

Ethanol Extract of *Elaeagnus glabra f. oxyphylla* Branches Alleviates the Inflammatory Response Through Suppression of Cyclin D3/Cyclin-Dependent Kinase 11^{p58} Coupled to Lipopolysaccharide-Activated BV-2 Microglia

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Hye-Sun Lim^{1,*}, Eunjin Sohn^{1,*} , Yu Jin Kim¹ , Bu-Yeo Kim¹,
Joo-Hwan Kim², and Soo-Jin Jeong¹

Abstract

Neuroinflammation plays a pivotal role in the pathogenesis of neurodegenerative diseases and is characterized by microglial dysregulation. Here, we explored the beneficial effects of a leaf extract of *Elaeagnus glabra f. oxyphylla* (EGFO), a native medicinal plant to Korea, South China, Japan, and Taiwan, on neuroinflammation using lipopolysaccharide (LPS)-stimulated BV-2 microglia. Levels of the inflammatory mediators were determined by enzyme-linked immunosorbent assays and reverse transcription–polymerase chain reaction. The phospho levels of mitogen-activated protein kinases, which are key kinase molecules in the inflammatory signaling pathway in microglia, were analyzed by Western blotting. Treatment with EGFO significantly suppressed the LPS-mediated induction of nitric oxide and prostaglandin E₂. Consistently, EGFO treatment in LPS-stimulated BV-2 cells markedly reduced the inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) levels. The best concentration of EGFO that could reduce TNF- α and IL-6 was 100 μ g/mL. EGFO relatively reduced the messenger RNA expression of TNF- α and IL-6 by 0.36 and 0.32-fold ratio, respectively, compared to LPS treatment. Moreover, EGFO markedly reduced the phospho levels of p38 and the c-Jun N-terminal kinase. Furthermore, antibody microarray and immunoblotting data revealed that the pharmacological mechanisms driving the antineuroinflammatory action of EGFO involve prevention of the cyclin D3/cyclin-dependent kinase 11^{p58} (CDK11^{p58}) interaction. In conclusion, our results demonstrate that EGFO alleviates the inflammatory response through the suppression of cyclin D3/CDK11^{p58} coupling in LPS-activated BV-2 microglia. We propose the potential of EGFO as a novel drug candidate for neurodegenerative diseases by targeting neuroinflammation.

Keywords

antibody array, cyclin D3/cyclin-dependent kinase 11^{p58}, *Elaeagnus glabra f. oxyphylla*, microglia, neuroinflammation, neurodegenerative diseases

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Introduction

The human brain consists of different cell types, including neurons, microglia, and astrocytes. Microglia, which are resident macrophages in the central nervous system, play key roles in neuronal immune response and serve as the headmost defense against inflammatory reactions and exogenous toxic substances.¹ Activated microglia significantly accelerate neuroinflammation by discharging various proinflammatory mediators and cytokines, including interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2).² These

neuroinflammatory responses are strongly associated with neuronal cell death, synaptic degeneration, and cognitive dysfunction in neurodegenerative conditions, such as Alzheimer's disease (AD).³

¹Korea Institute of Oriental Medicine, Daejeon, South Korea

²Gachon University, Seongnam, South Korea

*Equally contributed authors.

Corresponding Author:

Soo-Jin Jeong, KM Convergence Research Division, Korea Institute of Oriental Medicine, Daejeon 34054, South Korea.
Email: sjjeong@kiom.re.kr



Thus, controlling microglia-mediated neuroinflammation in the brain has been regarded as an attractive therapeutic strategy for treating or preventing neurodegenerative diseases.

Mitogen-activated protein kinases (MAPKs) are components of signal transduction pathways that are central to the management of cell proliferation and differentiation, cytokine production, and apoptosis.⁴ Among the MAPK proteins, p38 MAPK and c-jun N-terminal protein kinase (JNK) are potently induced by bacterial endotoxins, environmental stresses, and proinflammatory cytokines.⁵ Accumulating evidence suggests that increased activity of p38 and JNK signaling plays a crucial role in the inflammatory response in microglia.^{6–8} In particular, the use of molecular inhibitors that target p38 and JNK blocked the production of inflammatory cytokines, suggesting the involvement of p38 and JNK in the regulation of neuroinflammation.^{9,10}

Cyclin D3 is a cell-cycle protein that is specific to G1/S transition¹¹ and is fundamental for the kinase activity of the cyclin-dependent kinase 11^{p58} (CDK11^{p58}).¹² Increased expression of CDK11^{p58} and production of inflammatory factors were observed in lipopolysaccharide (LPS)-treated astrocytes. CDK11^{p58} silencing using specific RNA interference restrained the activation of p38 and JNK in LPS-induced astrocytes, indicating that CDK11^{p58} exerts its function in neuroinflammation via the activation of the p38/JNK pathway.¹³ Recently, Shen et al.¹⁴ reported that CDK11^{p58} stimulates microglial activation through the interaction with cyclin D3. However, no study has reported a link between CDK11^{p58} activation and the p38/JNK pathway in microglia.

