

# MicroRNA-Let-7a regulates the function of microglia in inflammation



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## ABSTRACT

Microglia have multiple functions in cerebrovascular and neurodegenerative diseases. Regulation of microglial function during inflammatory stress is important for treatment of central nervous system (CNS) diseases because microglia secrete various substances that affect neurons and glia. MicroRNA-Let-7a (miR-Let-7a) is a tumor suppressor miRNA that has been reported to target transcripts that encode proteins involved in apoptosis. In the present study, we examined the essential role of miR-Let-7a in inflammatory stress by over-expressing miR-Let-7a to investigate its role in determining the BV2 microglial phenotype, a cell line often used as a model of activated microglia. We found that inflammatory factors and Reactive Oxygen Species (ROS) production levels were altered according to miR-Let-7a expression level as measured by Western blot analysis, reverse transcription PCR, quantitative real time PCR, the measurement of nitrite (indicative of the nitric oxide (NO) pathway), and immunocytochemistry (ICC). Our results suggest that miR-Let-7a is involved in the function of microglia in the setting of inflammatory injury. In response to inflammation, miR-Let-7a participates in the reduction of nitrite production and the expression of inducible nitric oxide synthase (iNOS), interleukin (IL)-6 and is involved in increased expression of brain derived neurotrophic factor (BDNF), interleukin (IL)-10, and IL-4 in microglia. Thus, miRNA-Let-7a could act as a regulator of the function of microglia in inflammation.

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## 1. Introduction

Microglia are derived from primitive macrophages in the yolk sac and the resident macrophages of the central nervous system (CNS) (Ginhoux et al., 2010). In response to any stimulus, microglia indicates a variety of responses that includes morphological alterations (Minten et al., 2012), migration to the site of injury (Jolivel et al., 2014; Yu et al., 2014), and increased expression of various factors such as cytokines (Kim et al., 2014; Skaper et al., 2014). Moreover, microglia transform into phagocytic cells; removing dead cells, protein aggregates, and viral pathogens (Hanisch and Kettenmann, 2007). Microglia are fundamentally responsible for inflammatory changes (Lynch, 2009). For example, ischemic injury and oxidative stress are associated with rapid microglia activation and inflammatory changes (Lynch, 2009). This acute response is considered to be protective (Lynch, 2009). In contrast, persistent microglial activation triggers recruitment of peripheral cells into the brain and finally leads to chronic neuroinflammation (Lynch, 2009; Walker et al., 2014). Uncontrolled microglia activation is associated with the pathophysiology of several diseases including ischemic

stroke (Yrjanheikki et al., 1998), and neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (Frank-Cannon et al., 2009). Recent studies revealed that the activation states are broadly described in terms of macrophagic responses as the classically-activated, pro-inflammatory (Frankola et al., 2011; Hagar et al., 2013), neurotoxic phenotype (M1 phenotype), and the alternatively-activated, anti-inflammatory or M2 phenotype involved in phagocytosis and tissue repair (Fiorentino et al., 1989; Glocker et al., 2009). Exposure of microglia cell cultures to bacterial lipopolysaccharides (LPS) (Bhat et al., 1998; Chao et al., 1992), tumor necrosis factor-alpha (TNF- $\alpha$ ) (Takeuchi et al., 2006), interferon-gamma (IFN- $\gamma$ ) (Meda et al., 1995), or oligomers of amyloid beta (A $\beta$ ) (Maezawa et al., 2011) induces the M1 phenotype. On the other hand, the alternative, M2 phenotype is neuroprotective (Hjorth et al., 2013; Koenigknecht-Talboo and Landreth, 2005; Mandrekar-Colucci et al., 2012) and can be induced in primary microglial cells by interleukins (IL)-4 and IL-13 (Freilich et al., 2013). IL-4 was found to decrease inducible nitric oxide synthase (iNOS) activity, and superoxide and TNF- $\alpha$  production in LPS-activated microglia (Chao et al., 1993; Zhao et al., 2006). IL-4 also increases the phagocytic activity of microglia, including the uptake of oligomeric A $\beta$  species (Shimizu et al., 2008). In addition, IL-13 and IL-10 are secreted from M2-phenotype microglia which increases microglial secretion of neuroprotective transforming growth factor beta (TGF- $\beta$ ) (Miron et al., 2013). M2-phenotype microglia activation leads to secretion of brain-derived neurotrophic factor (BDNF) (Hains and Waxman, 2006; Lu et al., 2013; Ulmann et al., 2008), as well

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as its receptors (Heese et al., 1998; Neumann et al., 1998). Several studies demonstrate that BDNF acts as a neuronal survival factor (Ghosh et al., 1994; Lindvall et al., 1994). Moreover, microglial BDNF increases neuronal tropomyosin-related kinase receptor B (trkB) phosphorylation which is a key mediator of synaptic plasticity (Chao, 2003) and contributes to learning and memory function by promoting learning-related synapse formation (Parkhurst et al., 2013).

MicroRNAs (miRNAs) are a class of short, non-coding RNA molecules (19–25 nucleotides in length) that suppress target gene expression at the post-transcriptional level through incomplete base-pairing to the 3'-untranslated region (3'UTR) of target mRNAs (Skafnesmo et al., 2007; Valencia-Sanchez et al., 2006). MiRNAs are transcribed from intragenic or intergenic regions by RNA polymerase II or RNA polymerase III, originating large stem-loop hairpin structures designated pri-miRNAs (Filipowicz et al., 2008). MicroRNAs have been reported to play critical roles in a variety of processes such as neurogenesis (Choi et al., 2008; Kloosterman et al., 2004; Wienholds and Plasterk, 2005). MiRNA-mediated regulation of gene expression has been associated with various important biological processes, including inflammation, apoptosis, angiogenesis, and proliferation (Arora et al., 2013; Chen et al., 2011). Recent studies have shown that miR-155 is able to target microglia M2 phenotype-associated genes, such as that encoding SMAD2, a protein associated with the TGF- $\beta$  pathway (Louafi et al., 2010). Also, miR-92a was recently shown to be down-regulated in response to the activation of Toll-like Receptors (TLRs), and is necessary to promote the production of inflammatory cytokines in M1-phenotype macrophages (Lai et al., 2013). MiR-124 is involved in the secretion of M1 phenotype-associated cytokines (Ponomarev et al., 2011). MiR-Let7 was initially found to regulate cell proliferation and differentiation in *Caenorhabditis elegans* (Reinhart et al., 2000). Let-7 is a kind of miRNA found across species that is present in multiple genomic locations and there are 10 mature Let-7 isoforms with the same seed sequence from 13 precursor sequences (Roush and Slack, 2008). MiR-Let-7a mainly regulates anti-inflammatory properties through repression of specific genes targeting downstream signaling pathways (Chen et al., 2007). It is unknown whether miR-Let-7a plays an essential role in microglia function under inflammatory conditions. In the present study, we investigated whether differentially regulated miR-Let-7a expression affects the macrophagic phase and modulates secretion of factors associated with the M1 or M2 phenotype of microglia under inflammatory conditions.

