Molecules and Cells

The Effect of Annexin A1 as a Potential New Therapeutic Target on Neuronal Damage by Activated Microglia

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Brain disease is known to cause irrevocable and fatal loss of biological function once damaged. One of various causes of its development is damage to neuron cells caused by hyperactivated microglia, which function as immune cells in brain. Among the genes expressed in microglia stimulated by various antigens, annexin A1 (ANXA1) is expressed in the early phase of the inflammatory response and plays an important role in controlling the immune response. In this study, we assessed whether ANXA1 can be a therapeutic target gene for the initial reduction of the immune response induced by microglia to minimize neuronal damage. To address this, mouse-origin microglial cells were stimulated to mimic an immune response by lipopolysaccharide (LPS) treatment. The LPS treatment caused activation of ANXA1 gene and expression of inflammatory cytokines. To assess the biological function in microglia by the downregulation of ANXA1 gene, cells were treated with short hairpin RNA-ANXA1. Downregulated ANXA1 affected the function of mitochondria in the microglia and showed reduced neuronal damage when compared to the control group in the co-culture system. Taken together, our results showed that ANXA1 could be used as a potential therapeutic target for inflammation-related neurodegenerative diseases.

Keywords: annexin A1, brain diseases, immune response, microglia, neural damage

INTRODUCTION

Recent trends in the study of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) focus on the role and function of microglia and the relation with neuron (Arends et al., 2000; Perry et al., 2010). The main causes of this diseases are the high susceptibility of neurons to inflammatory damage, and the main role of microglia as mediators of the inflammatory process (Chan et al., 2001; Magnus et al., 2001). Various omics data suggest about the key role of microglia in the pathogenesis of brain diseases such as AD (Lewcock et al., 2020). There are evidences that neurodegenerative diseases, which are caused by misfolding and aggregation of proteins such as β-amyloid and tau tangles, could cause dementia (Ashrafian et al., 2020; Binder et al., 2005). Among brain disorders, neuroinflammation is a defense mechanism that initially protects the brain by removing or inhibiting diverse pathogens (Picca et al., 2020). This inflammatory response can have beneficial effects that activated microglia by pathogens involve immune regulation or injury resolution by acquiring phenotypes as well as participate in cell cytotoxic. However, when inflammatory responses continue, it become chronic inflammatory which are detrimental, and they inhibit regeneration as well as involve microglia and astrocytes and can lead to neurodegenerative diseases (Scheiblich et al., 2020).

Microglia, which are immune cells in the central nervous

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system (CNS) (Fan et al., 2017), responds to endogenous stimuli generated following infection or injury. Upon appropriate stimulation, classically activated microglia trigger the first line of host defense against pathogens, leading to a neuroprotective role (Graeber, 2010; Nimmerjahn et al., 2005). However, chronic activation of microglia leads to potential cytotoxicity (Franco and Fernandez-Suarez, 2015; Pascual et al., 2012), such as pro-inflammatory cytokines and reactive oxygen intermediates, which can cause various CNS disorders such as pain, neurodegeneration, and stroke that carry actual or potential risks (Dai et al., 2015). Consequently, the activated microglia in the brain attack the neurons, leading to dementia due to the damaged neurons (Eikelenboom and Veerhuis, 1996; Shaji et al., 2018).

Currently, active research in various fields has been conducted to find effective treatments, including gene therapy (Nilsson et al., 2010), stem cell-based cell therapy (Duncan and Valenzuela, 2017), and drug development. Among them, risperidone is the only treatment currently approved to treat agitation and aggression in patients with AD. Risperidone is a clinically preferred drug, because it has fewer side effects as compared to other drugs, and it effectively reduces the symptoms and works against the negative symptoms by blocking dopamine receptors (Katz et al., 1999). Although several other drugs such as citalopram, aripiprazole, and quetiapine are used, they are not recommended because of the greater risk of side effects than positive effects (Dai et al., 2015). Despite various treatments, including β-amyloid-inhibiting drugs, and studies conducted for dementia, no fundamental treatment or significant therapeutic effect has been reported. Therefore, in addition to the existing treatment methods, the development and approaches of new treatments that can cure the fundamental causes is required.

To address this issue, we focused on genetic material related to immune response as a way to treat the disease at its source in order to slow it down. Among the genes expressed in microglia stimulated by various antigens, annexin A1 (ANXA1) was considered as a candidate genetic material involved in inflammatory reactions that causes microglia to damage nerve cells. ANXA1 is a protein that is expressed and activated in the early phase of the inflammatory response and plays an important role in controlling the immune response (McArthur et al., 2010). It exists in the plasma membrane, extracellular region, and cytosol of cells (Han et al., 2020). Moreover, several studies have reported that the expression of ANXA1 is found in glial cells (specifically in microglia) of the normal adult human and rodent brain (Buckingham et al., 2006; Dreier et al., 1998). Thus, ANXA1 is observed to be constitutively expressed in cells related with the innate immune stage of the normal brain (Solito et al., 2008). Although ANXA1 is controversial because it is responsible for the functions of pro-inflammatory and anti-inflammatory in the CNS through various studies, enhanced ANXA1 expression has been found in brain lesions of patients with PD (Solito et al., 2008), and AD, which may specifically act in the inflammatory phase before dementia occurs.

Using this characteristic of ANXA1, we hypothesized that ANXA1 would engage in early immune responses and affect neuronal damage in the immune response by lipopolysaccharide (LPS)-stimulated microglia. We evaluated whether the neuronal damage was reduced when ANXA1, expressed in the microglia activated by antigens, was substantially down-regulated (Fig. 1).

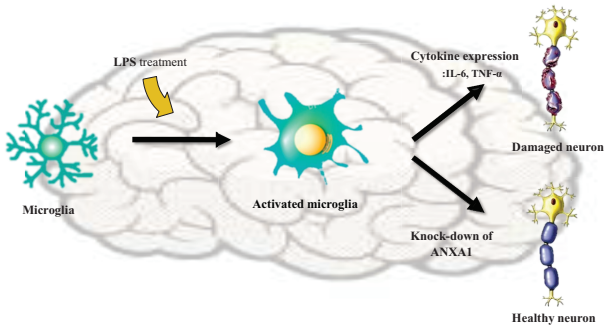


Fig. 1. Schematic representation of the proposed role in the control of microglial phagocytosis. Microglia is activated by LPS treatment, and in the CNS, the activated microglia damage the neuron.

MATERIALS AND METHODS

Cell cultures

BV2 microglial cells and SH-SY5Y neuron cell lines were provided by professor Seung-Ju Yang of Konyang University. Two cell lines were incubated at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium with high glucose (HyClone, USA) containing 10% fetal bovine serum (HyClone) and 1% penicillin G-streptomycin solution (Gibco, USA).

RNA extraction and relative quantitative real-time polymerase chain reaction or conventional polymerase chain reaction

Total RNA was isolated from BV2 microglial cells using Trizol reagent (Invitrogen, USA) by following the manufacturer's instructions. It was quantified using NanoDrop™ One (Thermo Fisher Scientific, USA), and the reverse transcription-polymerase chain reaction (RT-PCR) assay was performed with DiaStar 2X RT Pre-Mix (Solgent, Korea) on T100 (Bio-Rad, USA). The RT-PCR experiment using primers for the detection of each gene was performed as per the manufacturer's PCR conditions. The products were electrophoresed in 2% agarose gels (Agarose, Molecular Biology Grade; Vivantis Technologies, Malaysia) prepared in 1X tris-acetate-ethylenediaminetetraacetic acid buffer (Biosesang, Korea). Gel images were obtained using a Fusion SL (Vilber Lourmat, France). Real-time PCR was conducted in a CFX96™ real-time system employing the Solg™ 2X RT-PCR Smart mix (Solgent). Table 1 shows the primer sequences used in this study.