Elaeagnus glabra f. *oxyphylla* (Servett.) W.T. Lee (Elaeagnaceae) (EGFO) is an evergreen broad-leaved tree that grows at the foot of beech trees. *Elaeagnus umbellata* Thunb is the most cultivated medicinal plant in Korea, Pakistan, Japan, China, India, Afghanistan, Canada, and the USA.¹⁵ Other *Elaeagnus* species, that is, *Elaeagnus angustifolia* var. *turcica* Yild., *E. angustifolia* L., and *Elaeagnus rhamnoides* (L.) A. Nelson, are widely grown and their fruits are consumed in Turkey.^{16,17} *Elaeagnus* species were introduced as a food and ornamental plant to grow under natural conditions in Turkey.¹⁵ In particular, *Elaeagnus* species grow on seaside cliff areas and river banks in northeastern Turkey.¹⁸ The leaves of *Elaeagnus* species are traditionally used as a restorative drink or decoction for intestinal disorders in China and Japan.¹⁹ Ellagitannins and their derivatives in the leaves of *Elaeagnus* species possess clinically valuable activities, chemopreventive effects, and have nutraceutical and biological potential.²⁰ Previous reports indicated that the fruits of *Elaeagnus* species are consumed as healthy condiments and juice.²¹ The fruits, seeds, and flowers of *Elaeagnus* species have been used in the treatment of cough, pulmonary infections, cardiac ailments, and colon cancer.²²

Of note, we recently reported that EGFO has potential for the treatment of AD through its antioxidant and anti-amyloid beta aggregation activities.²³ Additionally, 16 chemical components in EGFO extract were isolated and identified, including 4-hydroxybenzoic acid, salicylic acid, vanillic acid, (+)-catechin, (–)-epicatechin, (+)-gallocatechin, (–)-epigallocatechin, procyanidin B3, procyanidin B4, kaempferol, astragalin, *trans*-tiliroside, helichryoside, betulinic acid-3-*O*-*trans*-caffeate, ursolic acid-3-*O*-*trans*-caffeate, and 1-mono(22-*O*-feruloyl-oxydocosanoyl)glycerol. For the quality

control of EGFO, a high performance liquid chromatographic method was developed.²³

In the present study, we explored the effects of an EGFO branch extract on the inflammatory response in microglia, one of the key events in AD pathogenesis,²⁴ by measuring the production of inflammatory mediators known as cytokines. We also examined the mechanisms responsible for the regulation of neuroinflammation using a phospho-specific antibody microarray analysis. Here, we demonstrated that EGFO exerted suppressive effects on the inflammatory response and the coupling of cyclin D3 and CDK11^{p58} through the down regulation of the p38/JNK pathway in microglia.

Materials and Methods

EGFO Preparation

The branches of EGFO were collected in Gongjeong-ri, Anseong-myeon, Muju-gun, Jeollabuk-do, South Korea by the Korean Seed Association (Seongnam, South Korea) and identified by Professor J-H Kim, Gachon University, South Korea. Voucher specimen SCD-A-112 was placed at the Clinical Medicine Division, Korea Institute of Oriental Medicine (Daejeon, South Korea). Dried materials of EGFO branches (2.9 kg) were extracted twice with 30 L of ethanol using MS-DM608 and MS-DM609 (MTOPS®; Yangu) heating mantles for 3 h. The extracted material was filtered, evaporated under vacuum (EYELA N-12; Rikakikai Co.), and freeze-dried. An extract powder (179.28 g) was finally obtained (yield, 6.18%).