## 2. Results

### 2.1. Microglia over-expressing Let-7a were less vulnerable to apoptotic cell death

First of all, by TaqMan assay we examined the change of miRNA level in microglia BV2 cells under LPS stimulation and miR-Let-7a was significantly reduced under LPS stimulated condition (Fig. 1A). This result gave us a hint that Let-7a, a well-known miRNA, may involve in inflammation by microglia BV2 cell. Under LPS, miR-Let-7a level decreased to 0.3 fold. When BV2 was transfected with miR-Let-7a mimic, the level of miR-Let-7a was over-expressed to over 3 fold. After then, to evaluate the role of miR-Let-7a on the death of microglia, we conducted Western blot analysis and immunochemical imaging analysis under conditions of over-expression of miR-Let-7a. We examined the protein level (Fig. 1B) and the immunochemistry (Fig. 1C) of cleaved caspase-3 as a marker of mitochondrial apoptosis. Our results showed that the protein level of cleaved caspase-3 and the number of cleaved caspase-3 immunopositive cells increased in the LPS treatment group compared with the normal control group (Fig. 1B, C). Moreover, overexpression of the miR-Let-7a with LPS treatment significantly attenuated the protein level of cleaved caspase-3 and the number of cleaved caspase-3 positive cells compared to the LPS-only treatment group (Fig. 1B, C). Our results showed that the protein level of cleaved caspase-3 and the

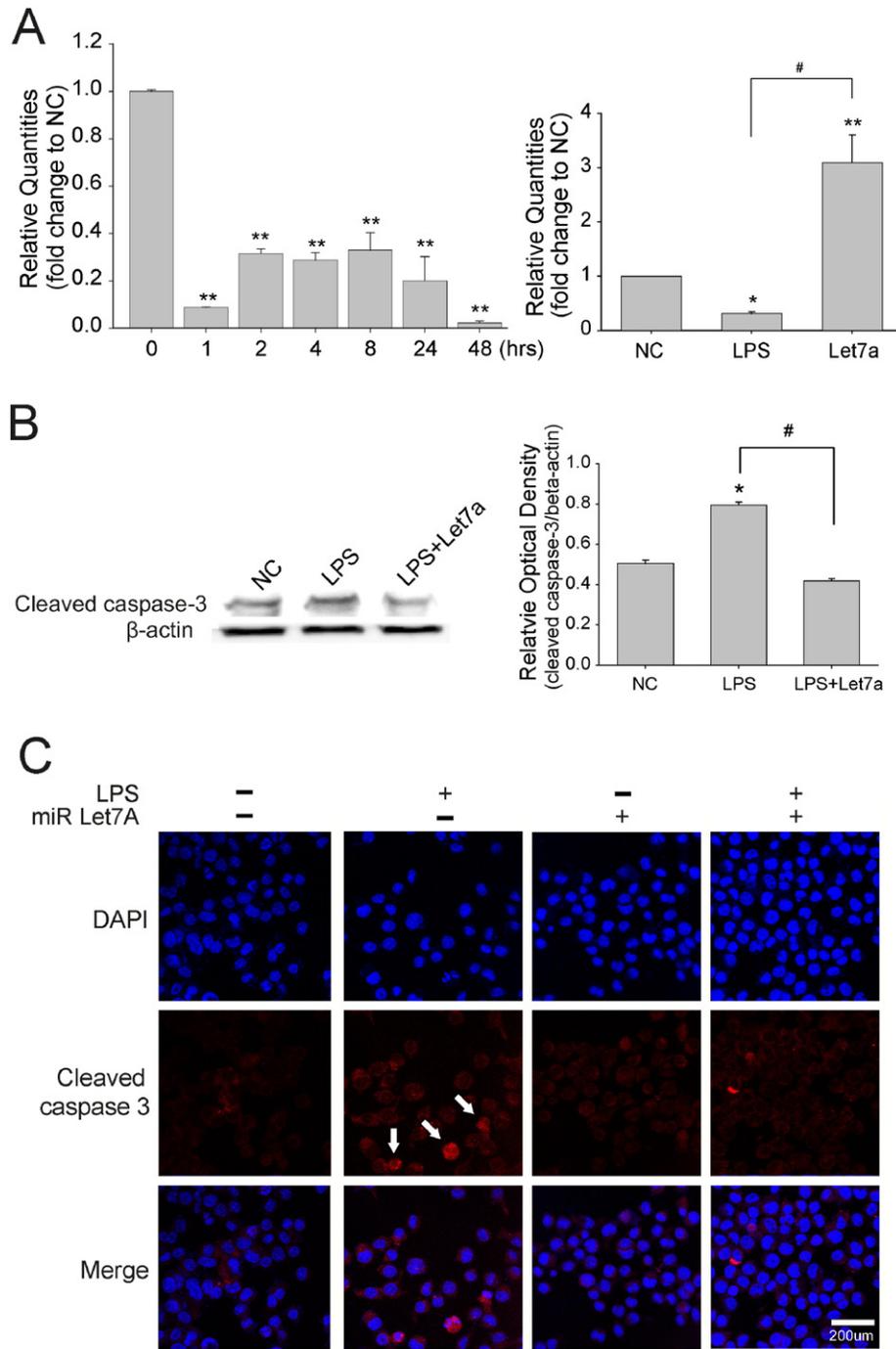
number of cleaved caspase-3 immunopositive cells increased in the LPS treatment group compared with the normal control group (Fig. 1B, C). MiR-Let-7a over-expression during LPS treatment significantly attenuated the protein level of cleaved caspase-3 and decreased the number of cleaved caspase-3 immunopositive cells compared to the LPS-only treatment group (Fig. 1B, C). Taken together, our results suggested that miR-Let-7a suppresses LPS-induced apoptosis through regulation of apoptosis-related gene expression.

### 2.2. Microglia over-expressing Let-7a present with an M2 phenotype rather than an M1 phenotype

To check the effect of miR-Let-7a on the phenotype of microglia, we conducted immunochemical analysis to detect CD68 as a marker of the M1 phenotype of microglia (Fig. 2A) or a mannose receptor CD206 as a marker of the M2 phenotype of microglia (Fig. 2B). Fig. 2A shows also that the number of CD68-positive BV2 microglia increased whereas over-expression of miR-Let-7a did not change the expression of CD68 in the same LPS-induced inflammation condition. On the contrary, our result showed that the number of CD206-immunopositive BV2 microglia decreased whereas miR-Let-7a over-expression did not change the expression of CD206 in the same LPS-induced inflammation condition (Fig. 2B). As a counter-part, these data suggest that miR-Let-7a may affect the phenotype of microglia and promote the M2 phenotype under inflammatory conditions through regulating the associated gene expression.