Transfection of annexin A1 short hairpin RNA to BV2 microglial cells

ANXA1 short hairpin RNA (shRNA) was constructed using the same sequences (NM_010730) (Supplementary Fig. S1). To establish the conditions of ANXA1 knockdown (Kd) and scrambles (Scr), a total of 1×10^5 BV2 cells were transfected

Table 1. List and sequences of primers used

Gene	Forward	Reverse
GAPDH	TCACCACCATGGAGAAGG	GCTAGGCAGTTGGTGGTGCA
IL-6	ACAAGTCCGGAGAGGAGACT	GGTCTTGGTCCTTAGCCACTC
TNF-α	CAAGGGACAAGGCTGCCCCG	GCAGGGGCTCTTGACGGCAG
ICAM1	AGCACCTCCCCACCTACTTT	AGCTTGCACGACCCTTCTAA
ANXA1	ACGTGAACGTCTTCACCACA	TCGGCAAAGAAAGCTGGAGT

with pGPU6/GFP/Neo vector with shRNA-ANXA1 (OriGene, USA) by using TransIT-X2 (Takara Bio, Japan) in serum-free medium according to the manufacturer's instructions. The same plasmid without any insert and a plasmid with a Scr vector were used as negative controls. After the transfection, green fluorescent protein (GFP)-expressing BV2 cells were observed after 48 h of incubation, and then RNA was extracted and subjected to conventional PCR to examine ANXA1 expression level.

Enzyme-linked immunosorbent assay for interleukin-6

In order to confirm the cytokine expression pattern of microglia activated by LPS and the Kd by shRNA, BV2 microglial cells were cultured in 48-well plates at 5×10^4 cells/ml for 24 h. The cells were transfected with ANXA1 shRNA or Scr shRNA using Mirus (Takara Bio) in a serum-free medium according to the manufacturer's instructions. In brief, culture supernatants were collected after transfection. Pro-inflammatory cytokine interleukin-6 (IL-6) secreted from microglia stimulated by LPS was confirmed using the Mouse IL-6 DuoSet ELISA (R&D Systems, USA) as per the manufacturer's instructions and expressed as pg/ml. All assays were performed thrice, and the absorbance of the solution was measured at 450 nm using VersaMax™ (Molecular Device, LLC, USA). This was calculated using the standard value obtained from a linear regression equation.

Cell viability test

Based on a 24-well plate, 2 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, UK) was added to each well for 300 μl and incubated at 37°C for 4 h and 30 min. After the formation of formazan salt, the supernatant was removed and treated with dimethyl sulfoxide at 37°C for 15 min in the same amount as the MTT reagent, and the absorbance was measured at 570 nm using VerxaMax.

In-cell Western assay

The in-cell Western (ICW) assay was performed using the Odyssey Imaging System (LI-COR Biosciences, USA) according to the manufacturer's instructions. Cells were seeded at 1×10^4 cells/ml for 24 h in 96-well plates (Falcon™, Cat. #161093; BD Biosciences, USA). The cells were then fixed and permeabilized with 3.7% formaldehyde for 20 min at room temperature (RT) and blocked with LI-COR Odyssey Blocking Solution (LI-COR Biosciences) for 90 min. The cells were incubated at RT overnight with nuclear factor-kappa-B p65 (NF-κB) (SC-514451; Santa Cruz Biotechnology, USA) premixed with a mouse immunoglobulin (Ig) G antibody against β-actin (1:500 dilution, 8432; Santa Cruz Biotechnology). After five washes with 0.1% phosphate-buffered saline with Tween®, the cells were stained with a goat anti-mouse IgG IRDye™ 680 antibody (1:800 dilution; LI-COR Biosciences) at RT for 1 h. The microplates were scanned using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). The integrated fluorescence intensities representing the protein expression levels were acquired using the software provided with the imager station (Odyssey Software Version 3.0; LI-COR Biosciences) (Ma et al., 2017). The relative amount of protein was obtained by normalizing to endogenous β-actin in all experiments.

Measurements of oxygen consumption rate (OCR)

BV2 microglial cell were assayed as described in the manufacturer's instructions in XF DMEM (containing 2 mM glutamine, Agilent Seahorse; Agilent Technologies, USA) supplemented with 10 mM pyruvate and 25 mM glucose. BV2 microglial cell were plated at 1×10^4 cells per well in 8-well Seahorse culture plates (Agilent Seahorse) in 200 μl of the appropriate growth medium. One day after plating, BV2 microglial cell was treated LPS 2 h. After then, we measured OCR on a Seahorse FXp as described by the manufacturer, with modifications (Shin et al., 2019). The concentrations of the inhibitors used in this study were the following: 1 mM oligomycin, 1 mM FCCP, and 0.5 mM each of antimycin A and rotenone.

Apoptosis assay

To clarify whether ANXA1 was downregulated in microglia treated with shRNA cell death (the apoptotic effect was caused by ANXA1 in microglial cells), apoptosis was detected using the FITC-Annexin V Apoptosis Detection Kit 1 (BD Biosciences) according to the manufacturer's protocol. Samples were assessed using a co-culture system. First, the microglia prepared in the upper insert were treated with LPS for 2 h. The SH-SY5Y neuron cells that had been seeded on the bottom plate were then harvested and washed twice with cold PBS and re-suspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. The cells were transferred to 100 μl of the solution (1×10^5 cells/ml), 5 μl of FITC-Annexin V, and 5 μl propidium iodide (PI) for 15 min in the dark at RT. Next, 400 μl of binding buffer was added to each sample. Finally, apoptotic levels were analyzed by flow cytometry (Novo Cyte flow cytometer; ACEA Bioscience, USA). Data were analyzed using Novoexpress software (ACEA Biosciences).

Statistical analysis

All data are presented as mean ± SEM. The values were obtained from at least three repetitive experiments. The exper-

iment results were determined by applying the paired *t*-test and one-way ANOVA. Significance of analysis is indicated in the figures ($*P < 0.05$, $**P < 0.02$, and $***P < 0.01$).

RESULTS

The expression of annexin A1 in microglia stimulated by lipopolysaccharides

First, we assessed the optimal conditions for checking whether the expression of ANXA1 was induced in the microglia by pathogenic stimuli. ANXA1 expression in BV2 microglia was examined according to the treatment concentration and time of LPS treatment. RNA extraction and cDNA synthesis by RT-PCR were used to confirm the expression of the ANXA1 gene in BV2 microglia. The total RNA isolated from these cells was normalized to glyceraldehyde 3-phosphate dehydrogenase. As shown in Fig. 2A, the treatment concentration of LPS for the induction of ANXA1 expression was significantly higher in the microglia group treated with 1,000 ng/ml LPS than in the untreated microglial cells. The quantitative data of the ANXA1 band showed a similar tendency (Fig. 2B). Using the optimal concentration conditions for LPS treatment from the results of Fig. 2A, the expression of ANXA1 was evaluated over time (Fig. 2C). The highest expression was observed after 2 h of LPS treatment (Figs. 2C and 2D). These data indicate that LPS is a potent inducer of ANXA1 expression in microglial cells.

Cytokine expression and M1 phenotype polarization of microglia by lipopolysaccharide stimulation

Microglia in the brain contribute to the immune response by switching to M1 or M2 types to function as macrophages, depending on the stimulation. Activated pro-inflammatory (M1) phenotype often act as the first line of defense of the innate immune system, which occurs within the first few hours or days (Tang and Le, 2016). The microglia secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and IL-6, and produce reactive oxygen species (ROS) and nitric oxide (NO). Therefore, we evaluated whether the LPS-stimulated microglia converted to M1 type and secreted pro-inflammatory cytokines in response. As shown in Figs. 3A and 3B, the expression of intercellular adhesion molecule-1 (ICAM1), which is an inflammatory protein that acts as a biomarker of the M1 type, was increased in the LPS-treated microglia when compared to the untreated microglia. This indicated that the LPS-stimulated microglia converted to the M1 type to function as macrophages in the brain. In addition, cytokine secretion, one of the major functions of macrophages, was expressed at high levels at 2 h, similar to the expression of ANXA1 in the microglia treated with LPS (Figs. 3C and 3D). These results indicate that microglia stimulated by LPS present ANXA1 as soon as they switch to the M1 type.