Cell Maintenance and Cytotoxicity Measurement

The BV-2 microglial cells line was purchased from AcceGen Biotechnology (cat no. ABC-TC212S). Cells were cultured with Dulbecco's Modified Eagle Medium (cat no. SH30021.01, Hyclone/Thermo) containing 10% heat-inactivated fetal bovine serum (cat no. SH30919.03, Hyclone/Thermo), streptomycin (100 µg/mL), and penicillin (100 U/mL) (cat no. 15140122, Gibco Fisher Scientific) in a humidified 5% CO₂ atmosphere incubator at 37 °C. Cytotoxicity was assessed by using CCK-8 (cat no. CK-04, Cell Counting Kit, Dojindo), as previously described.²⁵ In brief, cells were seeded onto 96-well plates (3 × 10⁴ cells/well) and treated with EGFO extract (0, 12.5, 25, 50, or 100 µg/mL) for 24 h. The CCK-8 solution (10 µL) was dropped into each well and kept for 4 h. The optical density (OD) was read using a microplate reader (Benchmark Plus, Bio-Rad Laboratories) at 450 nm. Cell viability was represented using the following formula:

$$\begin{aligned} &\text{Viability of cells (\% of control)} \\ &= \left(\frac{\text{mean OD in EGFO-treated cells}}{\text{mean OD in untreated control cells}} \right) \times 100 \end{aligned}$$

Measurement of Nitric Oxide

The amount of nitrite (NO₂[–]), a stable product of nitric oxide (NO), was measured by using the commercial Griess reagent

(cat no. G2930, Promega), as previously described.²⁶ Cells were pretreated with EGFO extract 2 h before the LPS stimulation and maintained for 24 h. An equal volume of culture supernatant medium was reacted with Griess reagent for 30 min. OD was assessed in a plate reader (Benchmark Plus, Bio-Rad Laboratories) with a filter at 530 nm. NO₂⁻ levels were calculated from a standard reference curve.

Enzyme-Linked Immunosorbent Assays for prostaglandin E₂, TNF- α , and IL-6

The levels of prostaglandin E₂ (PGE₂), TNF- α , and IL-6 were examined by using commercial enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemical (cat no. 514010) and R&D Systems (cat no. MTA00B), as previously described.²⁶ The cultured supernatants were applied to the ELISA reaction and the levels of PGE₂, TNF- α , and IL-6 in the samples were determined by extrapolation of the standard curve of each protein.

RNA Extraction, Complementary DNA Synthesis, and Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted by using TRIzol (cat no. 15596026, Invitrogen), as previously described.²⁶ Quantified RNAs were reverse transcribed using the iScript complementary DNA (cDNA) synthesis kit (cat no. 1708890, Bio-Rad Laboratories). PCR was performed using the template cDNA in a T100 thermal cycler (Bio-Rad Laboratories). The reaction conditions and primer sequences for iNOS, COX-2, IL-6, TNF- α , and β -actin are shown in Table 1. The PCR products were separated on a 1.5% to 2.0% agarose gel and visualized using the Azure C150 Gel Imaging Workstation (Azure Biosystems). β -Actin gene was used as an endogenous reference for each reaction.

Phospho-Specific Protein Antibody Array Analysis

Antibody microarray was performed as previously described.²⁷ Proteins were lysed by using a lysis buffer supplemented with 1% protease inhibitor cocktail (cat no. EXB050, Full Moon

BioSystems), and purified using the gel matrix column in the Full Moon BioSystems antibody array assay kit (cat no. KAS02). The column was mixed by vortexing for 5 s and hydrated for 1 h at room temperature. The column was centrifuged at 750 *g* for 2 min and placed in a collection tube. The protein sample was transferred onto the column, followed by centrifugation at 750 *g* for 2 min. The purified sample was confirmed by its ultraviolet spectrum on a NanoPhotometer (Implen). Protein concentration was determined using the bicinchoninic acid assay protein assay kit (cat no. 23225, Pierce). Antibody microarray was performed using the antibody array assay kit (cat no. PEX100, Full Moon BioSystems). The protein sample was mixed with labeling buffer and biotin/dimethylformamide solution for 90 min, and then the stop reagent was added and the mixture incubated for 30 min at room temperature. An antibody microarray slide was incubated with 30 mL of blocking solution for 30 min and washed 3 times with distilled water. The slide was incubated with a coupling mixture for 2 h at room temperature and rinsed with Milli-Q grade water for 5 min. Cy3–streptavidin (cat no. PA43001, GE Healthcare) was mixed with a detection buffer. The coupled array slide was treated with the detection mixture for 20 min at room temperature and rinsed with Milli-Q grade water. The dried slide within 1 to 2 days at a 10 μ m resolution was scanned using optimal laser power and a photomultiplier tube using a GenePix 4100A scanner (Axon Instruments), and the images were quantified by using GenePix 7.0 Software (Axon Instruments). Normalized data were analyzed using Genowiz 4.0 (Ocum Biosolutions). The normalization decides the average intensity of all samples numerically equivalent to the average intensity of all genes. One-sample *t*-tests were conducted using MeV software (version 4.9.0; TM4 Development Group). The protein data were annotated using UniProt DB and the phosphorylation ratio was calculated using the following equation:

$$\text{Phosphorylation ratio} = \left(\frac{\text{phospho}_{\text{experiment}}}{\text{unphospho}_{\text{experiment}}} \right) / \left(\frac{\text{phospho}_{\text{control}}}{\text{unphospho}_{\text{control}}} \right).$$

Table 1. Primer Sequences and PCR Conditions.