### 2.3. Let-7a was down-regulated by LPS-inflammation and IL-10 and IL-4 were enhanced by Let-7a over-expression

To examine and confirm the role of miR-Let-7a in regulating the inflammatory phase, expression of IL-10, IL-4 and IL-6 were assessed by quantitative real time RT-PCR (Fig. 3). The mRNA level of IL-10 that is anti-inflammatory interleukin was significantly decreased in LPS-treated microglia compared with the normal control group (Fig. 3A-a). With reverse correlation of IL-10, the mRNA level of IL-6 that is a representative pro-inflammatory factor was significantly increased in LPS-treated microglia compared with the normal control group (Fig. 3A-c). However, the decreasing level of IL-4 was not significant even though the IL-4 is also an anti-inflammatory factor same as IL-10 (Fig. 3A-b). Corresponding to the result of CD 206 immunohistochemistry, an anti-inflammatory phase M2 marker, the expression of IL-10 was increased in miR-Let-7a over-expressing cells with LPS treatment compared to the normal group as well as the LPS-only treatment group (Fig. 3A-a). In case of IL-6 expression level, over-expressing miR-Let-7a slightly attenuated the level of it (Fig. 3A-c). Although the level of IL-6 was still higher than normal, the reduced amount was significant comparing to LPS-only treatment. Under LPS-only treatment, IL-10 was significantly reduced over 5 fold (0.168 fold change), whereas IL-4 did not significantly decrease (0.76 fold change). However, when miR-Let-7a was over-expressed, both IL-10 and IL-4 were significantly enhanced up to over 3.5 fold. In addition, when miR-Let-7a was over-expressed during LPS treatment, IL-10 was increased 3.8 fold compared to the LPS-only treated group. In contrast to IL-10, the mRNA level of IL-4 showed to be the same, but differed only to the LPS treated microglia. To further evaluate the role of miR-Let-7a in regulating inflammatory factor, we used the miR-Let-7a mimic and also inhibitor (anti-Let-7a) and they were transfected in BV2 cells (Fig. 3B). The level of IL-10 transcript was significantly improved in BV2 cells over-expressing miR-Let-7a compared with normal while the BV2 cells down-regulating miR-Let-7a was significantly diminished to similar level of LPS group (Fig. 3B-a). The IL-6 mRNA level was shown the reverse correlative pattern of IL-10 but the down-regulating miR-Let-7a did not increased up to the level of LPS group (Fig. 3B-b). Thus, miR-Let-7a affects the expression of IL-10, IL-6 and IL-4 in microglia and is involved mainly in regulation of IL-10 under inflammatory conditions.

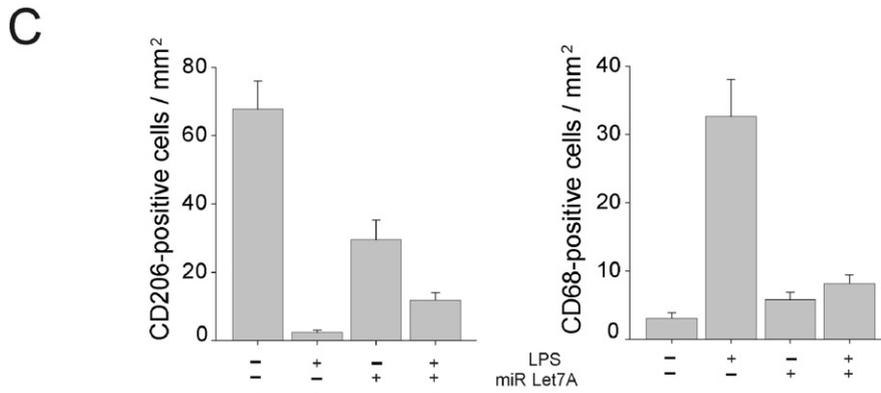
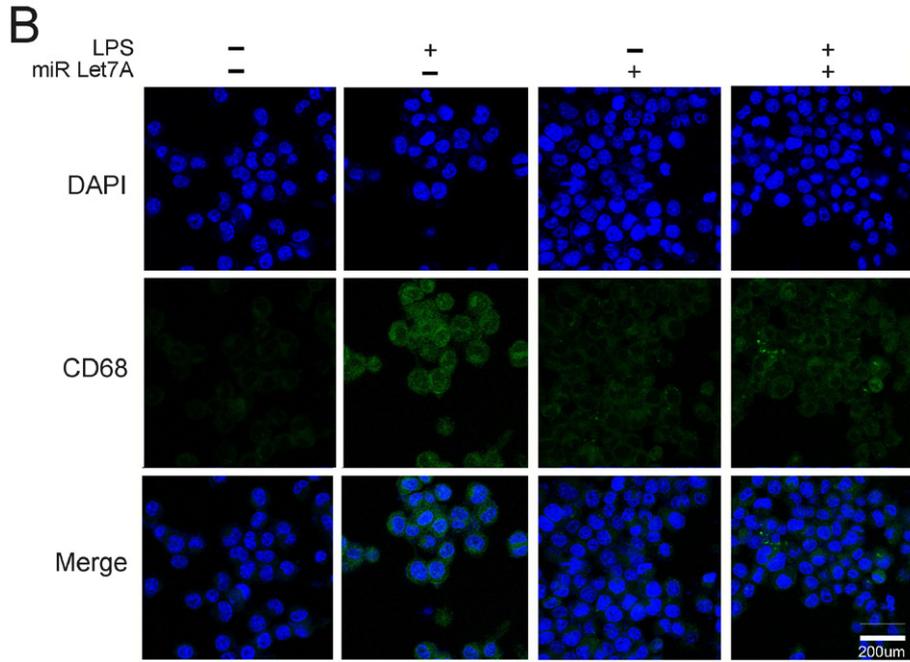
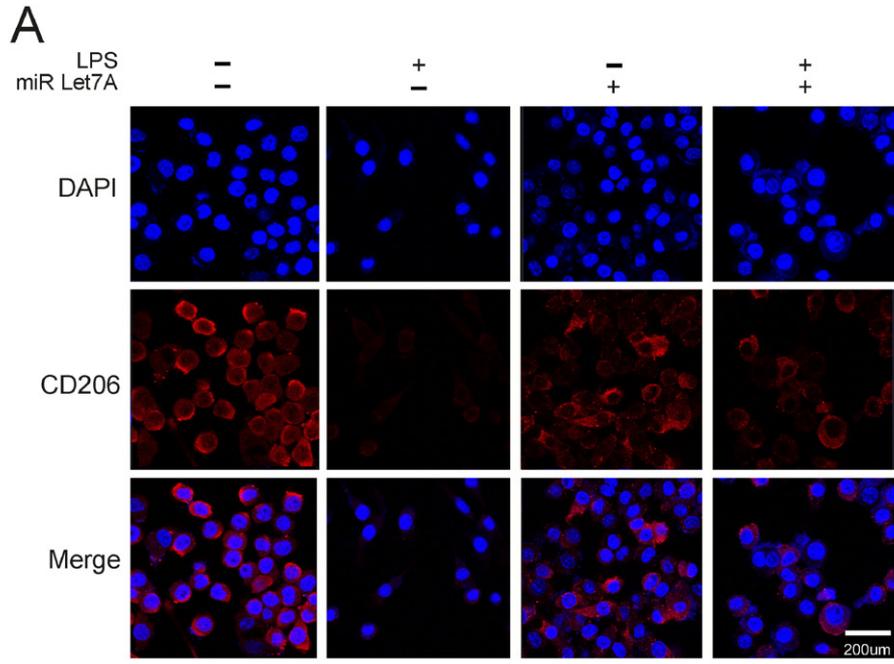


