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ATTENUATION OF CD11B EXPRESSION AND PRO-INFLAMMATORY RESPONSES VIA GLYCOGEN SYNTHASE KINASE-3 INHIBITION IN LIPOPOLYSACCHARIDE STIMULATED BV2 MICROGLIAL CELLS

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Abstract

Background: Excessive production of pro-inflammatory mediators by microglia may lead to neuroinflammation and neuronal death. Glycogen synthase kinase-3 (GSK-3) is a regulatory enzyme that involves in the mechanism of pro- and anti-inflammatory responses. Inhibition of GSK-3 potentially attenuates the aggravation of neuroinflammation. **Objective:** We aimed to investigate the effects of GSK-3 inhibition on the immune responses through assessment of the expressions of CD11b and production of immune mediators in lipopolysaccharide (LPS)-stimulated BV2 microglial activation. **Methods:** The activation status of BV2 stimulated with LPS was determined by the expression of CD11b through flow cytometry analysis. The production and expression of nitric oxide (NO) and pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) in the absence or presence of GSK-3 inhibitors namely Tideglusib (TG) and lithium chloride (LiCl) in LPS-stimulated BV2 cells were determined by using Griess assay, BD cytometric beads array mouse inflammatory kit, and quantitative RT-PCR (qPCR), respectively. **Results:** Activation of microglia through stimulation with LPS significantly increased the expression of CD11b and the production and expression of NO and pro-inflammatory cytokines. The expression of CD11b reduced approximately 17% and 23% (p<0.001) when treated with TG and LiCl, respectively. Analysis of NO production in response to GSK-3 inhibitor showed a significantly decreased by approximately 50% (p<0.001) indicated by changes in nitrite levels. Additionally, inhibition of GSK-3 significantly decreased the production of TNF-α, IL-6, and MCP-1 as observed in the presence of 10 mM and 15 mM of LiCl (p<0.05) or 5 µM and 10 µM of TG (p<0.001). The reduction of these pro-inflammatory cytokine levels is corroborated by a significant decline in inducible nitric oxide synthase (*iNOS), CD11b, TNF-α*, and *IL-6* gene expression. **Conclusion:** Inhibition of GSK-3 signaling molecule potentially downregulate the neuroinflammation through attenuation of the production and expression of NO, pro-inflammatory cytokines, and CD11b elicited by LPS in microglia.

INTRODUCTION

Microglia is a brain macrophage that regulates the central nervous system (CNS) innate immunity. It provides immune surveillance and protection from harmful foreign substances. Activation of microglia is the hallmark of the neuroinflammatory initiation response primarily in its quest to protect the CNS [1]. Activated microglia (M1 through the classical pathway or M2 through alternative pathway) has dual effector functions; to induce immune response and

neuroinflammation, or to promote CNS tissue recovery and repair [2]. Induced microglial activation *in vitro* by lipopolysaccharides (LPS) or interferon-gamma (IFN-γ) which promote M1 phenotype, produces various immune mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) [3–5]. These mediators play a major role in the elimination of pathogens. However, uncontrolled M1 microglial activation and constant exposure to the inflammatory mediators could be detrimental to the CNS tissue and environment. Excessive production of pro-inflammatory molecules potentially causes CNS tissue damage and exacerbates CNS injury [6]. This, in turn, will cause degeneration of neurons which contribute to the development of neuroinflammation and neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) [7].

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that plays an important role in regulating cellular immune functions [8,9]. This enzyme is constitutively active and highly expressed in the CNS. GSK-3 also plays a significant role in the regulation of microglial phenotypic functions including microglial migration, the release of inflammatory molecules, and inflammation-induced neurotoxicity [10]. A previous study had shown that GSK-3 inhibitor reduced the expression of CD11b, a macrophage activation marker, in a macrophage cell line, RAW264.7 [11]. This finding also suggests that inhibition of GSK-3 potentially downregulates the immune response in macrophages induced by LPS [11]. It has also been reported that inhibition of the GSK-3 signaling molecule could potentially alleviate neuroinflammation and promote disease recovery. In AD, inhibition of GSK-3 by LiCl prevents tau hyperphosphorylation, the disturbance of cytoskeleton, and the degeneration of neurofibrillary [12]. Besides, a significant improvement in patients' cognitive performance is seen in AD patients with Tideglusib (TG) treatment [13]. Here, we evaluated the activation of microglia and GSK-3 immunoregulatory activities through the expression of CD11b, pro-inflammatory cytokine production, and the expression of its related genes in the LPS-stimulated BV2 cells.

MATERIALS AND METHODS

Antibodies and Reagents

LPS extracted from *Escherichia coli* 0111: B4 and GSK-3 inhibitors, namely lithium chloride (LiCl) and Tideglusib (TG) (Sigma; St Louis, MO, USA), high glucose Dulbecco Modified Eagle Medium (DMEM) (Gibco; Grand Island, NY, USA), Cytometric Bead Array (CBA) mouse inflammatory kit (BD Biosciences, San Jose, CA, USA; Cat. No: 552364) and Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b (BD Biosciences; San Jose, CA, USA) and fixable viability dye eFluor 780 (eBioscience; Cat. No: 65-0865-14), RNA extraction kit, cDNA transcription kit and qPCR (Thermo Fisher Scientific; Waltham, MA, USA) were used in this study.