Target	Accession number	Primers	Annealing temperature (°C/cycles no.)	
iNOS	NM_010927.4	F: 5'-CCTCCTCCACCCTAGCAAGT-3' R: 5'-CACCCAAAGTGCTTCAGTCA-3'	52	28
COX-2	NM_011198.4	F: 5'-AAGACTTGCCAGGCTGAAC-3' R: 5'-CTTCTGCAGTCCAGGTTCAA-3'	52	28
IL-6	NM_001314054.1	F: 5'-AAGAGACTTCCATCCAGTTG-3' R: 5'-TCCAGGTAGCTATGGTACTC-3'	47	28
TNF- α	NM_013693.3	F: 5'-TGGGTAGAGAATGGATGAAC-3' R: 5'-GCCGATTTGGTATCTCATAC-3'	47	28
β -actin	NM_007393.5	F: 5'-TGTGATGGTGGGAATGGGTCAG-3' R: 5'-TTTGATGTCACGCACGATTTCC-3'	57	25

Abbreviations: iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL-6, interleukin-6; TNF- α , tumor necrosis factor-alpha.

Western Blot Analysis

Protein samples (30 μ g) were resolved by 4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (cat no. 3450060, Bio-Rad Laboratories) and transferred onto polyvinylidene fluoride membranes (cat no. 4561096, Bio-Rad Laboratories). The membranes were blocked with 5% skim milk in tris-buffered saline with Tween (TBST) and probed overnight at 4 °C with the desired primary antibodies; antiphospho-p38 MAPK, p38 MAPK, phospho-JNK, JNK (cat no. 9211, 9212, 9255, and 9252, Cell Signaling Technology), phospho-cyclin D3 (cat no. PA5-104862, Thermo Scientific), and CDK11^{p58} (cat no. SC-928, Santa Cruz Biotechnology). β -Actin (cat no. A5316, Sigma Aldrich) was used as an internal control. The washed membranes with TBST were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research) for 1 h at room temperature. After washing the membranes with TBST (cat no. HT2007, Biosesang), the Supersignal development solution (cat no. 46640, Thermo Scientific) was added to the membranes to develop the expression signals. Images were captured using an image analyzer (LAS 4000 mini, GE Healthcare Bio-Sciences).

Statistical Analyses

All data were calculated using Prism 8.0 (GraphPad Software). The differences were considered significant at $P < .05$. All quantitative experiments were performed at least 3 times. Tukey's test was used for multiple comparisons among the different groups. The data are represented as the mean $\pm$ standard error of mean (SEM). One-way analysis of variance was used to detect significant differences between the control and treatment groups.

Results

Cytotoxicity of EGFO Extract in BV-2 Microglia

To examine the cytotoxic effect of the EGFO extract in BV-2 microglia, we performed a CCK-8 assay. Cells were treated with various concentrations of the EGFO extract for 24 h. As shown in Figure 1, EGFO had no significant effect on the cell viability at doses ranging from 0 to 100 μ g/mL. A nontoxic concentration of EGFO extract (≤ 100 μ g/mL) was used in subsequent experiments.

Effects of EGFO Extract on Inflammatory Mediators in LPS-Stimulated BV-2 Microglia

Excessive generation of NO is a hallmark of inflammation.²⁸ The effect of EGFO on NO generation was evaluated in LPS-stimulated BV-2 cells in either the presence or absence of EGFO. Figure 2A shows that the NO production was significantly enhanced to 28.14 ± 0.38 μ M in cells treated with LPS alone compared with the untreated control (7.24 ± 0.38 μ M, $P < .001$). In contrast, pretreatment with the EGFO extract (prior to LPS stimulation) significantly decreased the