**Fig. 1.** Changing level of miR-Let-7a and anti-apoptotic role of Let-7a. (A) The profile of miR-Let-7a expression after LPS treatment was examined with TaqMan assay (left graph). The mRNA of miR-Let-7a was detected in all groups using TaqMan RT-PCR assay (right graph). In Let-7a group, miR-Let-7a mRNA was measured over 2 fold changes compared to the normal control group owing to the miR-Let-7a mimic treatment. In LPS treatment group, mRNA level of miR-Let-7a decreases over 2 fold changes compared to the normal control group. Data are expressed as mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.001$ . # $p < 0.05$ , comparison to LPS only treated group. (B) Western blotting showed that the protein level of cleaved caspase 3 was evidently increased in LPS treatment group compared to the normal control group. The protein level of cleaved caspase 3 was attenuated in miR-Let-7a with LPS treatment group, compared to only LPS treatment group. The bar graph shows the quantification of cleaved caspase 3 protein in all groups.  $\beta$ -Actin was used as an internal control. Data are expressed as mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.001$ . # $p < 0.05$ , comparison to LPS only treated group. (C) The expression of cleaved caspase-3 in the LPS treatment group was increased compared to the normal control group. MiR-Let-7a attenuated the LPS-induced increase in the number of cleaved caspase-3 positive cells. In miR-Let-7a with LPS treatment group, the expression of cleaved caspase-3 was lower than in only LPS treatment group. The expression of cleaved caspase-3 was attenuated in miR-Let-7a treatment group under LPS induced inflammatory condition. Scale bar: 200  $\mu$ m, cleaved caspase-3: red, 4', 6-diamidino-2-phenylindole (DAPI): blue. Normal: normal control group, LPS: LPS (100 nM) treatment group, Let-7a: miR-Let-7a mimic treatment group, LPS + Let-7a: miR-Let-7a mimic with LPS (100 nM) treatment group.

#### 2.4. NO and iNOS were suppressed in BV2 cells over-expressing Let-7a

To evaluate the expression of iNOS, we conducted (RT)-PCR assays, and measured nitrite using the Griess reagent (Fig. 4). Our results showed that LPS-induced inflammation leads to the increase of iNOS

mRNA and the increase of nitrite production (Fig. 4A) in microglia. In addition, miR-Let-7a over-expression decreased the production of nitrite and the expression of iNOS in LPS-treated microglia (Fig. 4A). And also to check and confirm the role of miR-Let-7a in iNOS and NO production, iNOS mRNA level and NO production were measured both



in miR-Let-7a over-expression by miRNA mimic and in miR-Let-7a down-regulation by miRNA inhibitor (Fig. 4B). The expression level of iNOS was not changed in miR-Let-7a over-expressing BV2 cells whereas the level of it was significantly raised up to level in LPS treated condition (Fig. 4B-a). Corresponding to the iNOS transcript level, production level of NO was not altered in miR-Let-7a over-expressing BV2 cells whereas the level of it was significantly increased up to level in LPS treated condition (Fig. 4B-b). Thus, miR-Let-7a may regulate transcripts involved in or encoding iNOS expression and may attenuate the production of nitrite in microglia under inflammatory conditions.

### 2.5. Upregulation of Let-7a prevented the decrease in BDNF expression

BDNF which is produced and secreted by microglial cells as well as neuron is a crucial signaling molecule between microglia and neurons. To examine the expression of BDNF, Western blot analysis and immunocytochemistry were performed (Fig. 5). Our results showed that the expression of BDNF (Fig. 5) was significantly decreased in LPS-treated microglia compared with the normal control group. The expression of BDNF was increased in miR-Let-7a over-expressing cells treated with LPS compared to the non-over-expressing cells (Fig. 5A, B). Therefore, miR-Let-7a may promote the expression of BDNF proteins in microglia under inflammatory conditions.

## 3. Discussion

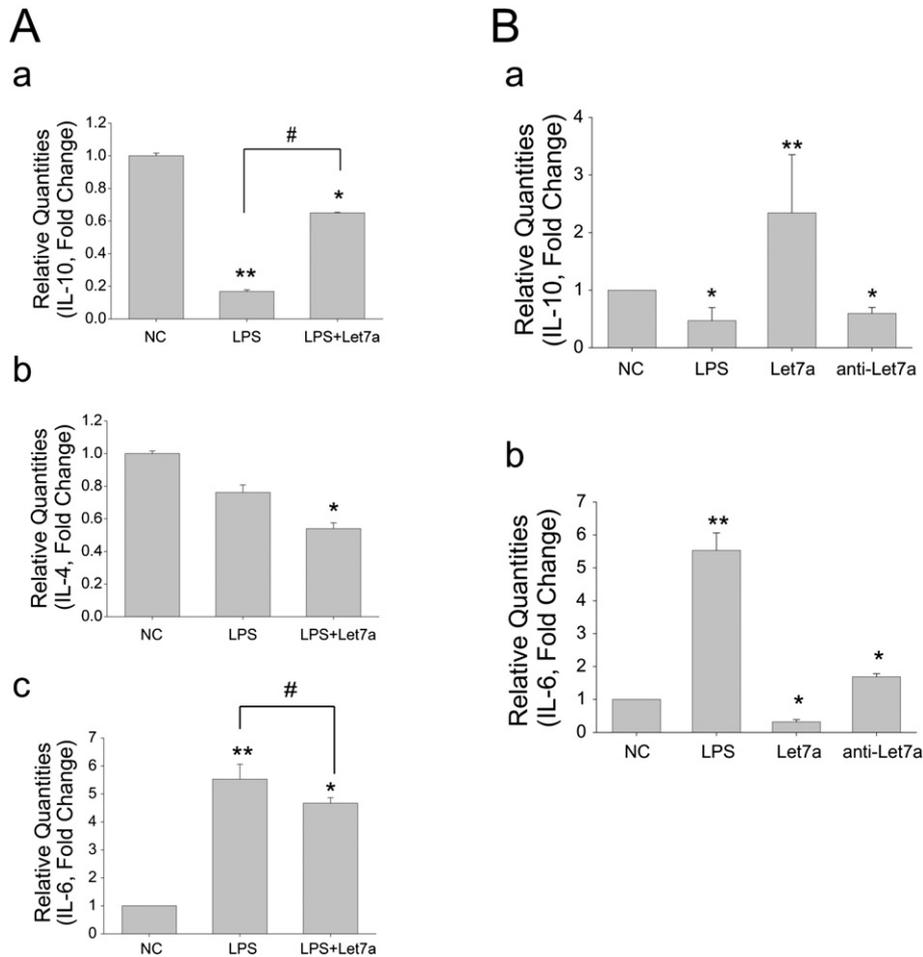
Our study was designed to test whether the miR-Let-7a modulates the phenotype of microglia and microglial BDNF levels under LPS-induced inflammatory conditions, which would mediate microglial function by controlling anti-inflammatory factors. This study provides two major findings: (1) Up-regulated miR-Let-7a in microglia increases anti-inflammatory factors and protects microglia from apoptotic damage; (2) Under inflammatory conditions, microglia over-expressing miR-Let-7a display the M2 phenotype.