Decreased cell viability of neuron by activated microglia

In previous experiments, we confirmed that microglia differentiate into cells that function as M1 phenotype by LPS stimulation (Fig. 3). Next, we assessed how the activated microglia affect neurons. To address this, co-culture was performed and stabilized by seeding microglia (BV2) cells in the upper well and neuronal (SH-SY5Y) cells in the down well. The wells were combined for 1 day after the activation of microglial cells by LPS for 4 h (Fig. 4A). As shown in the results of Fig. 4B, co-cultured neurons with microglia may be slightly affected by the unstimulated microglia, although some cytotoxicity was observed in neuron cells treated with LPS. However, neuronal cell viability was significantly reduced when co-cultured with LPS-activated microglia (Fig. 4B). This result

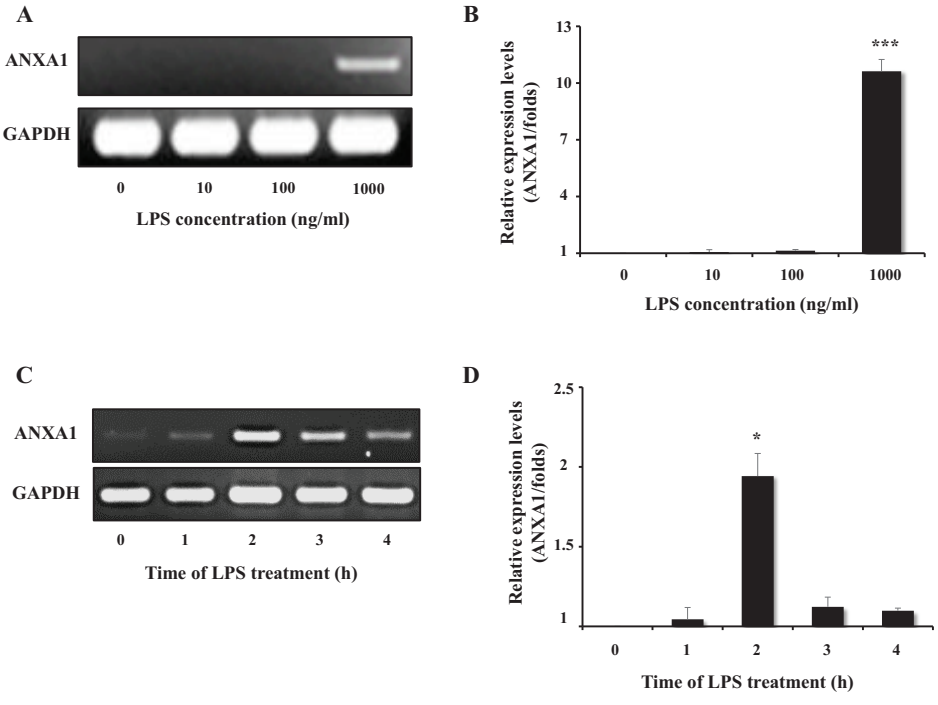


Fig. 2. Optimal conditions for the expression induction of ANXA1 in microglial cells by LPS. When LPS was treated to BV2 according to various concentration, ANXA1 was significantly increased at 1,000 ng/ml. (A and B) The expression of ANXA1 according to various LPS concentration (10-1,000 ng/ml) and its quantitative data. (C and D) The expression of ANXA1 according to the time of LPS treatment (1,000 ng/ml) and its quantitative data. Quantifications of ANXA1 of conventional PCR intensities normalized to their respective controls (defined as 1.0). Original gel images showed in Supplementary Fig. S2. $***P < 0.01$, as compared with control group. $*P < 0.05$, as compared with control group.

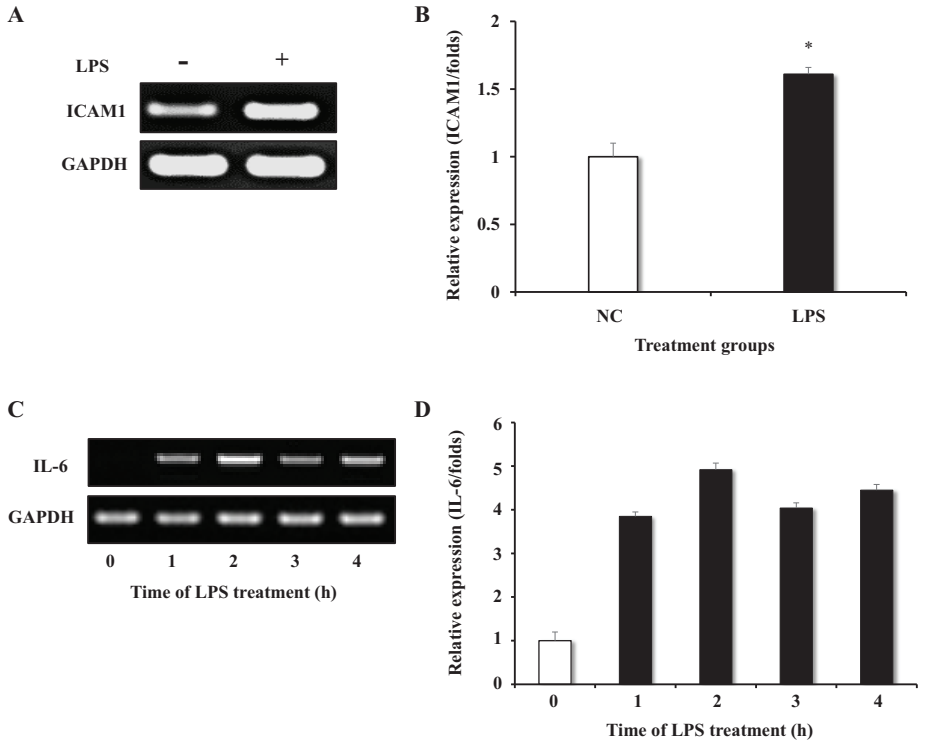
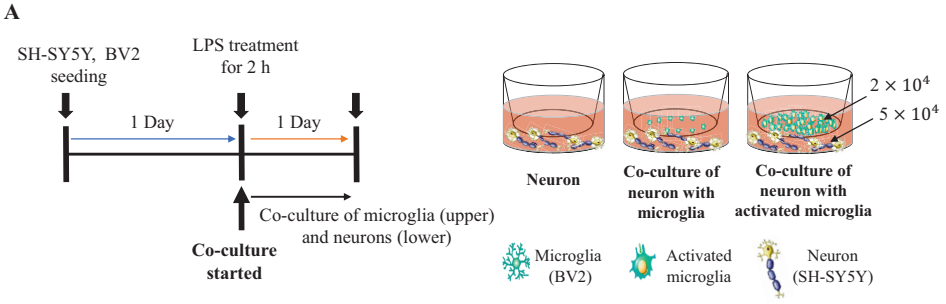


Fig. 3. The assessment of BV2 microglia stimulated by LPS to confirm the function as macrophage. (A and B) The confirmation of ICAM1 biomarker for the conversation of pro-inflammatory M1 macrophage of microglia stimulated with LPS. (C and D) The expression of pro-inflammatory cytokine IL-6 in activated microglial cells. Data are expressed as mean ± SEM. Original gel images showed in Supplementary Fig. S3. $*P < 0.05$, as compared with control group.