Cell Culture, LPS Stimulation, and GSK-3 Inhibition

Mouse-derived BV2 cell line (from ATCC) was cultured in 24-well plates at a density of $1x10^5$ cells/well and maintained in a high glucose DMEM supplemented with 5% heat-inactivated foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 ml/L gentamycin, 250 µg/mL fungizone, 1X non-essential amino acids, 6.25 µ/mL insulin and 1.5 g/L sodium bicarbonate. This cell line was cultured in a 95% humidified atmosphere containing 5% CO_2 at 37°C. Cells were harvested using a cell scraper. Following overnight incubation or when the BV2 cells have reached approximately 70% confluency, the cells were then stimulated with or without 1 µg/mL LPS for 24 hours prior to treatment with LiCl (10 mM and 15 mM) or TG (5 µg and 10 µg) for 24 hours at 37°C in 5% CO_2 incubator.

Griess Assay

Equal amount (50 µL each) of BV2 cell culture supernatant and Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) was combined in a 96-well plate for 10 minutes at room temperature (RT) in dark. The nitrite level was measured at 540 nm using a microplate reader (Dynex Technologies; USA). The concentration of nitrite was quantitated based on the nitrite standard curve constructed using sodium nitrite [14].

Expression of CD11b by Flow Cytometry

The surface expression of CD11b in BV2 cells was quantified using a flow cytometer. Briefly, about $5x10^5$ cells were harvested and washed with FACS buffer and spun down at 1500 rpm for 5 min. The cells were then added with 3 µg/mL Purified Rat Anti-Mouse CD16/CD32 (FC block) (BD Bioscience, USA) and stained with a fluorescent dye Zombie NIRTM Fixable Viability Kit (1:1000; Biolegend, San Diego, CA) for 5 min followed by FITC-conjugated anti-mouse CD11b antibody (1:100; BD Biosciences, USA) or FITC Rat IgG2a,κ Isotype control antibody (1:100; BioLegend, San Diego, CA) staining for 30 minutes at 4°C in dark. The cells were acquired using a FACS Canto flow cytometer (BD Biosciences, USA) attached with FACS Diva software. Data were analyzed using FlowJo V10 (Flowjo, LLC, USA).

Cytokine Profile Assay

The secretion of pro-inflammatory cytokines by BV2 cells was determined by using a CBA mouse inflammation kit according to the manufacturer's protocol. Briefly, 50 µL of

BV2 cell culture supernatant was incubated with capture beads conjugated with a specific antibody for IL-6, MCP-1, and TNF-α in dark at RT for 2 hours. Detection reagent which contains phycoerythrin (PE) was then added into each tube and acquired using the FACSCanto flow cytometer attached to FACSDiva software. Data were analyzed using FCAP Array software (BD Bioscience, USA).

Table 1: List of gene primers used for quantitative RT-PCR.

Gene	Direction	Sequence 5' → 3'	NCBI accession number
iNOS	Forward	CGAAACGCTTCACTTCCAA	M87039.1
	Reverse	TGAGCCTATATTGCTGTGGCT	M87039.1
CD11b	Forward	AAGGATTCAGCAAGCCAGAA	NM_008401.2
	Reverse	GGAGGGATGAGAGTCCACAT	NM_008401.2
TNF-α	Forward	TGTAGCCCACGTCGTAGCAA	NM_013693.3
	Reverse	AGGTACAACCCATCGGCTGG	NM_013693.3
IL-6	Forward	TAGTCCTTCCTACCCCAATTTCC	NM_031168.2
	Reverse	TTGGTCCTTAGCCACTCCTTC	NM_031168.2
GAPDH	Forward	TGCGACTTCAACAGCAACTC	BC023196.2
	Reverse	CTTGCTCAGTGTCCTTGCTG	BC023196.2
B2M	Forward	GCTATCCAGAAAACCCCTCAA	NM_009735.3
	Reverse	CGGGTGGAACTGTGTTACG	NM_009735.3

Quantitative Real Time-Polymerase Chain Reaction (qPCR)

Total RNA from the culture of LPS-stimulated BV2 cells with or without the inhibitor was extracted with the GeneJet RNA purification kit (ThermoFisher Scientific Inc.; NYSE, TMO) according to the manufacture's instruction. Concentration and quality of RNA were assessed by using Agilent 2100 Bioanalyzer with RNA 600 Nano Chip (Agilent Technologies Inc.; Santa Clara, CA, USA). Total RNA (1 μg) was subjected to reverse transcription using the Maxima First Strand cDNA synthesis kit according to the manufacturer's protocol. The qPCR was performed in triplicate using Maxima SYBR Green qPCR Master Mix according to the manufacturer's protocol. Primers of the gene of interest are listed in Table 1.

Statistical Analysis

Data in triplicate from three independent experiments are displayed as mean ± standard deviation (SD) and assessed by one-way analysis of variance (ANOVA) followed by Dunnet test for multiple comparisons. The significant differences of the data were considered statistically significant at $p<0.05$(* or #), $p<0.01$(** or ##), and $p<0.001$(*** or ###). Statistical analysis was conducted using GraphPad Prism version 8 (GraphPad Software Inc., USA).