MiRNAs, regulatory molecules involved in many physiological processes, have been shown to regulate cellular differentiation and activation of cells in the immune system (Ambros, 2004). Specifically, in the central nervous system (CNS), miRNAs have been identified as crucial regulators for determining cell types such as neuronal cells and myeloid cells (Bi et al., 2009; Johnnidis et al., 2008). One of the miRNAs, miR-124, is expressed in normal microglia and also identified as a key regulator of proliferation of microglia. Additionally, miR-124 differentiates immature CNS cells into mature cells via down-regulation of the active microglia (Ponomarev et al., 2011). Let-7A was the first identified miRNA and is relatively well-known. The alteration of miR-Let-7a levels has been reported to regulate genes related to cell cycle and cell proliferation such as cyclin A2, CDC34, and E2F (Klemke et al., 2010); apoptosis is also modulated by miR-Let-7a and mediated by caspase-3, bcl-2 and the MAP3 kinase pathway (Mu et al., 2010). In the immune response, it has been suggested that Let-7a is involved in post-translational regulation of innate immunity and in the decrease of IL-6 and IL-10 (Ricarte-Filho et al., 2009; Trang et al., 2010).

Microglia are macrophages of the CNS and participate in innate inflammatory responses. Microglia are heterogenic and have two distinct phenotypes according to their activation (Freilich et al., 2013). One is the classical M1 phenotype associated with inflammation and tissue damage, and the other is an alternative M2 phenotype (Freilich et al., 2013). MiRNAs have emerged as crucial regulators of the post-transcriptional control of gene expression and modulators for decisive

cascades occurring thereafter (Yao et al., 2014). The major goal of this study was to investigate the contribution of a specific miRNA, Let-7a, to the modulation of the microglia-mediated inflammatory response. Our investigation showed that miR-Let-7a was significantly reduced in LPS-treated microglia BV2 cells (Fig. 1A) and indicated that modulation of the microglia phenotypes by miR-Let-7a might be related to triggering the inflammatory pathway by LPS. We over-expressed miR-Let-7a levels by transfecting the Let-7a miRNA mimic. Prior to modulation of inflammation by miR-Let-7a, we validated the effect of miR-Let-7a on apoptotic cell death (Fig. 1). In this study, miR-Let-7a attenuated the expression of cleaved caspase-3 in microglia in inflammatory conditions. The results suggested that miR-Let-7a suppresses the apoptosis of microglia in response to LPS-induced inflammation. Although our study could not differentiate the possibility that over-expression of miRNA-Let-7a caused microglial proliferation, at least microglia were less irrigated by LPS inducing inflammatory condition. Also, when cells such as microglia are exposed to LPS, inflammation occurs as a responsive reaction by increasing the macrophage 1 (M1) phase instead of macrophage 2 (M2) phase. In our study, miR-Let-7a affected the phenotype of microglia toward the anti-inflammatory M2 phenotype in physiologic conditions (normal state). Moreover, over-expression of miR-Let-7a promoted the M2 phenotype in microglia under inflammatory conditions (Fig. 2). These data suggest that miR-Let-7a not only deactivates microglia but also tilts their polarization from an M1 phenotype toward an M2 phenotype. This result is consistent with the report that inactive microglia show properties of M2 phenotype, which are important in the suppression of experimental autoimmune encephalomyelitis (EAE) (Ponomarev et al., 2007). Transfection of microglia with a miR-Let-7a mimic resulted in down-regulation of M1-positive phenotype cells whereas the activated M2 phenotype microglia increased and was followed by up-regulation of BDNF. Although the anti-inflammatory microglia M2 phenotype has been demonstrated to play an important role in the regulation of the allergic immune responses (Freilich et al., 2013), studies are needed to examine the mechanisms of regulation and the beneficial role of the M2 phenotype. Innate immunity is the first defense system against both external and internal insults in the brain, and microglia are regarded as a decisive mediator of the process. In a study using microglia cultures (Zhao et al., 2006), inflammation produced by LPS increased nitric oxide (NO) and superoxide ( $O^{2-}$ ) levels, and interaction of IL-4 with microglial IL-4 receptors inhibited NO release. NO is one of the free radicals detected in microglia, and over-production of NO in microglia results in immune-mediated brain injury (Chao et al., 1992). In our study, miR-Let-7a suppressed the production of nitrite and the expression of iNOS in microglia treated with LPS which normally leads to an increase in iNOS mRNA and protein level (Fig. 4). MiR-Let-7a may regulate the expression of iNOS and may attenuate the production of nitrite in microglia under inflammatory conditions. Alternatively, studying on translationally suppressed genes targeted by specific miRNA, miRNAs containing let-7 family are generally implicated in regulating TLR/NF- $\kappa$ B signal pathway (Mueller et al., 2014; Choudhury and Li, 2012). SIRT1 known as Sirtuin 1 or NAD-dependent deacetylase sirtuin-1 has been reported to promote p65 deacetylation and deacetylated p65 to inhibit NF- $\kappa$ B activity and also recently to be identified a target for some kind of miRNAs (Xie et al., 2014). Recently Let-7 miRNA family is also reported to target SIRT1 expression: especially let-7i is harnessing SIRT1 in epithelial

**Fig. 2.** Differential regulation of Let-7a on macrophagic condition. (A) Immunofluorescent staining conducted to check CD206 expression known as a M2 phenotype marker. LPS stimulated microglia shows increased expression of CD206 compared to the normal control microglia. MiR-Let-7a suppresses the expression of CD206 in LPS stimulated microglia. Scale bar: 200  $\mu$ m, 4', 6-diamidino-2-phenylindole (DAPI); blue, CD206; red. (B) Immunofluorescent staining conducted to check mannose receptor CD68 expression known as a M1 phenotype marker. LPS stimulated microglia shows decreased expression of CD68 compared to the normal control microglia. MiR-Let-7a promotes the CD68 expression in LPS stimulated microglia. (C) The graphs present the quantity of the CD206-positive cells (left) and CD68-positive cells (right). Scale bar: 200  $\mu$ m, 4', 6-diamidino-2-phenylindole (DAPI); blue, CD68; green.