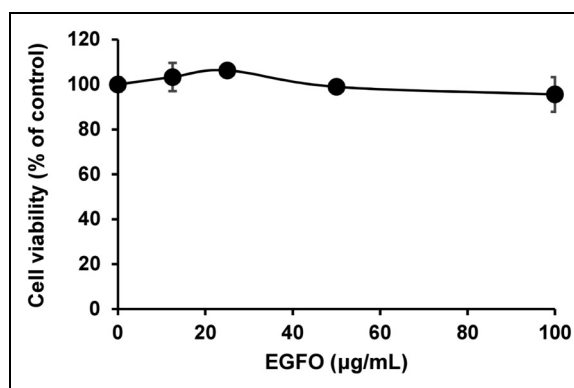


Figure 1. Cytotoxic effects of EGFO against BV-2 microglia. Cells were plated onto 96-well microplates and treated with the EGFO extract (0, 12.5, 25, 50, or 100 μ g/mL). Cell viability was measured using the CCK-8 assay kit. The values are expressed as the mean $\pm$ SEM of three independent experiments. Abbreviations: EGFO, *Elaeagnus glabra* f. *oxyphylla*; SEM: standard error of mean.

LPS-stimulated NO production in a dose-dependent manner ($P < .01$ at 25 μ g/mL; $P < .001$ at 50 and 100 μ g/mL). The NO level was decreased to 24.36 ± 0.40 , 22.85 ± 0.35 , and 15.10 ± 0.83 μ M at 25, 50, and 100 μ g/mL of EGFO, respectively. Subsequently, the effect of the EGFO extract on PGE₂ production was determined using a competitive ELISA kit. The production of PGE₂ is an essential event in the response to inflammation and swelling in microglia.²⁹ LPS stimulation yielded a significant 1.8-fold increase in PGE₂ production compared with the untreated control ($P < .001$). In contrast, the EGFO extract significantly inhibited the levels of PGE₂ in LPS-stimulated BV-2 cells in a dose-dependent manner ($P < .01$ at 25 μ g/mL; $P < .001$ at 50 and 100 μ g/mL, Figure 2B). Next, we evaluated the expression of iNOS and COX-2 at the messenger RNA (mRNA) level after treatment with the EGFO extract in LPS-stimulated BV-2 cells using reverse transcription–polymerase chain reaction (RT–PCR). Exposure of BV-2 cells to LPS markedly increased the expression of iNOS and COX-2 compared with the untreated control ($P < .001$). Consistent with the ELISA results, the EGFO extract suppressed the LPS-stimulated upregulation of the iNOS and COX-2 mRNAs compared with LPS treatment alone (Figure 2C and D).

Effects of EGFO Extract on Inflammatory Cytokines in LPS-Stimulated BV-2 Microglia

Inflammatory cytokines are soluble mediators of the intercellular/intracellular communication that occurs during inflammatory responses.³⁰ The effects of the EGFO extract on inflammatory cytokine release were investigated in BV-2 cells treated with LPS in either the presence or absence of EGFO. TNF- α production was also increased to 442.44 ± 5.72 pg/mL in the LPS-stimulated cells compared with the untreated control (95.02 ± 10.78 pg/mL).