RESULTS

GSK-3 Inhibitor Reduced the Production of NO in LPS-Stimulated BV2 cells

The effects of GSK-3 inhibition on NO levels in the LPS-stimulated BV2 cells were determined by Griess assay through quantification of the production of nitrite. Production of NO in BV2 cells was increased in a concentration-dependent manner (Fig. 1A). The unstimulated microglia produced a very low amount (1.41±0.61 μM) of NO. Stimulation of the cells with 1 ug/mL of LPS is sufficient to increase a significant amount of NO (29.01±1.07 μM; $p<0.001$). Treatment of LPS-stimulated BV2 cells with various concentrations of LiCl and TG significantly reduced the production of NO in a concentration-dependent manner (Fig. 1B). In figure 1C, two concentrations of each GSK-3 inhibitor that showed a significant decrease ($p<0.001$) in the production of NO

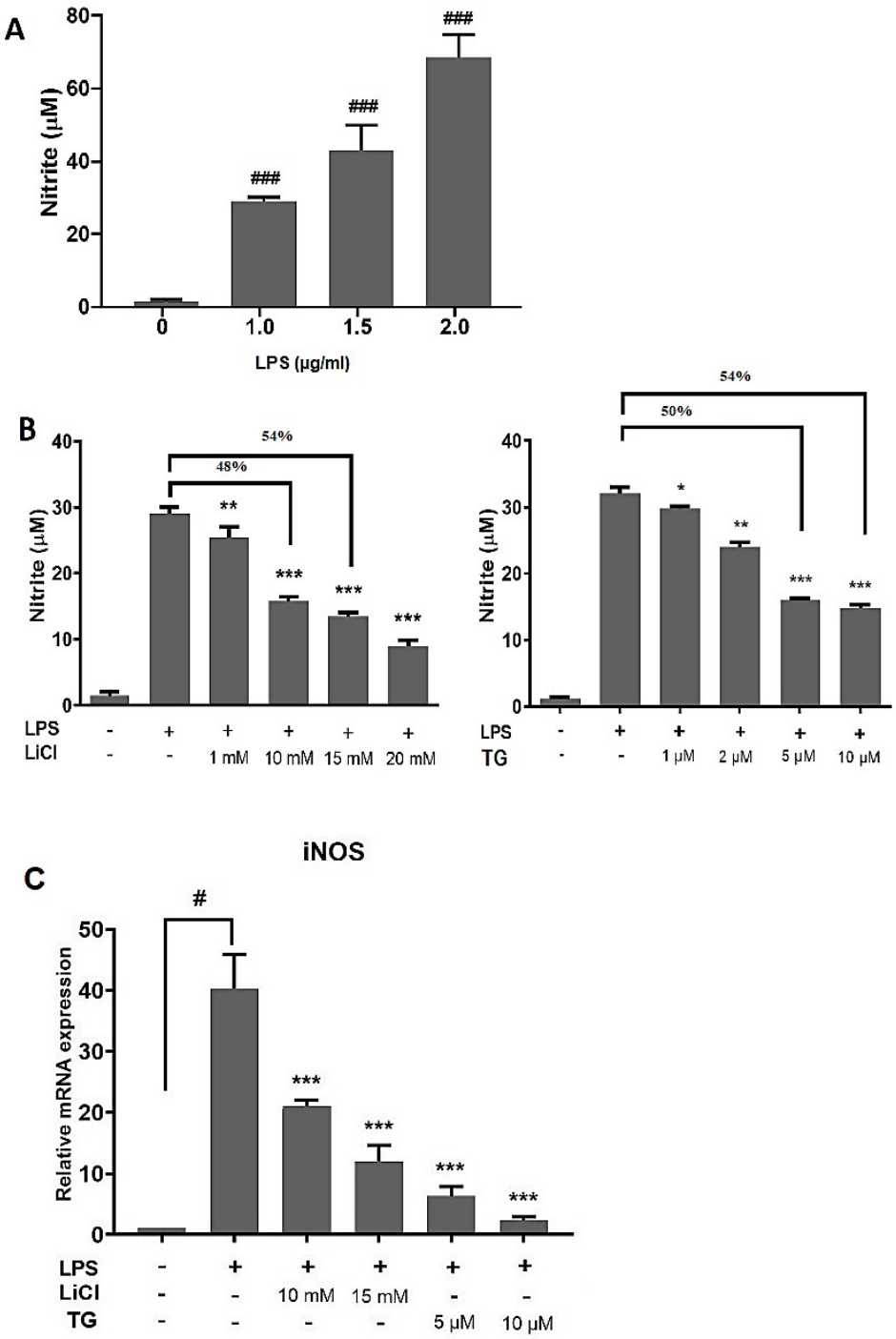

Figure 1: Effects of LiCl and TG on NO production in LPS-stimulated BV2 cells. A) BV2 cells were cultured in 24-well plates at a density of $1x10^5$ cells/well and stimulated with various concentrations of LPS for 24 hours. The amount of NO was determined by the quantification of nitrite production in BV2 cells. B) BV2 cells were cultured in 24-well plates at a density of $1x10^5$ cells/well in the presence or absence of 1 μg/mL LPS. Following 24 hours incubation, the cells were treated with different concentrations of LiCl or TG for 24 hours. C) The mRNA level of *iNOS* was analyzed by quantitative qPCR. Values are mean ±SD of three independent experiments. #p<0.05, ##p<0.01, and ###p<0.001 indicate statistically significant in comparison with the unstimulated control. *p<0.05, **p<0.01, and ***p<0.001 indicate statistically significant in comparison with the LPS alone.

were selected and further evaluated on the expression of *iNOS* mRNA. The result showed that the mRNA expression of *iNOS* greatly increased after stimulation with LPS. On the other hand, *iNOS* expression showed a significant decrease (p<0.001) in the presence of GSK-3 inhibitors.