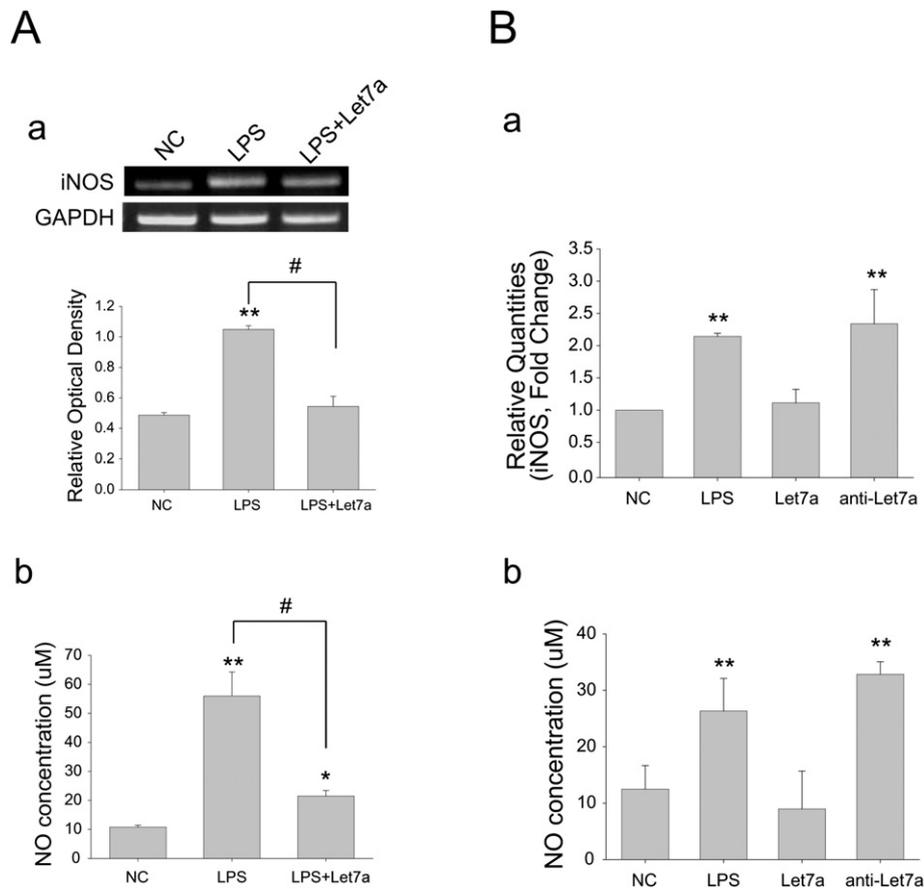
**Fig. 3.** Anti-inflammatory role of over-expressing Let-7a in microglia under inflammation. (A) The mRNA level of IL-10 (a), IL-4 (b) after miR-Let-7a treatment in LPS-induced inflammation was measured by using quantitative real time (RT)-PCR. The LPS treatment group showed lower mRNA levels of IL-10 (a), IL-4 (b) compared to the normal control group. MiR-Let-7a with LPS treatment was almost the same IL-10 (a), IL-4 (b) mRNA levels compared to the only LPS treatment group. The LPS treatment group showed higher mRNA levels of IL-6 (c) compared to the normal control group. MiR-Let-7a with LPS treatment was slightly decreased IL-6 (c) mRNA levels compared to the only LPS treatment group. \* $p < 0.05$ , comparison to normal control (NC). # $p < 0.05$ , comparison to LPS only treated group. (B) The mRNA level of IL-10 (a), IL-6 (b) after miR-Let-7a mimic (Let7a) or inhibitor (anti-Let7a) treatment in LPS-induced inflammation was measured by using quantitative real time (RT)-PCR. The LPS treatment group showed lower mRNA levels of IL-10 (a) compared to the normal control group. Under LPS treatment, miR-Let-7a over-expression was shown to higher level of IL-10 than NC, and the level of IL-10 treated with anti-Let-7a was converted to the level of LPS single treated group. The LPS treatment group showed higher mRNA levels of IL-6 (b) compared to the normal control group. miR-Let-7a with LPS treatment was slightly decreased IL-6 (b) mRNA levels compared to the only LPS treatment group. \* $p < 0.05$ , comparison to normal control (NC). NC: normal control group, LPS: LPS (100 nM) treatment group, Let-7a: LPS with miR-Let-7a mimic treated group, anti-Let7a: LPS with miR-Let-7a inhibitor treated group.

cells of host, moreover via TLR/NF- $\kappa$ B pathway when host is infected by *Cryptosporidium parvum* (Xie et al., 2014). We did not provide the gene or pathway directly targeted by let-7a in our study. Referred above reports, it might be inferred that let-7a consisted in let-7 family involves in TLR/NF- $\kappa$ B pathway via targeting SIRT1.

Under LPS-induced inflammatory conditions, our results showed that reduction of BDNF was prevented in BV2 microglia over-expressing miR-Let-7a. MiR-Let-7a may promote the expression of BDNF in microglia under inflammatory conditions. During inflammation, microglia undergo changes in cell proliferation and morphology, and consequently synthesize and secrete both pro-inflammatory and anti-inflammatory components (Gomes et al., 2013). The neurotrophic factor, BDNF, has been reported to prevent axonal and neuronal damage due to various pathological insults (Dougherty et al., 2000). BDNF is also involved in neuro-inflammation via several modulators (Gomes et al., 2013). BDNF in the CNS is supplied by immune cells and increased GP145-TrkB has been suggested as a candidate for mediating a neuro-protective role in multiple sclerosis (Stadelmann et al., 2002). BDNF, which is produced and secreted by microglial cells, is a crucial signaling molecule between microglia and neurons (Coull et al., 2005). Blocking of this signaling pathway between microglia and neurons has been

suggested as a possible therapeutic strategy for neuropathic pain (Coull et al., 2005). Our results also demonstrated that the effect of miR-Let-7a over-expression on BV2 microglia-derived BDNF was not inhibited by LPS-triggered inflammation. Consistent with previous reports, our results imply that miR-Let-7a is involved in the anti-inflammatory role of microglia under active inflammatory conditions.

Up-regulated miR-Let-7a boosted the transcriptional expression level of the anti-inflammatory factors IL-10 and IL-4 in LPS-treated microglia. There have been previous reports examining the effects of miRNA on microglia or neuro-inflammation. MicroRNA miR-124, over-expressed in microglia, is reported to induce the M2 phenotype, and three miR-124 precursors induced IL-4 expression on a transcriptional level (Freilich et al., 2013; Ponomarev et al., 2011). Another report found that microglia with miR-9 enhanced pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and MCP-1, and suggested that miR-9 was involved in LPS-derived microglial activation (Yao et al., 2014). In the case of inflammation, the anti-inflammatory cytokine IL10 was preferentially reduced instead of IL-4. In this study, miR-Let-7a expression led to increases in the anti-inflammatory cytokines IL-10 and IL-4. When microglia over-expressing miR-Let-7a were treated with LPS, IL-10 was significantly increased and the transcriptional level of IL-10 increased



**Fig. 4.** Inflammatory regulation of Let-7a via iNOS. (A) (a) iNOS mRNA expression after miR-Let-7a treatment in LPS-induced inflammation. iNOS mRNA level was measured by using reverse transcription (RT)-PCR. The LPS treatment group showed higher mRNA levels of iNOS compared to the normal control group. MiR-Let-7a with LPS treatment resulted in lower iNOS mRNA levels compared to the only LPS treatment group. \* $p < 0.05$ , \*\* $p < 0.001$ . (b) Nitrite production from LPS-treated microglia was measured by using Griess reagent. Nitrite concentration is approximately 55  $\mu\text{M}$  in the LPS treatment group whereas upon the addition of miR-Let-7a, the nitrite concentration was 25  $\mu\text{M}$ . \* $p < 0.05$ , \*\* $p < 0.01$ . # $p < 0.05$ , comparison to LPS only treated group. (B) (a) iNOS mRNA level was measured by using quantitative real time (RT)-PCR. The LPS treatment group showed almost 2 fold changes higher mRNA levels of iNOS compared to the normal control group. MiR-Let-7a with LPS treatment resulted in lower iNOS mRNA levels compared to the only LPS treatment group. In anti-Let-7a treatment group, iNOS mRNA level is the same of the normal control group. \* $p < 0.05$ , \*\* $p < 0.001$ . (b) Nitrite concentration is approximately 28  $\mu\text{M}$  in the LPS treatment group. Upon the addition of miR-Let-7a, the nitrite concentration decreases over 2 fold changes compared to those of the LPS treatment group. In anti-Let-7a treatment group, iNOS mRNA level is the same of the normal control group. Data were expressed as the mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.001$ . NC: normal control group, LPS: LPS (100 nM) treatment group, Let-7a: miR-Let-7a mimic treatment group, LPS + Let-7a: miR-Let-7a with LPS (100 nM) treatment group, anti-Let-7a: anti-miR-Let-7a treatment group.