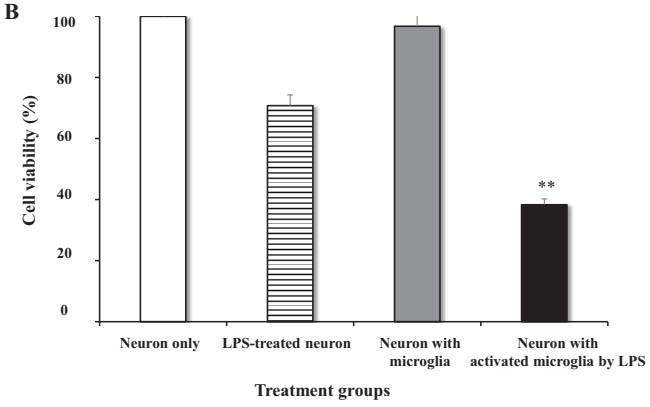


Fig. 4. The evaluation of biological linkage between activated BV2 microglia and neuron cells. (A) Co-culture was stabilized by seeding microglia (BV2) in the upper well and neuron (SH-SY5Y) in the down well, and then the wells were combined after 24 h. (B) Although co-culture of neurons may be affected by unstimulated microglia to some extent, neuron viability was significantly reduced by activated microglia. This confirmed that activated microglia can damage neurons. Data are presented as mean ± SEM for three independent experiments. $**P < 0.02$, as compared with neuron with microglia.

demonstrated that microglia activated by foreign stimulation have a serious effect on neurons.

Characterization change of activated BV2 microglia by annexin A1 silencing

To investigate the role of ANXA1 in the regulation of M1 phenotype polarization and function in activated microglia, various shRNA were generated to induce the knock-down (Kd) of ANXA1 at the transcriptome level. Successful cell transfection efficiency of shRNA series was confirmed by the expression of GFP protein inserted into the shRNA expression vector in Fig. 5A. Among the shRNA used, ANXA1 gene expression in BV2 microglia treated with shB successfully showed the best gene silencing efficiency through markedly decreased mRNA expression levels of ANXA1 (Fig. 5B). As shown in Fig. 5C, the mRNA levels of ICAM1, a good indicator of the pro-inflammatory state, were decreased as low as that of microglia not activated by the ANXA1 Kd. These results demonstrate that ANXA1 Kd can reduce the immune reaction to foreign pathogens in microglia.

Downregulated nuclear factor-kappa B activity of microglia in protein level by annexin A1 knockdown suppression

It is well known that many pro-inflammatory cytokines such as IL-6 and TNF-α induction mainly depend on the NF-κB activity with the onset of an immune response. Among various cytokines, NF-κB is a protein complex that controls transcription of DNA, cytokine production, and cell survival. NF-κB not only plays a key role in regulating the immune response to infection, but is also a major transcription factor that regulates the genes responsible for innate and adaptive immune responses. When NF-κB is activated, various cytokines are secreted, causing damage to neurons. Thus, we evaluated how the Kd of ANXA1 in microglia affects NF-κB activity using the ICW assay. As shown in Fig. 6, the expression of phosphorylated NF-κB increased with cytokine secretion after LPS treatment. The shScr-treated group also showed similar results to those of the LPS-treated group. However, microglia treated with shB-ANXA1 showed significantly decreased NF-κB expression after LPS treatment as compared to the LPS- or shScr-treated cells (Fig. 6A). The quantitative data of NF-κB in the Odyssey image results showed a similar tendency (Fig.

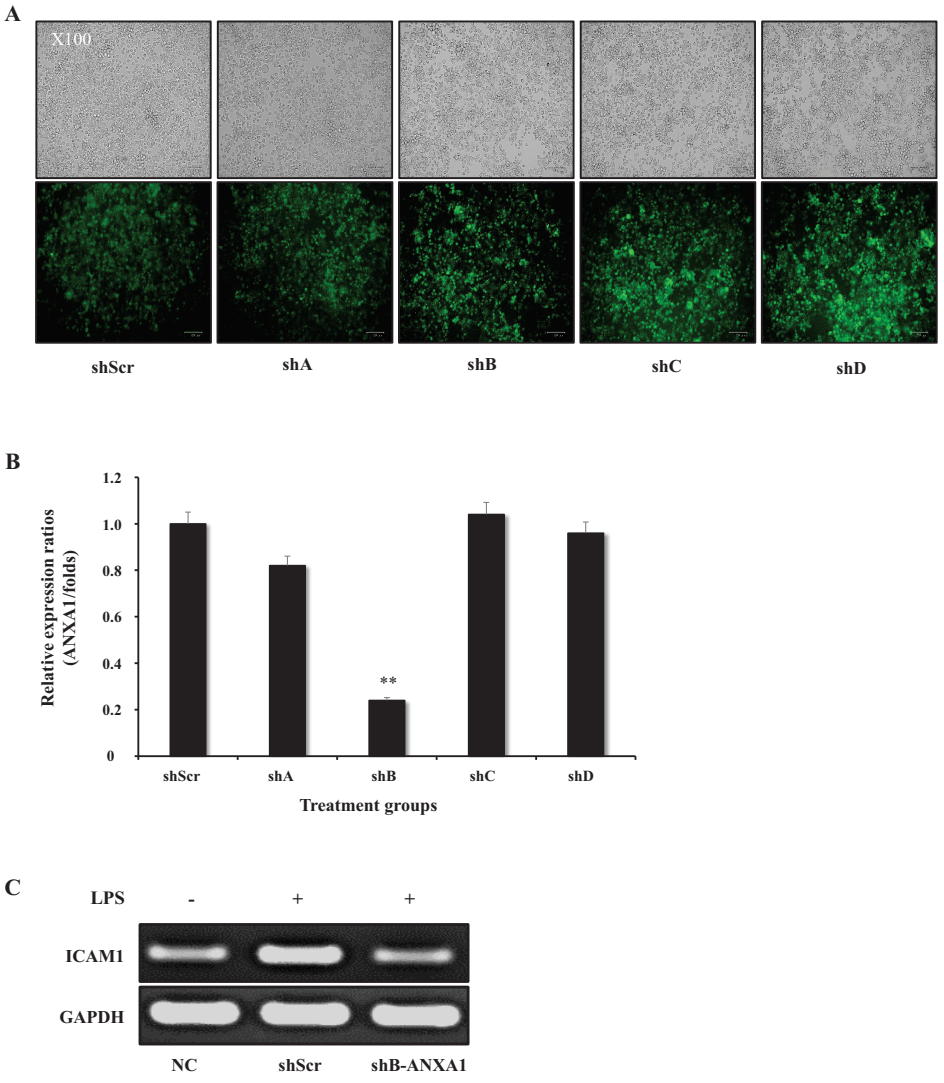


Fig. 5. Knock-down of ANXA1 and deactivation in BV2 microglia. Short hairpin RNA (shRNA) series were constructed in pGPI6/GFP/Neo vector to effectively knock-down ANXA1 gene in the activated microglia. (A and B) Knock-down efficiency of ANXA1 by constructed shRNAs through GFP expression and qPCR experiment. $^{**}P < 0.02$, as compared with shScr group. (C) The assessment of M1 type depolarization of activated microglial cells treated with shRNA. Original gel images showed in Supplementary Fig. S4.

6B). The decreased activity of NF-κB by ANXA1 Kd could also be caused by the downregulation of cytokines. To address this, we examined the expression level of pro-inflammatory cytokine IL-6 following shRNA treatment. In BV2 microglial cells stimulated by LPS, the secretory levels of IL-6 in the supernatant detected by ELISA were significantly increased as compared to the unstimulated cells (NC group). Conversely, shB-ANXA1 treated BV2 microglia cells showed reduced IL-6 expression as compared to the LPS- or shScr-treated groups (Fig. 6C). In conjunction with the results seen in Fig. 6C, we examined whether ANXA1 Kd could affect cytokine expression at the transcriptome level. Consistent with the protein level results, the increase in TNF-α and IL-6 mRNA levels induced by LPS was significantly alleviated by ANXA1 Kd (Figs. 6D and 6E). These data indicate that ANXA1 Kd could decrease LPS-induced inflammatory cytokine expression in BV2 microglial cells, leading to neuronal cell survival.