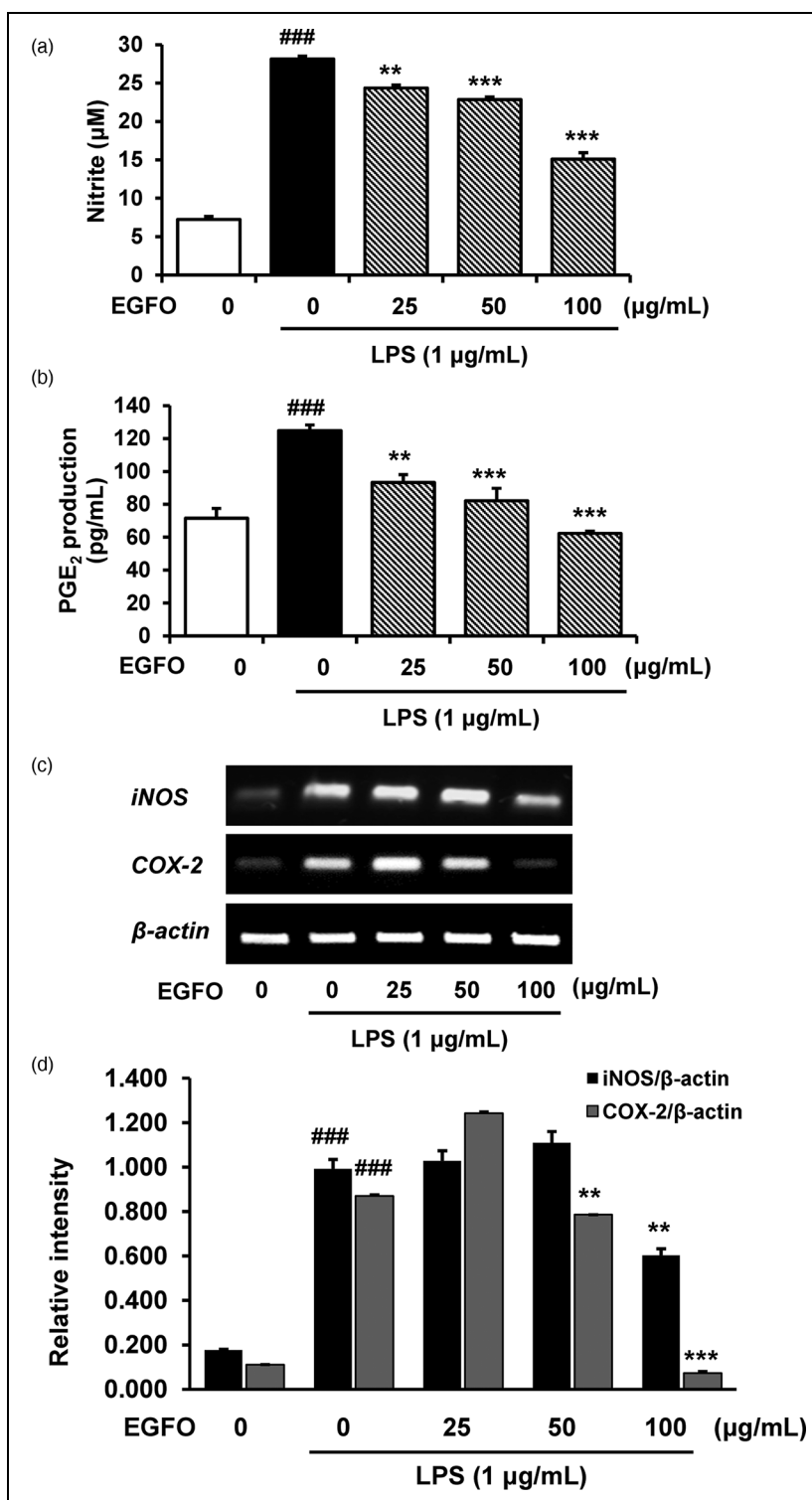


Figure 2. Inhibitory effects of EGFO on inflammatory molecules in LPS-stimulated BV-2 microglia. (A and B) Cells were pretreated with EGFO 2 h before LPS stimulation (1 µg/mL) and maintained for 24 h. The concentrations of nitrite (A) and PGE₂ (B) released into the culture media were determined using the Griess reagent for (A) and a PGE₂ ELISA kit for (B). (C) Cells were pretreated with EGFO 1 h before LPS stimulation (1 µg/mL) and maintained for 6 h. RT-PCR was performed to determine the expression of the iNOS and COX-2 mRNAs. (D) The bar graphs represent the relative intensity of the iNOS and COX-2 expression levels adjusted to the β-actin gene. The values are expressed as the mean ± SEM of three independent experiments. ###*P* < .001 versus untreated control. ***P* < .01 or ****P* < .001 versus LPS-treated cells.

Abbreviations: EGFO, *Elaeagnus glabra* f. *oxyphylla*; LPS, lipopolysaccharide; PGE₂, prostaglandin E₂; RT-PCR, reverse transcription-polymerase chain reaction; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; SEM, standard error of mean.