TG and LiCl Inhibit the Surface and mRNA Expression of CD11b in Activated Microglia

CD11b is ubiquitously expressed in microglia. During a resting state (unstimulated), the BV2 cells expressed

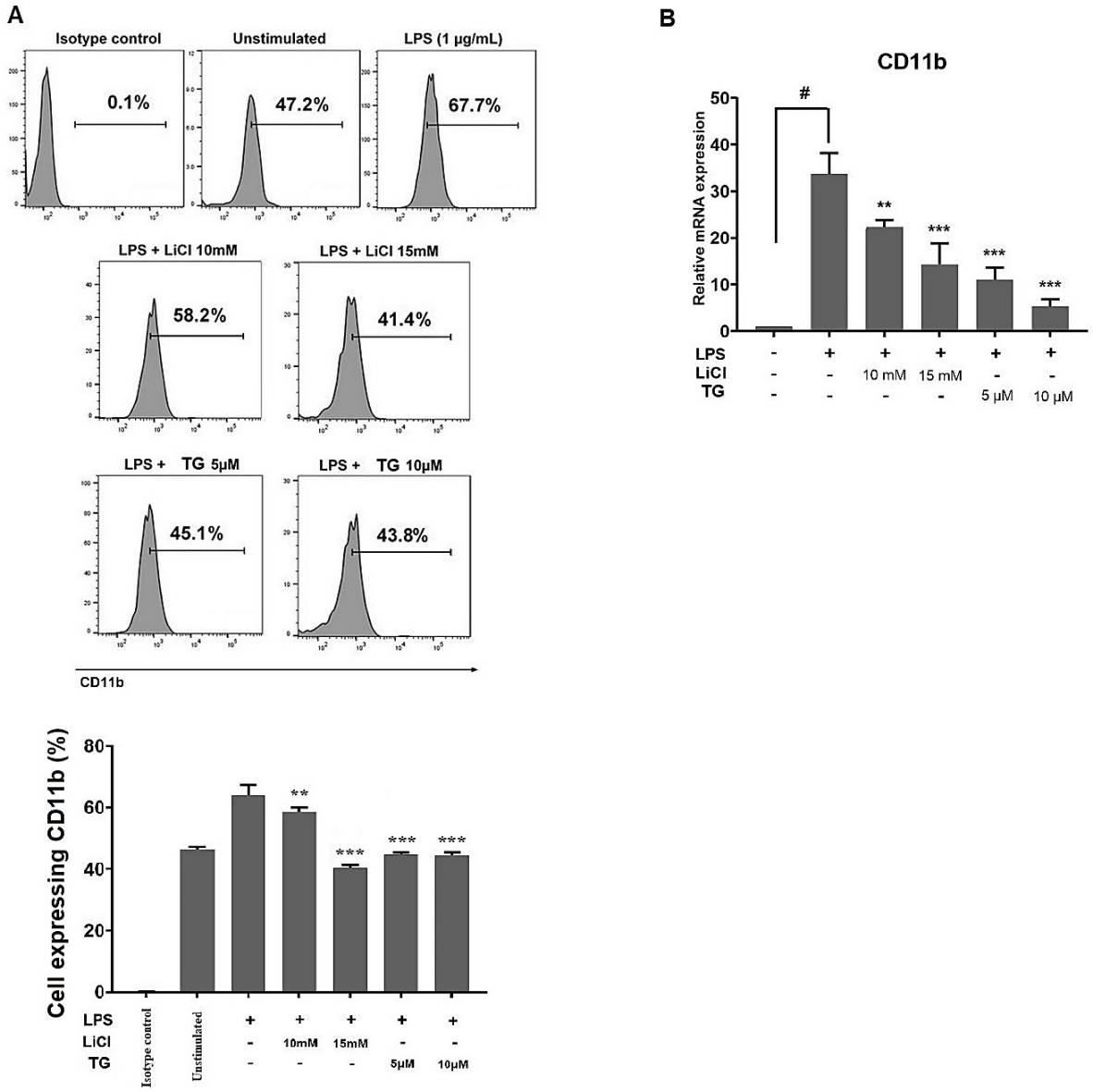

Figure 2: Effect of GSK-3 inhibition on the surface expression of CD11b in LPS-stimulated BV2 cells. BV2 cells were cultured in 24-well plates at a density of 1x10^5 cells/well in the presence or absence of 1 µg/mL LPS. Following 24 hours incubation, the cells were treated with different concentrations of LiCl or TG for 24 hours. A) The surface staining of BV2 cells with FITC-conjugated CD11b or FITC-conjugated IgG2a,κ isotype control was carried out followed by acquisition on the flow cytometry. The histogram depicts the representative of CD11b expression from three independent experiments that normalized with isotype control. Cumulative cell expressing CD11b (%) is shown in the bar graph with values are mean ±SD of three independent experiments. B) The mRNA level of *CD11b* was analyzed by quantitative qPCR. Values are mean ±SD of three independent experiments. #p<0.05 indicates statistically significant in comparison with the unstimulated control. *p<0.05, **p<0.01 and ***p<0.001 compared to the LPS group.

moderate (47.2%) levels of CD11b on the cell surface (Figure 2A). Stimulation of the cells with LPS increased by approximately 20% of the CD11b expression. However, inhibition of GSK-3 by 10 mM and 15 mM LiCl reduced the surface expression of CD11b by approximately 9% (p<0.01) to 23% (p<0.001), respectively. Similarly, inhibition of GSK-3 by both 5 µM and 10 µM TG is reduced approximately 23% (p<0.001) of CD11b expression in BV2 cells.