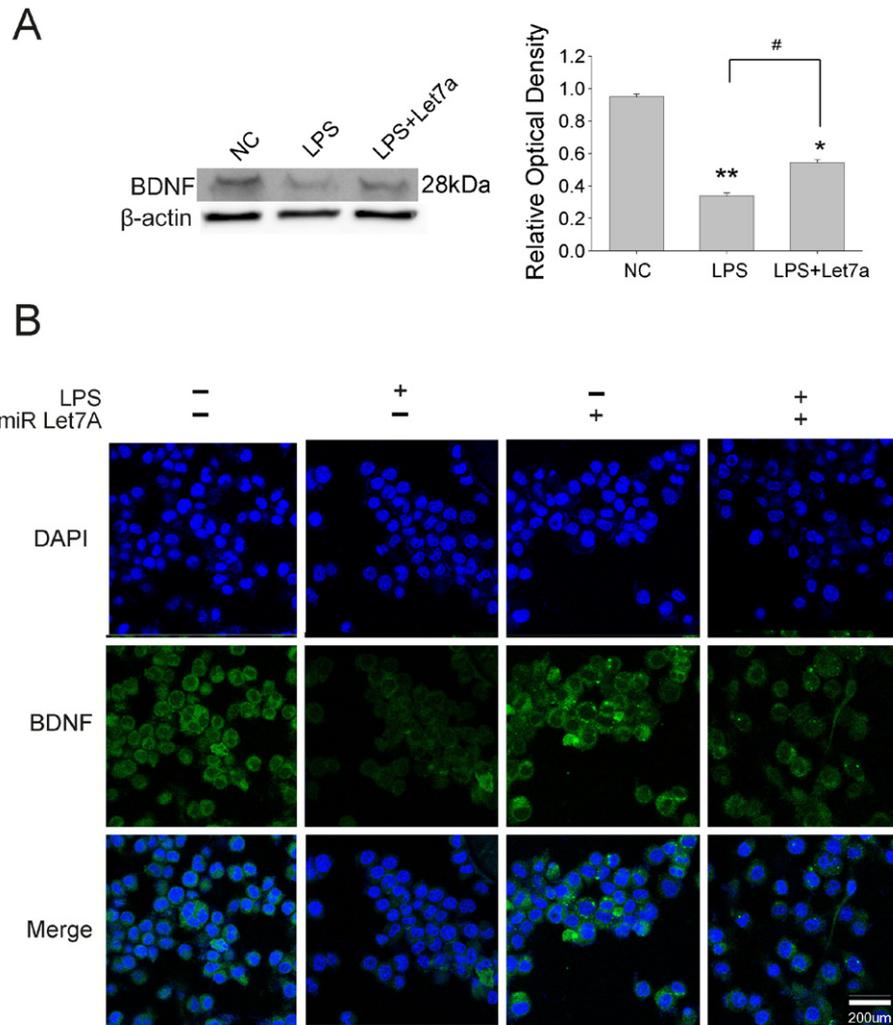
3.8 fold compared to LPS-only treated microglia. However, the level of IL-4 was found to be unchanged compared to LPS only treatment. It has been suggested that IL-4 is an important immuno-modulator that can protect against microglia-derived neurotoxicity by suppressing Reactive Oxygen Species (ROS) overproduction and release (Zhao et al., 2006). However, our study suggests that IL-10 is more highly regulated than IL-4 by miR-Let-7a in microglia. Nevertheless, in contrast to resting BV2 microglia, over-expressing miR-Let-7a triggered the up-regulation of both IL-10, and IL-4 at the transcriptional level in normal microglia. It is possible that miR-Let-7a acts on IL-10 transcription specifically, and not on IL-4.

Although this study did not evaluate the direct protective effect of BV2 microglia on neurons or investigate the relationship between microglia and neurons, the results suggest a possible beneficial and novel role of miR-Let-7a in microglia. Overall, our results showed that miR-Let-7a helped BV2 microglia to express the anti-inflammatory M2 phenotype by up-regulating mainly IL-10 transcription in response to the LPS-induced inflammatory conditions. In conclusion, in response to inflammation, miR-Let-7a represses the production of nitrite and the expression of iNOS, IL-6 in microglia, and activates the expression of BDNF, and anti-inflammatory factors such as IL-10 and IL-4. Our study suggests that miR-Let-7a is involved in modulating the anti-inflammatory function of microglia following inflammation.

## 4. Experimental methods

### 4.1. Cell culture and drug treatment

Murine BV2 microglial cells were obtained from Prof. Eun-hye Joe (Ajou University School of Medicine, Chronic Inflammatory Disease Research Center) and cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100  $\mu\text{g}/\text{mL}$  penicillin-streptomycin (Gibco, Grand Island, NY, USA) at 37  $^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ . Used miRNAs were purchased from Ambion (Ambion, Austin, TX, USA) and followed the manufacturer's protocol. Two kinds of miRNA are used in this study; Let-7a mimic (cat#, 4464066; sassy ID, MC10050) and Let-7a inhibitor (cat#, 4464084; assay ID, MH 10050). The expression and inhibition level was validated with negative (cat#, 4464058) and positive-control miRNA (cat#, 4464062) and their time-dependent profile was performed also. The cells were cultured for 2 days and were then treated with miR-Let-7a and miRNA negative control for each group. The cells were treated with lipopolysaccharide (LPS [1 $\mu\text{g}/\text{mL}$ ]) and anti-Let-7a (Ambion, Austin, TX, USA) for 48 h after miRNA treatment; proteins and RNAs were obtained from these cells 24 h later.



**Fig. 5.** Prevention of BDNF decrease by over-expressing Let-7a. (A) Western blotting showed that the protein level of BDNF was evidently decreased in the LPS treatment group compared to the normal control group. The protein level of BDNF was increased in miR-Let-7a with LPS treatment group compared with the only LPS treatment group. The bar graph shows the quantification of BDNF in all groups.  $\beta$ -Actin was used as an internal control. Data are expressed as mean  $\pm$  S.E.M. \* $p < 0.05$ . # $p < 0.05$ , comparison to LPS only treated group. (B) Immunofluorescent staining conducted to check BDNF expression. LPS stimulated microglia shows decreased expression of BDNF compared to the normal control microglia. MiR-Let-7a promotes the BDNF expression in LPS treated microglia. Scale bar: 200  $\mu$ m, 4',6'-diamidino-2-phenylindole (DAPI): blue, BDNF: green. Normal: normal control group, LPS: LPS (100 nM) treatment group, LPS + Let-7a: miR-Let-7a mimic with LPS (100 nM) treatment group.