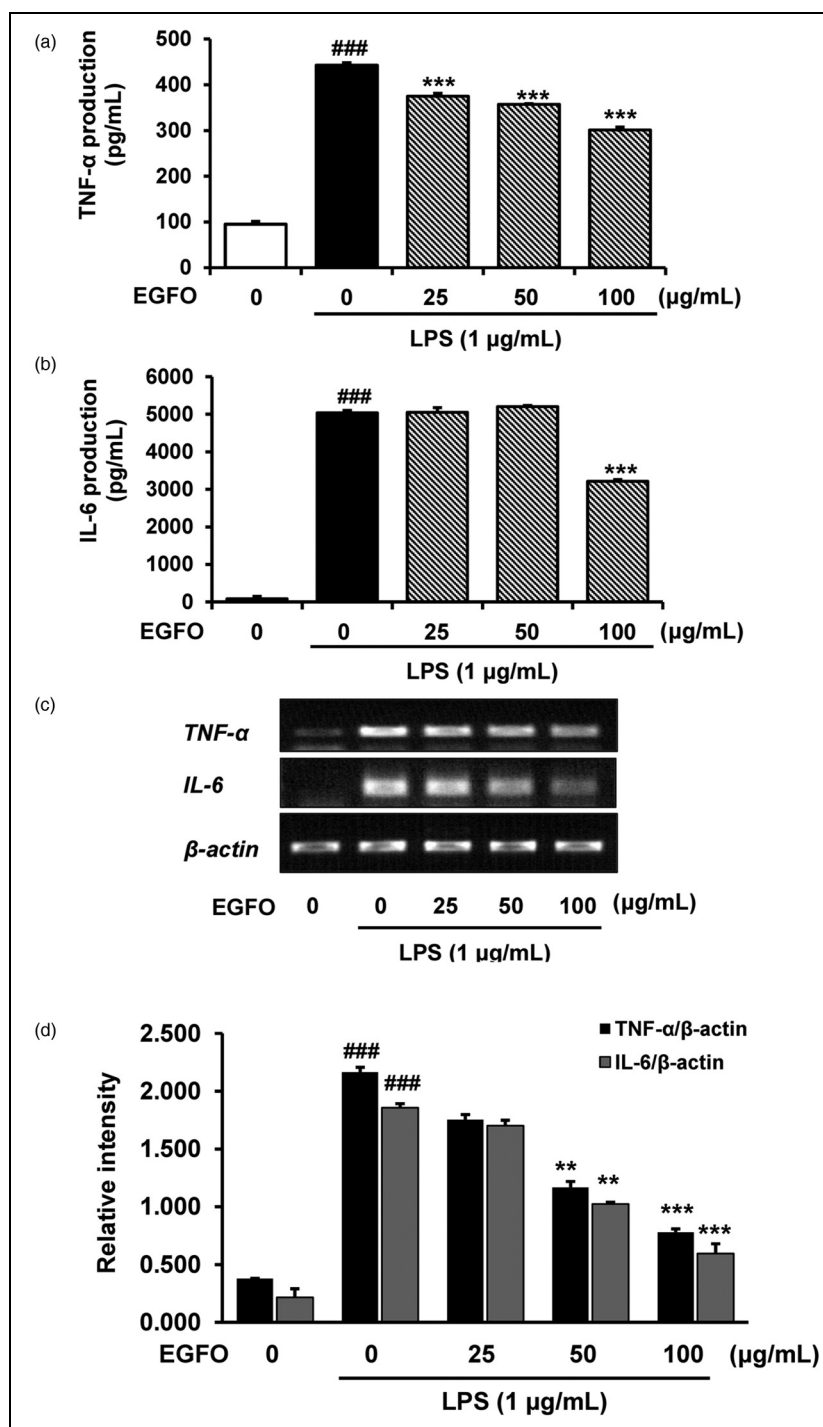


Figure 3. Inhibitory effects of EGFO on the inflammatory cytokines in LPS-stimulated BV-2 microglia. (A and B) Cells were pretreated with EGFO extract 2 h before LPS stimulation (1 μ g/mL) and maintained for 24 h. The amounts of TNF- α (A) and IL-6 (B) released into the culture media were determined using commercial ELISA kits. (C) Cells were pretreated with EGFO extract 1 h before LPS stimulation (1 μ g/mL) and maintained for 6 h. RT-PCR was performed to determine the expression of the TNF- α and IL-6 mRNAs. (D) The bar graphs represent the relative intensity of TNF- α and IL-6 expression level adjusted to the β -actin gene. The values are expressed as the mean $\pm$ SEM of three independent experiments. ### P < 0.001 versus untreated control. ** P < .01 or *** P < .001 versus LPS-treated cells.

Abbreviations: EGFO, *Elaeagnus glabra* f. *oxyphylla*; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger RNA; SEM, standard error of mean.