Inhibition of mitochondria dysfunction in annexin A1-downregulated microglia

Metabolic reprogramming has been tightly correlated with macrophage polarization and function. For instance, pro-inflammatory M1 phenotype display a metabolic transition towards aerobic glycolysis. Since mitochondria play a key role in this transition, we assessed whether the downregulation of ANXA1 affected mitochondrial function. To address this, a cellular respiratory assay using the Seahorse XF analyzer was used to assess mitochondrial function in ANXA1 Kd. Microglia were exposed to glycolytic stress compounds in the following order: oligomycin, FCCP, and rotenone & antimycin A during the real-time OCR measurement. The OCR of cells, an important indicator of normal cellular function, is used as a parameter to study mitochondrial function. As shown in Fig. 7, ANXA1 Kd showed a significantly decreased OCR than that of the LPS-treated microglial cells. However, OCR was restored in normal conditions like in the negative control (NC) group, or when BV2 microglia were treated with shB-ANXA1 (Fig. 7A). These results indicate that the activation of ANXA1 impairs mitochondrial function in microglia. Moreover, the baseline respiratory rate and energy demand of the cell under baseline conditions did not differ between the NC and ANXA1 Kd groups after 2 h of LPS stimulation (Fig. 7B). Impairment in mitochondrial respiration was more pronounced in LPS-induced microglial cell, as demonstrated by

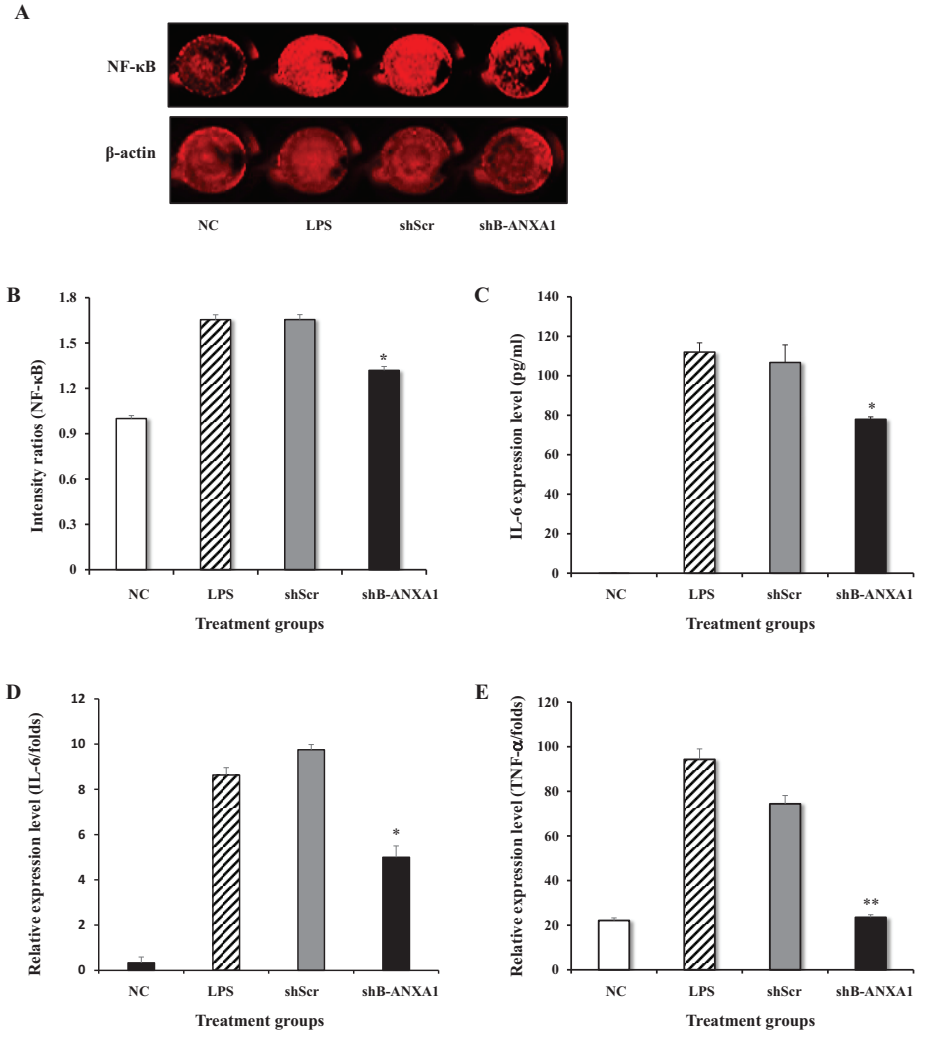


Fig. 6. The effect of on the decreased NF-κB activity in LPS-stimulated microglial cells via knock-down of ANXA1. BV2 cells were transfected with ANXA1 shRNA for 48 h and then incubated with LPS (1 μg/ml) for 2 h. (A) The scanned imaging results and (B) quantitative intensity ratio (ANXA1/β-action) by ICW analysis for NF-κB activation. (C) The secretory levels of IL-6 in activated BV2 microglial cells treated with shRNA-B using ELISA. (D and E) The expression of TNF-α and IL-6 in the transcriptomic levels. Data were presented as mean ± SEM of three independent experiments. $*P < 0.05$ and $**P < 0.02$, as compared with LPS group.

a significant reduction in maximal respiration, spare respiratory capacity, and coupling efficiency (Fig. 7C-7E). While no significant difference in proton leak was observed between the NC and BV2 cells treated with shB-ANXA1 after LPS stimulation, adenosine triphosphate (ATP) production was significantly lower in LPS-treated BV2 cells than in the ANXA1 Kd (Figs. 7F and 7G). These results suggest that microglial cell activated by LPS may have impaired mitochondrial function. Thus, these results elicit the downregulation of ANXA1 in microglia under the stimulatory conditions induced by LPS. The data suggested that in BV2 cells, a switch to glycolysis appears to serve as a survival response to maintain ATP levels