To validate the result, mRNA expression of CD11b was evaluated by quantitative qPCR. The mRNA expression of *CD11b* is detected at a very low level in the unstimulated BV2 cells (Figure 2B). A significant increase *(#p<0.05*) in the *CD11b* mRNA expression was highly exhibited after stimulation with LPS. Inhibition of GSK-3 by LiCl and TG significantly (p<0.01 and p<0.001) abolished the mRNA expression of *CD11b* in LPS-stimulated BV2 cells.

GSK-3 Inhibitors Limit the Production and mRNA Expression of TNF-α, IL-6, and MCP-1 in LPS-stimulated BV2 Cells

To investigate the effects of GSK-3 inhibitors on the production of pro-inflammatory cytokines, the cell culture supernatant of the LPS-stimulated BV2 cells in the presence or absence of GSK-3 inhibitor was collected and the pro-inflammatory cytokine levels were determined by CBA acquisition on the flow cytometry. Figure 3A demonstrates the changes in pro-inflammatory cytokine secretion upon LPS stimulation and GSK-3 inhibition in

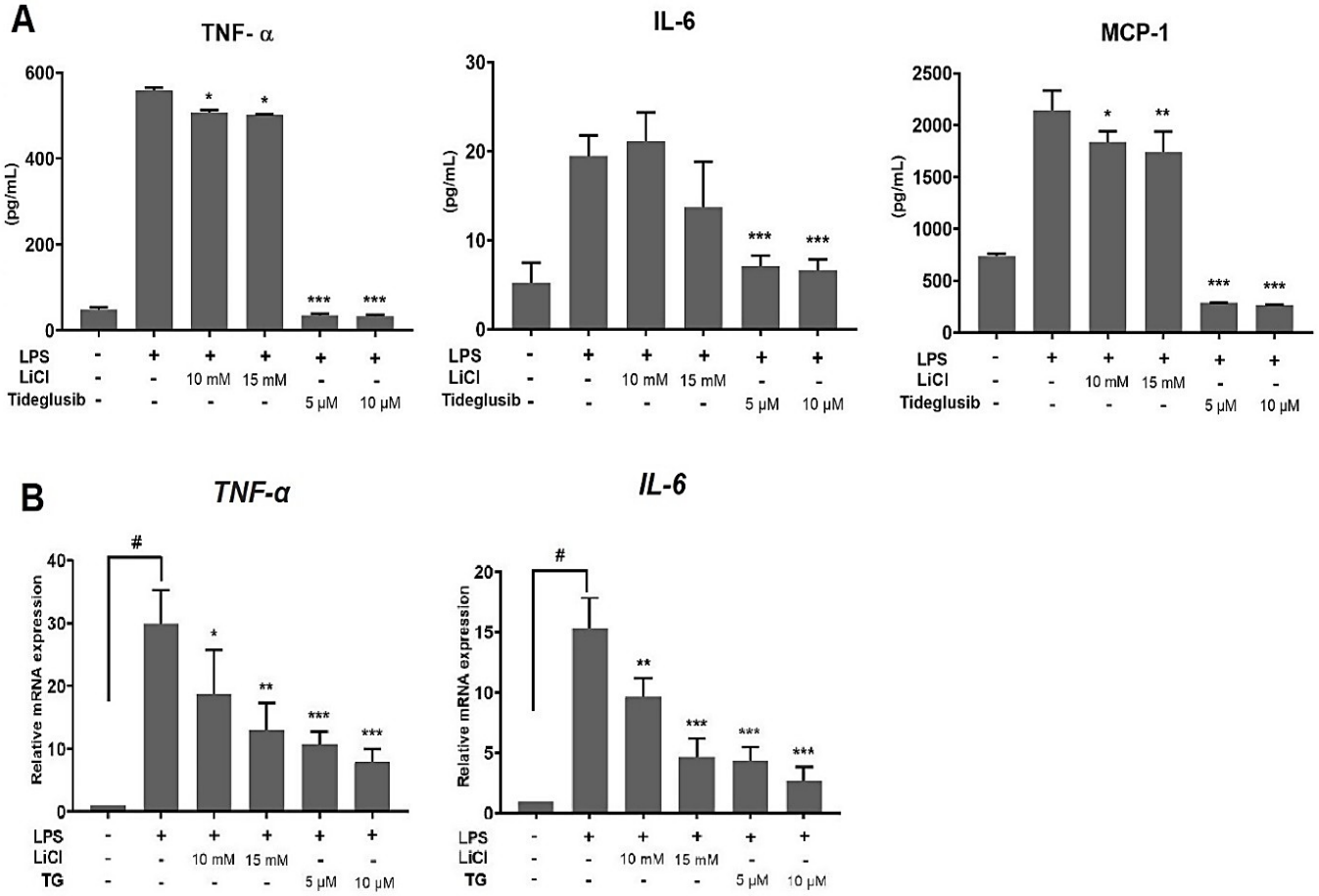

Figure 3: Inhibition of GSK-3 by LiCl and TG reduces the secretion and expression of pro-inflammatory cytokines in LPS-stimulated BV2 cells. BV2 cells were cultured in 24-well plates at a density of 1x10^5 cells/well in the presence or absence of 1 µg/mL LPS. Following 24 hours incubation, the cells were treated with different concentrations of LiCl or TG for 24 hours. A) The changes in the pro-inflammatory cytokine production in the cell supernatant of GSK-treated LPS-stimulated BV2 cells were evaluated by using a BD CBA mouse inflammatory kit on flow cytometry. B) The mRNA of the cells was extracted and the level of *TNF-α* and *IL-6* genes was analyzed by quantitative qPCR. Values are mean ±SD of three independent experiments. #p<0.05 indicates statistically significant in comparison with the unstimulated control. *p<0.05, **p<0.01 and ***p<0.001 compared to the LPS group.

