less organ injury [34]. Recent studies demonstrated that pro-inflammatory factors trigger the macrophages to secrete BGN in the innate immune system and strongly influence the activity of macrophages [35]. For instance, BGN acts as a pro-inflammatory factor mediating immunity by activating p38 and Erk1/2 in macrophages. Interestingly, other cancer studies also showed that deletion of BGN could inhibit tumor endothelial cell migration [36].

TLRs are known to be ligated by different pathogen associated molecules. A recent study reported that BGN is closely related to macrophages through binding to TLR2 [37]. However, the mechanisms between TLR2 and BGN in β-amyloid-induced microglial activation are poorly understood. Our finding reported that knockdown of TLR2 reversed the β-Amyloid-induced overexpression of CD11b and viability of BV2 cells. The present study has indicated that BGN regulates the β-amyloid-induced microglial activation through TLR2. Increasing studies have shown that BGN promotes in-

flammation through TLR4 and TLR2 signaling in various diseases [33,37,38]. These results are consistent with our studies, showing that BGN promoted β-amyloid-mediated activation of microglia through TLR2 signaling in the pathology of AD.

## CONCLUSION

In summary, this study demonstrated that β-amyloid could trigger BGN overexpression and promote inflammatory responses and microglial activation. Knockdown of BGN in BV2 cells could reverse the β-amyloid-induced microglial activation and neurodegenerative injury. Those results suggested that TLR2 signaling may be involved in the regulatory effects of BGN on β-amyloid-induced microglial activation.

**Source of Funding:**
This work was supported by the Natural Science Foundation of Guangdong Province (No. 2017A030313904, No. 2018A030310643), Science and Technology Foundation of Shenzhen City (No. JCYJ20170818142019 191), and Medical Scientific Research Foundation of Guangdong Province (No. A2016618).

**Declaration of Interest:**
The authors declare that no conflict of interest is associated with this work.

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