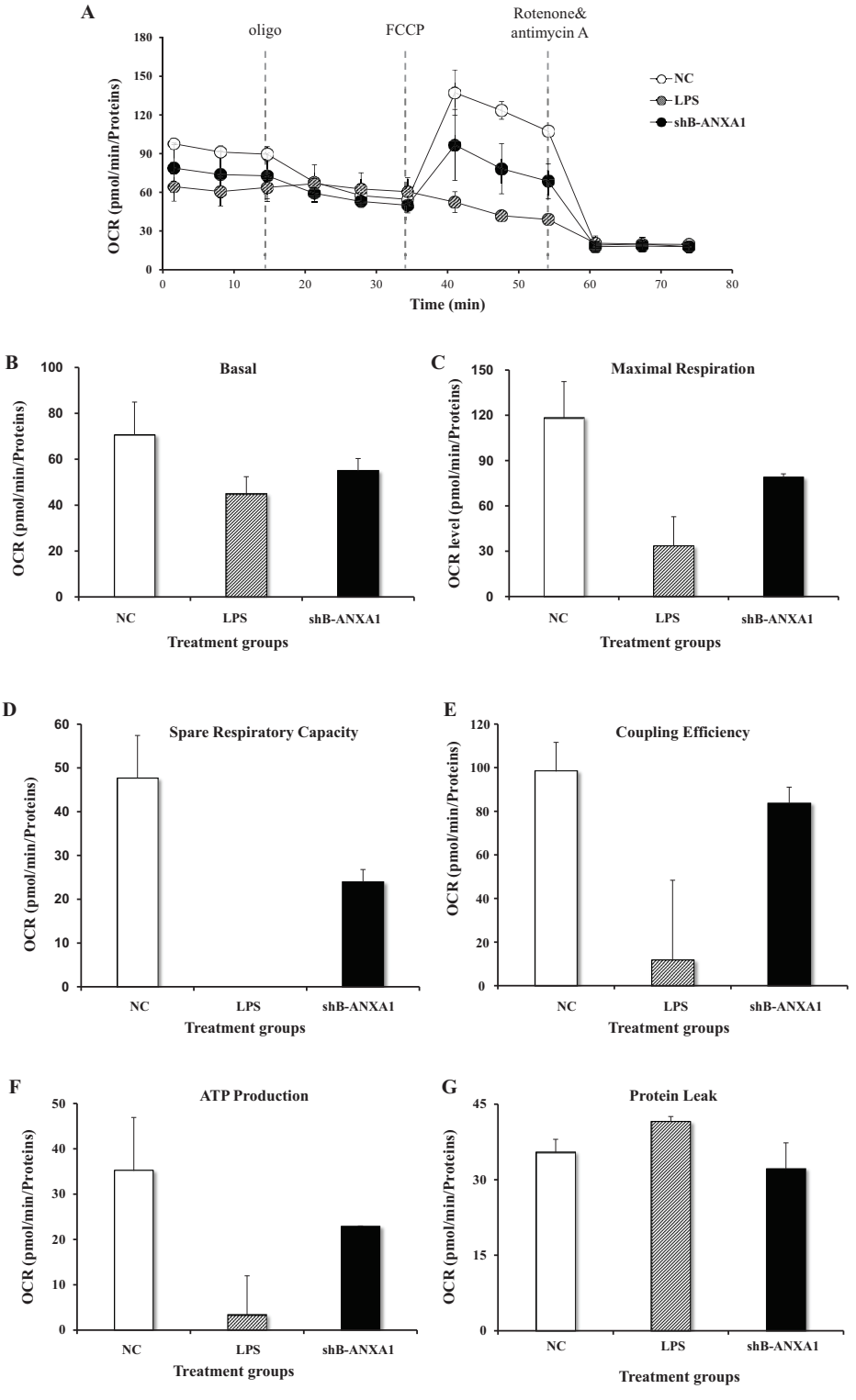


Fig. 7. Effect of ANXA1 down-regulation on mitochondrial function. BV2 microglial cells were seeded in 8-well Seahorse plate. Representative example of bioenergetics profile (Supplementary Fig. S5) shows a normal response pattern for control BV2 cells for basal respiration, and following addition of the mitochondrial stressors oligomycin (oligo; 1.0 μM), FCCP (1.0 μM), and Rotenone & antimycin A (0.5 μM). (A) Calculation of OCR as a percentage of control demonstrated a significant difference between controls and LPS-exposed cells suggesting an increased extracellular acidification of the media. (G) While no respectable reduction in proton leak, (B-F) LPS-exposed cells showed a decrease in basal respiration (B), maximal respiration (C), spare respiratory capacity (D), coupling efficiency (E), and ATP production (F) and were unresponsive to the different mitochondrial stressors, suggesting an impairment of mitochondrial function.

after inhibition of oxidative phosphorylation by NO.

Increased survival effects of neuron on annexin A1 down-regulation in BV2 microglia

We examined how the decrease in ANXA1 expression in microglia affects the cell viability of neurons. As shown in the results of Fig. 8A, the lowest cell survival rate was observed via MTT assay in the neurons co-cultured with activated microglia when compared to untreated neurons. The viability of neurons co-cultured with activated microglia reduced to twice as low as that of neurons co-cultured with non-activated microglia. In this result, as shown in Fig. 4, LPS treatment showed some cytotoxicity to neuron cells, but, not as shown in activated microglia by LPS group. On the other hand, in the case of neurons co-cultured with shB-ANXA1-treated microglia, the cell survival of neurons significantly improved when compared to that of neurons co-cultured with LPS-activated microglia.

Flow cytometry experiments to clarify the improved cell viability and cell death mechanism showed results similar to those of MTT experiments. It showed that BV2 cells significantly enhanced SH-SY5Y apoptosis (annexin V-positive) upon LPS stimulation, and this effect was significantly suppressed by the shRNA Kd of ANXA1 in BV2 cells. SH-SY5Y neuron cells were evaluated by double-staining fluorescence-activated cell sorting using FITC-annexin-V and PI. The cell death population was lower in neurons treated with shB-ANXA1 (annexin-V positive and PI negative, 0.18%; annexin-V positive and PI positive, 0.95%) as compared to those treated with LPS (annexin-V positive and PI negative, 0.92%; annexin-V positive and PI positive, 4.15%) or those of other groups (Figs. 8B and 8C). These results demonstrate that ANXA1 Kd expression by LPS stimulation in microglia elicits enhanced cell survival.

DISCUSSION

With the increase in life expectancy, the neurodegenerative diseases, such as AD (Jeong, 2017; Masters et al., 2015), PD (Davie, 2008), and Tay-Sachs disease (Ramani and Sankaran, 2020), is increasing considerably. They are involved by several factors including genetic, environmental, and endogenous factors, however, the exact mechanism underlying neurodegenerative diseases are not fully known. Among the various

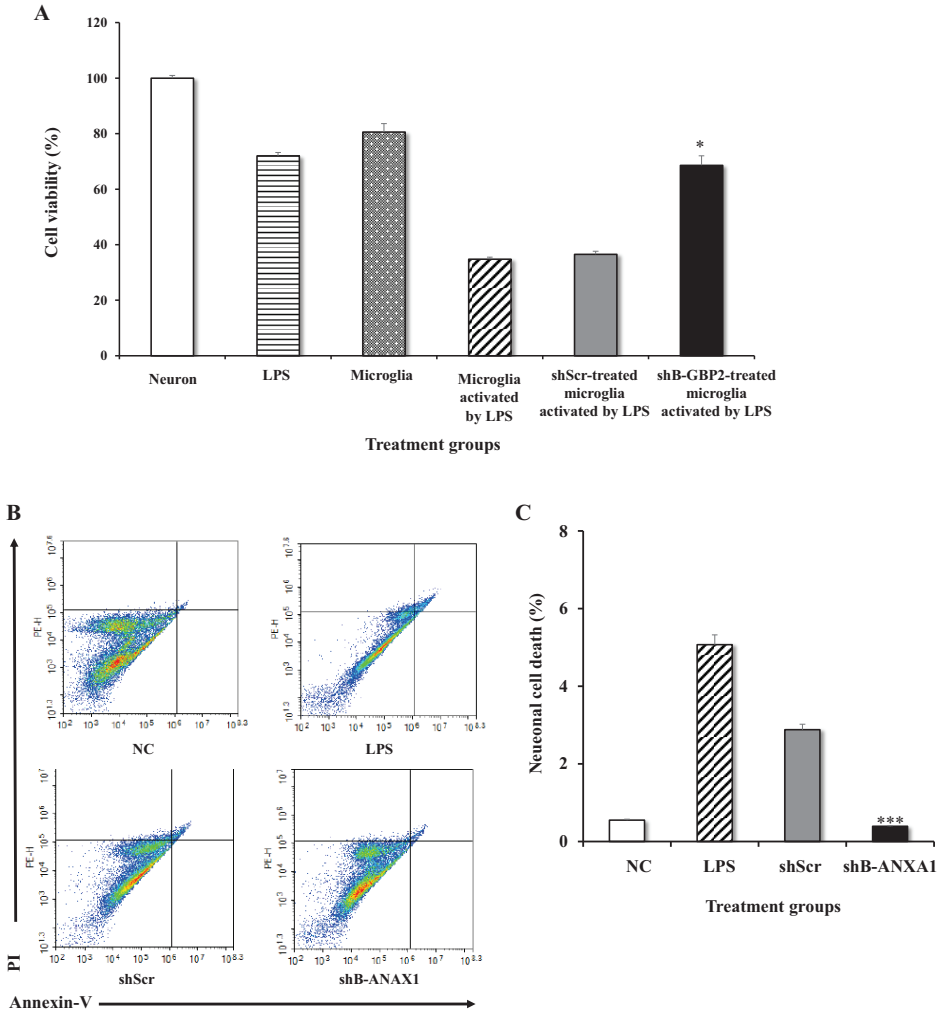


Fig. 8. Enhanced cell survival of neuron by down-regulation of ANXA1 in microglial cell. BV2 microglial cells were transfected with shB-ANXA1 or scramble vector for 48 h in co-culture system followed by LPS incubated for 2 h. (A) SH-SY5Y neuron cell viability by the MTT assay. $*P < 0.05$, as compared with LPS group. (B and C) FACS analysis of Annexin-V and PI double staining in SH-SY5Y neuron cell co-cultured with microglial cells. Data are representative of at least three independent experiments. $***P < 0.01$, as compared with LPS group.