BV2 cells. The levels of pro-inflammatory cytokines in the unstimulated cells were quantified at 48.93±5.32 pg/mL (TNF-α), 739.27±21.19 pg/mL (MCP-1), and 5.25±2.22 pg/mL (IL-6). The production of these pro-inflammatory cytokines increased to 558.44±6.79 pg/mL (TNF-α), 2145.76±189.21 pg/mL (MCP-1), and 19.46±2.31 pg/mL (IL-6) in the post-24 hours exposure to LPS. This data also indicated that the inhibition of GSK-3 by both LiCl and TG significantly reduced the production of pro-inflammatory cytokines. However, compared to LiCl, TG exhibited more potent and significant (p<0.001) inhibitory activity of GSK-3 in LPS-stimulated BV2 cells.

The inhibitory effects of GSK-3 inhibitors on *TNF-α* and *IL-6* genes in LPS-stimulated BV2 cells were evaluated at the mRNA expression levels. Figure 3B showed that the mRNA expression of these genes was at a very low level in the unstimulated condition. A significant *($p<0.05$)* increased was detected in both *TNF-α* and *IL-6* genes after stimulation with LPS. On the other hand, the expression of *TNF-α* and *IL-6* mRNA was decreased significantly (p<0.01 and p<0.001) in the presence of GSK-3 inhibitors.

DISCUSSION

Microglia plays important role in the immune surveillance and neuroinflammatory process of the CNS. Chronic activation of microglia has been associated with excessive production of inflammatory mediators and the pathogenesis of many neurodegenerative diseases [15]. Neurotoxic mediators that are produced during microglial activation which could induce neuroinflammation are including NO, pro-inflammatory cytokines, and reactive oxygen species (ROS) [16,17]. Therefore, immunomodulation of the CNS immune response via suppression of pro-inflammatory mediators secreted from microglial activation may contribute to the neurodegenerative disease therapy, which possibly through inhibition of GSK-3 signaling.

The present study showed that inhibition of GSK-3 signaling reduced the inflammatory responses that were previously induced by LPS in the BV2 cell line, which in line with a previous finding [18]. Microglial responses to extracellular stimuli are mediated by kinase and phosphate cascades [8,9]. Stimulation of microglia with LPS initiates inflammatory responses through the activation of nuclear factor-kappa light chain enhancer of activated B cells (NF-$_K$B) and GSK-3 signaling. On the other hand, the presence of a GSK-3 inhibitor modulates the inflammation state of microglia by inhibiting the transcriptional activity of NF-$_K$B [9,19]. The mechanism by which GSK-3 inhibitor blocks activation of NF-$_K$B is through inhibition of the toll-like receptor (TLR) that subsequently reduced the production of pro-inflammatory cytokines and other immune mediators such as NO [20]. In this study, selective GSK-3 inhibitors such as LiCl and TG are chosen due to their efficacy in reducing NO, glutamate, and pro-inflammatory cytokines in microglia [14]. Thus, our study is aimed to further investigate the effects of GSK-3 inhibition by TG and LiCl on the production of NO, pro-inflammatory cytokines, and also the expression of CD11b and their related genes.

Nitric oxide is an important molecule that plays a major role in many neurobiological processes such as supporting cell communication in the brain [21]. However, overproduction of NO is detrimental to the CNS as it may cause neuronal cell death [21]. Production of NO in activated microglia is dependent on the increase of *iNOS* expression [10]. In the present study, the production of NO that was previously increased by LPS stimulation was significantly reduced in the presence of GSK-3 inhibitor (Figure 1A). This finding is associated with the decline in the mRNA expression of *iNOS* in LPS-stimulated BV2 cells (Figure 1B).

Macrophage activation marker, CD11b, is ubiquitously expressed on the surface of many leukocytes including microglia [10,22]. Although CD11b is not a highly specific marker, immunocytochemical reactions for this protein are often used as the supplementary method of detecting activated microglia [23]. Elevation of CD11b expression is associated with the activation of microglia during neuroinflammation [24]. In many neuroinflammatory diseases, the severity of microglial activation is associated with an increase in the expression of CD11b [25]. In the context of the morphology, vigorous ramification, and cytoskeletal rearrangement of the cells, changes in the shape and motility are also much related to CD11b expression [25]. Our flow cytometry analysis data showed that CD11b is ubiquitously expressed in the unstimulated BV2 cells (Figure 2A). A marginal increase of CD11b-expressing microglia during LPS stimulation was inhibited in the presence of GSK-3 inhibitors. The mRNA expression of *CD11b* on the other hand was detected at a very low level in the unstimulated condition of BV2 cells (Figure 2B). This result showed that the CD11b constitutively expressed on the cell surface but not in the nucleus. However, like CD11b surface expression, a similar trend was observed in the expression of *CD11b* mRNA in the presence of LPS which shown a mark increased. A previous finding has revealed that increased production of NO was associated with the maintenance of CD11b expression in LPS-stimulated microglia [24], which in line with our findings. The effects of GSK-3 inhibition on *iNOS* expression are accompanied by the decline in *CD11b* levels [10]. Our results showed that treatment of BV2 cells with GSK-3 inhibitor significantly decreased the expression level of *CD11b* mRNA in LPS-stimulated BV2 cells. Thus, these data suggest that the expression of CD11b is associated with microglial activation and NO production, in which, the inhibition of GSK-3 induces negative feedback to CD11b expression.