#### 4.2. Determination of nitrite

In order to determine the level of nitrite production in the BV2 microglia cultures, the supernatant (100  $\mu$ L) of each sample treated with miRNA-Let-7a, microRNA negative control, or LPS was transferred to 96-well plates. The Griess reagents (100  $\mu$ L) were added to the plate and incubated for 15 min at room temperature (RT). Subsequently, the absorbance was measured at 540 nm. Standards were prepared with nitrite solution instead of the sample. The nitrite concentration in the sample was calculated by comparing the absorbance of the sample with a nitrite standard curve.

#### 4.3. Western blot analyses

Protein was extracted from BV2 microglia cultures, and equal amounts (50  $\mu$ g) were electrophoresed on 10%–12% SDS-polyacrylamide gels. Separated proteins were electrotransferred to immunobilon-NC membranes (Millipore, Massachusetts, MA, USA), which were blocked for 1 h at RT with 5% skim milk in Tris-buffered saline and 0.1% Tween-20 (TBST). The primary antibodies used were BDNF (1:2000, Abcam, Cambridge,

UK), cleaved caspase-3 (1: 2000, Santa Cruz, CA, USA), and  $\beta$ -actin (1:1000, Santa Cruz, CA, USA). Blots were incubated with the primary antibodies overnight at 4  $^{\circ}$ C. Membranes were washed 3 times (5 min each) with TBST. The secondary antibodies were anti-rabbit and anti-mouse (1:2000, New England Biolabs, Ipswich, MA, USA) and were incubated for 1 h at RT. After washing with TBST (0.05% Tween 20) 3 times, immunoreactive signals were detected using chemiluminescence and an ECL detection system (Amersham Life Science, Buckinghamshire, UK) with the LAS 4000 program.

#### 4.4. Reverse transcription-PCR (RT-PCR)

To examine the expression of iNOS in BV2 cells under LPS induced inflammation condition, RT-PCR was performed using iNOS primers. Briefly, samples were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's protocol. cDNA synthesis from mRNA and sample normalization were performed. PCR was performed using the following thermal cycling conditions: 10 min at 95  $^{\circ}$ C; 35 cycles of denaturing at 95  $^{\circ}$ C for 15 s, annealing for 30 s at 70  $^{\circ}$ C, elongation at 72  $^{\circ}$ C for 30 s; final extension for 10 min at 72  $^{\circ}$ C, and held at 4  $^{\circ}$ C. PCR was performed using the

following primers (5' to 3'); iNOS forward (F): CCCTCCGAAGTTCTGG CAGCAGC, reverse (R): GCCTGTACAGAGCTCGTGGCTTTGG, GAPDH (F): GGCATGGACTGTGGTCATGAG, (R): TGCACCACCAACTGCTTAGC. PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide.

#### 4.5. Quantitative real time-PCR

To examine the amount of IL-10, IL-6 and IL-4 mRNA in BV2 cells under LPS-induced inflammation conditions, quantitative real time-PCR was performed using IL-10, IL-6 and IL-4 primers. Total cellular RNA was extracted from the BV2 microglia cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Poly (A) was added using poly (A) polymerase (Ambion, Austin, TX, USA). One Step SYBR® Prime Script™ RT-PCR Kit II (Takara, Japan) was used to conduct qRT-PCR. PCR was performed using the following primers (5' to 3'); IL-10 forward (F): CCAAGCCTTATCGGAAATGA, reverse (R): TTTTACAGGGGAGAAATCG, IL-6 (F): AACGATGATGCACTTGCAGA, (R): CTCTGAAGGACTCTGGCTTTG, IL-4 F: TCAACCCCA GCTAGTTGTC, R: TGT TCTTCGTGTCTGAGG, GAPDH F: GGCATGGACT GTGGTCATGAG, R: TGCACCACCAACTGCTTAGC. Denaturing was carried out at 95 °C for 3 min; 40 cycles of 95 °C for 20 s; annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. At each extension step at 72 °C, fluorescence was detected at 585 nm. The expression of IL-10, IL-6, and IL-4 was assessed using an ABI prism 7500 Real-Time PCR System (Life Technologies Corporation, CA, USA) and analyzed with comparative Ct quantification. GAPDH was amplified as an internal control. The  $\Delta$ Ct values of GAPDH were subtracted from the Ct values of the IL-10, IL-6 and IL-4 genes ( $\Delta$ Ct). The  $\Delta$ Ct values of LPS-treated or miRNA-transfected cells were compared with the  $\Delta$ Ct values of untreated normal microglia BV2 cells. The fold change of each transcript was calculated with method from  $2^{-\Delta\Delta Ct}$  and the values were presented by relative quantity (RQ).

#### 4.6. TaqMan assay for miRNA

For quantitative analysis of miR-Let-7a, reverse transcription (RT) was first performed using the TaqMan Micro RNA Reverse Transcription kit (Applied Biosystems, Waltham, Massachusetts, USA) according to the manufacturer's instructions with total RNA of 10 ng. PCR reactions were then performed according to the manufacturer's instructions to quantitate the expression levels of miRNA-Let-7a using TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems, USA), and TaqMan microRNA assay (Applied Biosystems, Waltham, Massachusetts, USA) for the miR-Let-7a of interest. PCR amplification was performed in ABI 7500 Real Time PCR (Bio Rad, Philadelphia, PA, USA) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR incubation profile was extended to 40 cycles for miR-Let-7a. The differential expression level was analyzed with ddCt method. Each miRNA expression level was presented to relative quantity (RQ) to NC normalized with U6. PCR reactions were performed in triplicate. MiR-Let-7a expression was normalized to the expression of U6.

#### 4.7. Immunocytochemistry

The expression of cleaved caspase-3, CD206, CD68, and BDNF in BV2 cells was examined by immunocytochemistry. Cells in all experimental groups were washed three times with PBS, fixed with 4% paraformaldehyde for 3 h, and then washed with PBS. BV2 cells were permeabilized with 0.025% Triton X-100 and blocked for 1 h at RT with dilution buffer (Invitrogen, Carlsbad, CA, USA). Primary antibodies, anti-rabbit-CD206 (1:500, Santa Cruz, CA, USA), and anti-rabbit-cleaved caspase-3 (1:500, Santa Cruz, CA, USA), anti-rabbit-BDNF (1:500, Abcam, Cambridge, UK), or anti-mouse-CD 68 (1:500, Millipore, Massachusetts, MA, USA) were prepared in dilution buffer, added to samples, and incubated for 3 h at RT. Primary antibody was then removed, and cells were

washed 3 times for 3 min each with PBS. Next, samples were incubated with Rhodamine-conjugated goat anti-rabbit (1:200, Jackson ImmunoResearch), or Texas red-conjugated donkey anti-mouse (1:200, Jackson ImmunoResearch) for 2 h at RT. Cells were washed again three times for 3 min each with PBS and stained with 1  $\mu$ g/mL 4',6-diamidino-2-phenylindole (DAPI) (1:100, Invitrogen, Carlsbad, CA, USA) for 10 min at RT. Fixed samples were imaged using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

#### 4.8. Statistical analysis

Statistical analyses were carried out using SPSS 18.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as mean  $\pm$  S.E.M. Significant intergroup differences were determined by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* multiple comparison test. Statistical significance with the LPS treatment group was determined by t-test. Each experiment included 3 replicates per condition. Differences were considered significant at  $p < 0.05$  (\*) or  $p < 0.001$  (\*\*).

#### Conflicts of interest

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

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