researches regarding treatment of dementia (Summers et al., 1986), the fundamental treatment using genetic material is in the spotlight (Duncan and Valenzuela, 2017). Among them, ANXA1 play a potential anti-inflammatory functions such as glucocorticoid action, removal of apoptotic leukocytes in peripheral nervous system (Buckingham et al., 2006). However, the exact mechanism and action of microglia in controlling immune response in CNS remains poorly understood.

Here, we elucidated that ANXA1 Kd plays a protective role against inflammation induced by LPS in BV2 microglia to maintain mitochondrial function and prevent neuronal damage. BV2 microglial cells activated by LPS showed a remarkable up-regulation in the ANXA1 expression (Figs. 2 and 3). These characteristics are important results indicating that the expression of ANXA1 is induced in relation to the immune response in microglial cell. Microglia, as macrophage-like resident immune cell in the CNS (Block et al., 2007), are considered the first line of defense in the CNS and provide tissue defense and protection (Ransohoff and Perry, 2009). However, severe neuronal damage could cause by excessive acute or chronic microglial activation via the release of cytotoxic factors such as NO, ROS, TNF-α, and IL series (Colonna and Butovsky, 2017). Thus, microglia may work as a double-edged sword because it is responsible for neurotoxic and neuroprotective effects (Dai et al., 2015).

In the injured brain, the activated microglia participate in the course of inflammation (Liu and Hong, 2003), a process that includes actions of various cytokines by foreign stimulation (Gordon and Taylor, 2005), leading to particularly harmful effects on the neurons. Various materials secreted from activated microglia, such as IL-6 and TNF-α, can form a vicious cycle of pro-inflammatory responses that continuously damage neurons and other important nervous system structures (Hanisch and Kettenmann, 2007; Henkel et al., 2009). As shown in the results of Figs. 3C and 4, neuronal damage was observed to be induced by the activated microglia. The release and accumulation of pro-inflammatory factors secreted by activated microglia are thought to induce neurotoxicity, especially in neurodegenerative diseases (Colton and Wilcock, 2010). Additionally, the damaged neurons secrete neurotoxic soluble factors that in turn induce microglial reactivation. Thus, a vicious cycle causing prolonged neuroinflammation and progressive neurodegeneration is created. Therefore, it is important to clarify the cellular crosstalk signaling pathways between microglia and neurons to identify future therapeutic targets of CNS disorders, including pain, infection, neurodegeneration, and stroke.

For this, ANXA1 is a good genetic material that can control the crosstalk between microglia and neurons. The increase of ANXA1 in LPS-activated microglia indicates that external stimuli act as inducers of ANXA1 expression (Fig. 2). Although ANXA1 works in the early stages of external stimuli, it induces the differentiation of microglia into M1 type to function as a macrophage (Fig. 3), i.e., conversion into cells that damage neurons. Hence, the upregulation of ANXA1 could damage the adaptive response of microglia during inflammatory conditions. It is an attempt of the system to reduce inflammation in the early stages of the disease, since downregulation of ANXA1 effectively resolves the inflammatory phase.

Stimulation like LPS activates NF-κB pathway, which leads to further production of pro-inflammatory cytokines (McArthur et al., 2010). It is well known that pro-inflammatory M1 phenotype undergo metabolic re-programming typically characterized by increased glycolysis (Choi et al., 2020) and decreased oxidative phosphorylation (Ransohoff, 2016; Wang et al., 2014). Interestingly, oxidative phosphorylation was significantly decreased in ANXA1 Kd. This speculation is supported by previous reports that increased glucose uptake and enhanced glycogen accumulation were seen in M1 polarized phenotype (Qiu et al., 2018). As shown in the results of the energy map (Fig. 7), shRNA-ANXA1-treated microglial cell tended to return the OCR of reduced mitochondria to their original state (Fig. 7), suggesting that these cells may favor entering the G_0 cell cycle and transform into quiescent status. Quiescent microglial cell are usually deactivated and have a steady-state phenotype. Further research may be needed, but the downregulation of ANXA1 showed a mitochondrial function similar to that of non-activated microglia. Eventually, the ANXA1 Kd can inhibit intracellular energy production, and microglia can differentiate into M1 type based on the pattern of oxygen consumption in the mitochondria.

These results indicate that activated microglia revert to the deactivated state and eventually reduce neuronal cell damage. The ANXA1 Kd suggests that the damage and viability of neuronal cells are affected. To address this, we also assessed the cell viability of neurons in the co-culture system when the ANXA1 gene was downregulated. As a result, the cell survival of neurons by ANXA1 Kd microglia showed decreased cell death with reduced apoptotic cell population (Fig. 8). Overall, our study suggests that downregulation of ANXA1 can improve neuronal survival to clear damaged microglia caused by pro-inflammatory cytokine stimulation. Thus, this indicates the possibility that ANXA1 could be the new regulator of neuroinflammation by external stimulation for activating the microglia.

In the CNS, microglia act as macrophages when foreign pathogens invade, causing elimination of foreign substances by the induction of immune responses as the first step in defense. However, hyperactive microglia in the brain have a negative effect on neurons, which can also cause dementia. In this study, we used ANXA1 genetic material to reduce the side effects of activated BV2 microglia on neurons and prevent neuronal damage. When the “resting microglia” become “activated microglia” by pathogens such as LPS, the NF-κB signal is activated in them. Therefore, we conducted a study on the effects of Kd ANXA1 genes overexpressed in the activated microglia. Collectively, our data indicated that the downregulation of ANXA1 resulted in decreased secretion of cytokines from the LPS-activated microglia. In a co-culture system between activated microglia and SH-SY5Y neurons, microglia treated with shRNA-ANXA1 induced decreased damage and apoptosis in neurons. Taken together, our study suggest that ANXA1 could be used as a new potential target on neuronal damage by activated microglia.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS
P.H.K. designed experiment, coordinated the project and helped in manuscript preparation and writing. J.E.Y. and S.H.J. performed the experiments and contributed to the data collection and analysis. J.E.Y. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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REFERENCES

Arends, Y., Duyckaerts, C., Rozemuller, J., Eikelenboom, P., and Hauw, J. (2000). Microglia, amyloid and dementia in Alzheimer disease: a correlative study. Neurobiol. Aging *21*, 39-47.

Ashrafian, H., Zadeh, E.H., and Khan, R.H. (2020). Review on Alzheimer's disease: inhibition of amyloid beta and tau tangle formation. Int. J. Biol. Macromol. *167*, 382-394.

Binder, L.I., Guillozet-Bongaarts, A.L., Garcia-Sierra, F., and Berry, R.W. (2005). Tau, tangles, and Alzheimer's disease. Biochim. Biophys. Acta *1739*, 216-223.

Block, M.L., Zecca, L., and Hong, J.S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat. Rev. Neurosci. *8*, 57-69.