Overexpression of pro-inflammatory cytokines can be the main factor that exacerbates the neuroinflammatory condition. The pro-inflammatory cytokines such as TNF-α acts predominantly on their acute effects on cell activation

and inflammation, whereas MCP-1 activates macrophage and endothelial cells [26,27]. IL-6, on the other hand, plays an important role in mediating innate and adaptive immune responses [28]. Thus, inhibition of these pro-inflammatory cytokines may alleviate some inflammatory conditions. One of the important roles of the GSK-3 inhibitor such as LiCl and TG is its ability to suppress the pro-inflammatory cytokines while inducing the production of anti-inflammatory cytokines such as IL-10 [11,14]. Thus, the reduction of pro-inflammatory cytokine levels by GSK-3 inhibitor could due to the production of IL-10 [29]. Excessive production of pro-inflammatory cytokines can be a major contributor to neuroinflammation in neurodegenerative diseases [30]. In this study, the effect of GSK-3 inhibition on the expression TNF-α, IL-6, and MCP-1 was investigated in LPS-activated microglia. Upon inhibition of GSK-3, the production of secreted TNF-α, IL-6, and/or MCP-1 (Figure 3A) and their mRNA gene expression (Figure 3B) were abrogated which were correlated with other studies [11,14,18,31]. These data suggest that blocking microglial activation by inhibition of GSK-3 signaling potentially lessens the effects of inflammation.

CONCLUSION

In conclusion, activation of microglia by LPS stimulation increased CD11b expression and subsequently promoted the pro-inflammatory responses through the production of NO, TNF-α, IL-6, and MCP-1. Reduction of CD11b, NO, and pro-inflammatory cytokine expression through GSK-3 inhibition potentially alleviate the neuroinflammatory responses in activated microglia.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. U.-K. Hanisch, H. Kettenmann, Microglia: active sensor and versatile effector cells in the normal and pathologic brain, Nat. Neurosci. 10 (2007) 1387–1394. https://doi.org/10.1038/nn1997.
2. M. Kawabori, M.A. Yenari, The role of the microglia in acute CNS injury, Metab. Brain Dis. 30 (2015) 381–392. https://doi.org/10.1007/s11011-014-9531-6.
3. I.C.M. Hoogland, C. Houbolt, D.J. van Westerloo, W.A. van Gool, D. van de Beek, Systemic inflammation and microglial activation: systematic review of animal experiments, J. Neuroinflammation. 12 (2015) 114. https://doi.org/10.1186/s12974-015-0332-6.
4. H. Takeuchi, J. Wang, J. Kawanokuchi, N. Mitsuma, T. Mizuno, A. Suzumura, Interferon-γ induces microglial-activation-induced cell death: A hypothetical mechanism of relapse and remission in multiple sclerosis, Neurobiol. Dis. (2006). https://doi.org/10.1016/j.nbd.2005.09.014.
5. S.R. Subramaniam, H.J. Federoff, Targeting Microglial Activation States as a Therapeutic Avenue in Parkinson's Disease, Front. Aging Neurosci. 9 (2017). https://doi.org/10.3389/fnagi.2017.00176.
6. S.-M. Lucas, N.J. Rothwell, R.M. Gibson, The role of inflammation in CNS injury and disease, Br. J. Pharmacol. 147 (2006) S232–S240. https://doi.org/10.1038/sj.bjp.0706400.
7. D. Kempuraj, R. Thangavel, G.P. Selvakumar, S. Zaheer, M.E. Ahmed, S.P. Raikwar, H. Zahoor, D. Saeed, P.A. Natteru, S. Iyer, A. Zaheer, Brain and Peripheral Atypical Inflammatory Mediators Potentiate Neuroinflammation and Neurodegeneration, Front. Cell. Neurosci. 11 (2017). https://doi.org/10.3389/fncel.2017.00216.
8. Y. Fan, L. Xie, C.Y. Chung, Signaling Pathways Controlling Microglia Chemotaxis, Mol. Cells. 40 (2017) 163–168. https://doi.org/10.14348/molcells.2017.0011.
9. J.-S. Park, M.-S. Woo, D.-H. Kim, J.-W. Hyun, W.-K. Kim, J.-C. Lee, H.-S. Kim, Anti-Inflammatory Mechanisms of Isoflavone Metabolites in Lipopolysaccharide-Stimulated Microglial Cells, J. Pharmacol. Exp. Ther. 320 (2007) 1237–1245. https://doi.org/10.1124/jpet.106.114322.
10. C.J. Yuskaitis, R.S. Jope, Glycogen synthase kinase-3 regulates microglial migration, inflammation, and inflammation-induced neurotoxicity, Cell. Signal. 21 (2009) 264–273. https://doi.org/10.1016/j.cellsig.2008.10.014.
11. N.F.Z Zailan, N. Jaganathan, T. Sandramuti, S.N.E. Sarchio, M. Hassan, Inhibition of GSK-3 by Tideglusib Suppresses Activated Macrophages and Inflammatory Responses in Lipopolysaccharide-stimulated RAW 264.7 Cell Line, Malaysian J. Med. Heal. Sci. 16 (2020).
12. J.R. Muñoz-Montaño, F.J. Moreno, J. Avila, J. Díaz-Nido, Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons, FEBS Lett. 411 (1997) 183–188. https://doi.org/10.1016/S0014-5793(97)00688-1.
13. P. Williams, A. Sorribas, M.-J.R. Howes, Natural products as a source of Alzheimer's drug leads, Nat. Prod. Rep. 28 (2011) 48–77. https://doi.org/10.1039/C0NP00027B.
14. Z.M. Zain, S. Vidyadaran, M. Hassan, GSK3 inhibition reduces inflammatory responses of microglia and upregulates IL-10 production, Malaysian J. Med. Heal. Sci. 13 (2017) 1–8.
15. C.K. Glass, K. Saijo, B. Winner, M.C. Marchetto, F.H. Gage, Mechanisms Underlying Inflammation in Neurodegeneration, Cell. 140 (2010) 918–934. https://doi.org/10.1016/j.cell.2010.02.016.
16. K. Thompson, S. Tsirka, The Diverse Roles of Microglia in the Neurodegenerative Aspects of Central Nervous System (CNS) Autoimmunity, Int. J. Mol. Sci. 18 (2017) 504. https://doi.org/10.3390/ijms18030504.
17. S. Thameem Dheen, C. Kaur, E.-A. Ling, Microglial Activation and its Implications in the Brain Diseases, Curr. Med. Chem. 14 (2007) 1189–1197. https://doi.org/10.2174/092986707780597961.
18. W.C. Huang, Y.S. Lin, C.Y. Wang, C.C. Tsai, H.C. Tseng, C.L. Chen, P.J. Lu, P.S. Chen, L. Qian, J.S. Hong, C.F. Lin, Glycogen synthase kinase-3 negatively regulates anti-inflammatory interleukin-10 for lipopolysaccharide-induced iNOS/NO biosynthesis and RANTES production in microglial cells, Immunology. 128 (2009) 275–286. https://doi.org/10.1111/j.1365-2567.2008.02959.x.