Buckingham, J.C., John, C.D., Solito, E., Tierney, T., Flower, R.J., Christian, H., and Morris, J. (2006). Annexin 1, glucocorticoids, and the neuroendocrine–immune interface. Ann. N. Y. Acad. Sci. *1088*, 396.

Chan, A., Magnus, T., and Gold, R. (2001). Phagocytosis of apoptotic inflammatory cells by microglia and modulation by different cytokines: mechanism for removal of apoptotic cells in the inflamed nervous system. Glia *33*, 87-95.

Choi, E.J., Jeon, C.H., Park, D.H., and Kwon, T.H. (2020). Allithiamine exerts therapeutic effects on sepsis by modulating metabolic flux during dendritic cell activation. Mol. Cells *43*, 964-973.

Colonna, M. and Butovsky, O. (2017). Microglia function in the central nervous system during health and neurodegeneration. Annu. Rev. Immunol. *35*, 441-468.

Colton, C.A. and Wilcock, D.M. (2010). Assessing activation states in microglia. CNS Neurol. Disord. Drug Targets *9*, 174-191.

Dai, X.J., Li, N., Yu, L., Chen, Z.Y., Hua, R., Qin, X., and Zhang, Y.M. (2015). Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. Cell Stress Chaperones *20*, 321-331.

Davie, C.A. (2008). A review of Parkinson's disease. Br. Med. Bull. *86*, 109-127.

Dreier, R., Schmid, K.W., Gerke, V., and Riehemann, K. (1998). Differential expression of annexins I, II and IV in human tissues: an immunohistochemical study. Histochem. Cell Biol. *110*, 137-148.

Duncan, T. and Valenzuela, M. (2017). Alzheimer's disease, dementia, and stem cell therapy. Stem Cell Res. Ther. *8*, 111.

Eikelenboom, P. and Veerhuis, R. (1996). The role of complement and activated microglia in the pathogenesis of Alzheimer's disease. Neurobiol. Aging *17*, 673-680.

Fan, Y., Xie, L., and Chung, C.Y. (2017). Signaling pathways controlling microglia chemotaxis. Mol. Cells *40*, 163.

Franco, R. and Fernandez-Suarez, D. (2015). Alternatively activated microglia and macrophages in the central nervous system. Prog. Neurobiol. *131*, 65-86.

Gordon, S. and Taylor, P.R. (2005). Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. *5*, 953-964.

Graeber, M.B. (2010). Changing face of microglia. Science *330*, 783-788.

Han, P.F., Che, X.D., Li, H.Z., Gao, Y.Y., Wei, X.C., and Li, P.C. (2020). Annexin A1 involved in the regulation of inflammation and cell signaling pathways. Chin. J. Traumatol. *23*, 96-101.

Hanisch, U.K. and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. *10*, 1387-1394.

Henkel, J.S., Beers, D.R., Zhao, W., and Appel, S.H. (2009). Microglia in ALS: the good, the bad, and the resting. J. Neuroimmune Pharmacol. *4*, 389-398.

Jeong, S. (2017). Molecular and cellular basis of neurodegeneration in Alzheimer's disease. Mol. Cells *40*, 613.

Katz, I.R., Jeste, D.V., Mintzer, J.E., and Clyde, C. (1999). Comparison of risperidone and placebo for psychosis and behavioral disturbances associated with dementia: a randomized, double-blind trial. J. Clin. Psychiatry *60*, 107-115.

Lewcock, J.W., Schlepckow, K., Di Paolo, G., Tahirovic, S., Monroe, K.M., and Haass, C. (2020). Emerging microglia biology defines novel therapeutic approaches for Alzheimer's disease. Neuron *108*, 801-821.

Liu, B. and Hong, J.S. (2003). Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J. Pharmacol. Exp. Ther. *304*, 1-7.

Ma, H.W., Ye, W., Chen, H.S., Nie, T.J., Cheng, L.F., Zhang, L., Han, P.J., Wu, X.A., Xu, Z.K., and Lei, Y.F. (2017). In-cell western assays to evaluate Hantaan virus replication as a novel approach to screen antiviral molecules and detect neutralizing antibody titers. Front. Cell. Infect. Microbiol. *7*, 269.

Magnus, T., Chan, A., Grauer, O., Toyka, K.V., and Gold, R. (2001). Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. J. Immunol. *167*, 5004-5010.

Masters, C.L., Bateman, R., Blennow, K., Rowe, C.C., Sperling, R.A., and Cummings, J.L. (2015). Alzheimer's disease. Nat. Rev. Dis. Primers *1*, 15056.

McArthur, S., Cristante, E., Paterno, M., Christian, H., Roncaroli, F., Gillies, G.E., and Solito, E. (2010). Annexin A1: a central player in the anti-inflammatory and neuroprotective role of microglia. J. Immunol. *185*, 6317-6328.

Nilsson, P., Iwata, N., Muramatsu, S., Tjernberg, L.O., Winblad, B., and Saido, T.C. (2010). Gene therapy in Alzheimer's disease–potential for disease modification. J. Cell. Mol. Med. *14*, 741-757.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science *308*, 1314-1318.

Pascual, O., Achour, S.B., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. Proc. Natl. Acad. Sci. U. S. A. *109*, E197-E205.

Perry, V.H., Nicoll, J.A., and Holmes, C. (2010). Microglia in neurodegenerative disease. Nat. Rev. Neurol. *6*, 193.

Picca, A., Calvani, R., Coelho-Junior, H.J., Landi, F., Bernabei, R., and Marzetti, E. (2020). Mitochondrial dysfunction, oxidative stress, and neuroinflammation: intertwined roads to neurodegeneration. Antioxidants (Basel) *9*, 647.

Qiu, X., Guo, H., Yang, J., Ji, Y., Wu, C.S., and Chen, X. (2018). Down-regulation of guanylate binding protein 1 causes mitochondrial

dysfunction and cellular senescence in macrophages. Sci. Rep. *8*, 1-12.

Ramani, P.K. and Sankaran, B.P. (2020). Tay-Sachs disease. In StatPearls [Internet], B. Abai, ed. (Treasure Island: StatPearls Publishing).

Ransohoff, R.M. (2016). A polarizing question: do M1 and M2 microglia exist? Nat. Neurosci. *19*, 987.

Ransohoff, R.M. and Perry, V.H. (2009). Microglial physiology: unique stimuli, specialized responses. Annu. Rev. Immunol. *27*, 119-145.

Scheiblich, H., Trombly, M., Ramirez, A., and Heneka, M.T. (2020). Neuroimmune connections in aging and neurodegenerative diseases. Trends Immunol. *41*, 300-312.

Shaji, K., Sivakumar, P., Rao, G.P., and Paul, N. (2018). Clinical practice guidelines for management of dementia. Indian J. Psychiatry *60*(Suppl 3), S312-S328.

Shin, S.J., Jeon, S.G., Kim, J.I., Jeong, Y.O., Kim, S., Park, Y.H., Lee, S.K., Park, H.H., Hong, S.B., Oh, S., et al. (2019). Red ginseng attenuates Aβ-induced mitochondrial dysfunction and Aβ-mediated pathology in an animal model of Alzheimer's disease. Int. J. Mol. Sci. *20*, 3030.

Solito, E., McArthur, S., Christian, H., Gavins, F., Buckingham, J.C., and Gillies, G.E. (2008). Annexin A1 in the brain–undiscovered roles? Trends Pharmacol. Sci. *29*, 135-142.

Summers, W.K., Majovski, L.V., Marsh, G.M., Tachiki, K., and Kling, A. (1986). Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer type. N. Engl. J. Med. *315*, 1241-1245.

Tang, Y. and Le, W. (2016). Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol. Neurobiol. *53*, 1181-1194.

Wang, N., Liang, H., and Zen, K. (2014). Molecular mechanisms that influence the macrophage M1–M2 polarization balance. Front. Immunol. *5*, 614.