19. R. Cortés-Vieyra, A. Bravo-Patiño, J.J. Valdez-Alarcón, M.C. Juárez, B.B. Finlay, V.M. Baizabal-Aguirre, Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens, J. Inflamm. (United Kingdom). (2012). https://doi.org/10.1186/1476-9255-9-23.

20. Y.-T. Chang, C.-L. Chen, C.-F. Lin, S.-L. Lu, M.-H. Cheng, C.-F. Kuo, Y.-S. Lin, Regulatory Role of GSK-3 β on NF- κ B, Nitric Oxide, and TNF- α in Group A Streptococcal Infection, Mediators Inflamm. 2013 (2013) 1–10. https://doi.org/10.1155/2013/720689.

21. E. Džoljić, Why is nitric oxide important for our brain?, Funct. Neurol. (2015). https://doi.org/10.11138/FNeur/2015.30.3.159.

22. S. Gordon, A. Plüddemann, F. Martinez Estrada, Macrophage heterogeneity in tissues: phenotypic diversity and functions, Immunol. Rev. 262 (2014) 36–55. https://doi.org/10.1111/imr.12223.

23. H.-K. Jeong, K. Ji, K. Min, E.-H. Joe, Brain Inflammation and Microglia: Facts and Misconceptions, Exp. Neurobiol. 22 (2013) 59–67. https://doi.org/10.5607/en.2013.22.2.59.

24. A. Roy, Y.K. Fung, X. Liu, K. Pahan, Up-regulation of microglial CD11b expression by nitric oxide, J. Biol. Chem. 281 (2006) 14971–14980. https://doi.org/10.1074/jbc.M600236200.

25. R.B. Rock, G. Gekker, S. Hu, W.S. Sheng, M. Cheeran, J.R. Lokensgard, P.K. Peterson, Role of Microglia in Central Nervous System Infections, Clin. Microbiol. Rev. 17 (2004) 942–964. https://doi.org/10.1128/CMR.17.4.942-964.2004.

26. S.H. Park, K.-H. Park-Min, J. Chen, X. Hu, L.B. Ivashkiv, Tumor necrosis factor induces GSK3 kinase–mediated cross-tolerance to endotoxin in macrophages, Nat. Immunol. 12 (2011) 607–615. https://doi.org/10.1038/ni.2043.

27. C. Ziraldo, Y. Vodovotz, R.A. Namas, K. Almahmoud, V. Tapias, Q. Mi, D. Barclay, B.S. Jefferson, G. Chen, T.R. Billiar, R. Zamora, Central Role for MCP-1/CCL2 in Injury-Induced Inflammation Revealed by In Vitro, In Silico, and Clinical Studies, PLoS One. 8 (2013) e79804. https://doi.org/10.1371/journal.pone.0079804.

28. E. Choy, S. Rose-John, Interleukin-6 as a Multifunctional Regulator: Inflammation, Immune Response, and Fibrosis, J. Scleroderma Relat. Disord. 2 (2017) S1–S5. https://doi.org/10.5301/jsrd.5000265.

29. E. V. Hill, T.H.S. Ng, B.R. Burton, C.M. Oakley, K. Malik, D.C. Wraith, Glycogen synthase kinase-3 controls IL-10 expression in CD4+effector T-cell subsets through epigenetic modification of the IL-10 promoter, Eur. J. Immunol. (2015). https://doi.org/10.1002/eji.201444661.

30. W.-Y. Wang, M.-S. Tan, J.-T. Yu, L. Tan, Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease., Ann. Transl. Med. 3 (2015) 136. https://doi.org/10.3978/j.issn.2305-5839.2015.03.49.

31. M.-J. Wang, H.-Y. Huang, W.-F. Chen, H.-F. Chang, J.-S. Kuo, Glycogen synthase kinase-3β inactivation inhibits tumor necrosis factor-α production in microglia by modulating nuclear factor κB and MLK3/JNK signaling cascades, J. Neuroinflammation. 7 (2010) 99. https://doi.org/10.1186/1742-2094-7-99.