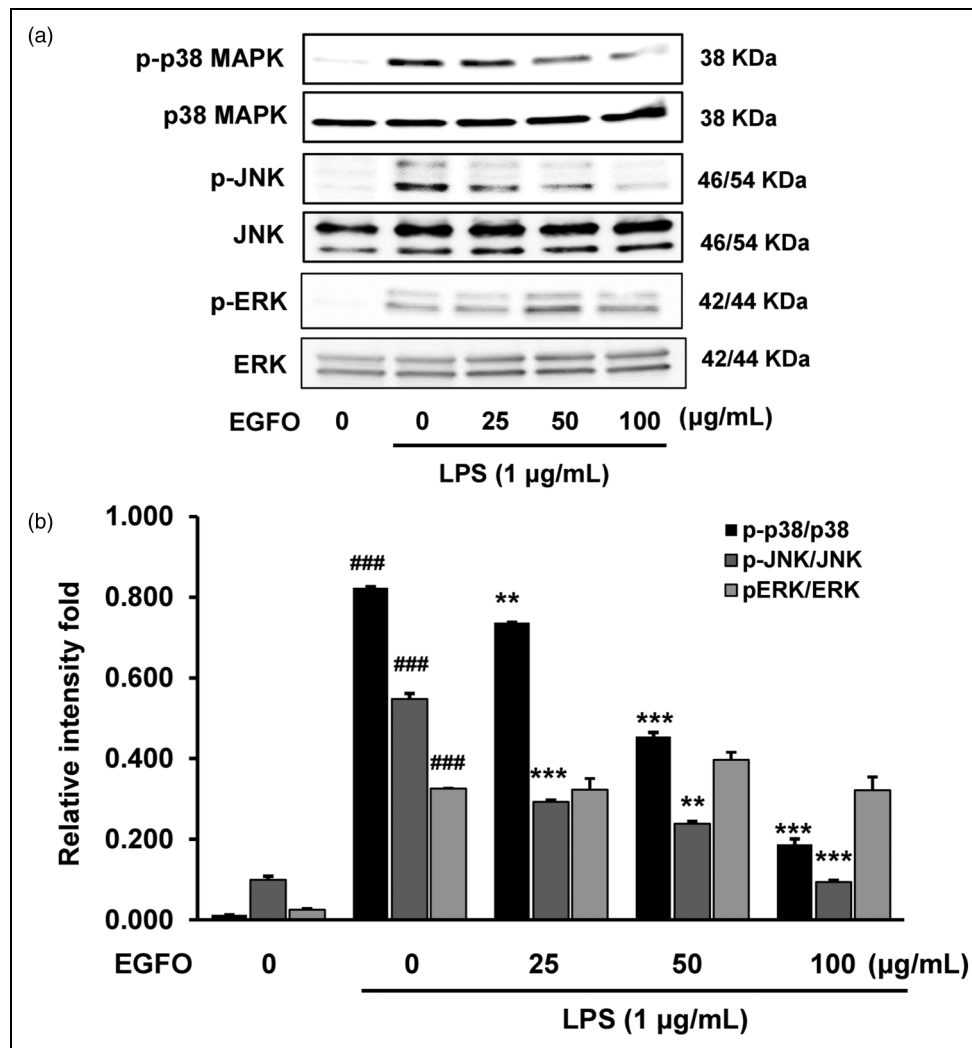


Figure 4. Inhibitory effects of EGFO on the p38 MAPK, JNK, and ERK pathways in LPS-stimulated BV-2 microglia. (A) Cells were pretreated with EGFO extract for 1 h, followed by LPS stimulation (1 μg/mL) for 30 min. Western blot analysis was performed to determine the phosphorylation levels of p38 MAPK, JNK, and ERK. (B) The bar graphs represent the relative levels of p38 MAPK, JNK, and ERK phosphorylation adjusted to the total protein expression of p38 MAPK, JNK, and ERK. The values are expressed as the mean ± SEM of three independent experiments. ### $P < .001$ versus untreated control. ** $P < .01$ or *** $P < .001$ versus LPS-treated cells. Abbreviations: EGFO, *Elaeagnus glabra* f. *oxyphylla*; MAPK, mitogen-activated protein kinase; JNK, c-jun N-terminal protein kinase; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide.

In contrast, the EGFO extract markedly suppressed the LPS-stimulated TNF- α production in a dose-dependent manner to 374.81 ± 6.20 , 357.20 ± 1.18 , and 300.98 ± 6.69 pg/mL at 25, 50, and 100 μg/mL of the extract, respectively ($P < .001$, Figure 3A). IL-6 released into the culture medium was markedly increased to 5035.97 ± 67.05 pg/mL in the LPS-stimulated cells compared with the untreated control, while this was reduced significantly in the EGFO extract-treated cells with LPS to 3212.76 ± 44.23 pg/mL at 100 μg/mL of the extract, compared with the LPS treatment alone ($P < .001$, Figure 3B). In addition, in parallel, the effects of EGFO on the mRNA expression of TNF- α and IL-6 were studied using RT-PCR. As shown in Figure 3C and D, the

enhanced levels of the TNF- α and IL-6 mRNAs in LPS-treated BV-2 cells were significantly decreased by EGFO treatment at 50 and 100 μg/mL of the extract, respectively ($P < .01$ at 50 μg/mL; $P < .001$ at 100 μg/mL), which was consistent with the ELISA or cytokine results.

Effects of EGFO Extract on the Phosphorylation of MAPKs in BV-2 Microglia

The MAPK pathway plays a crucial role in controlling the synthesis and release of inflammatory factors in activated microglia.³¹ Thus,

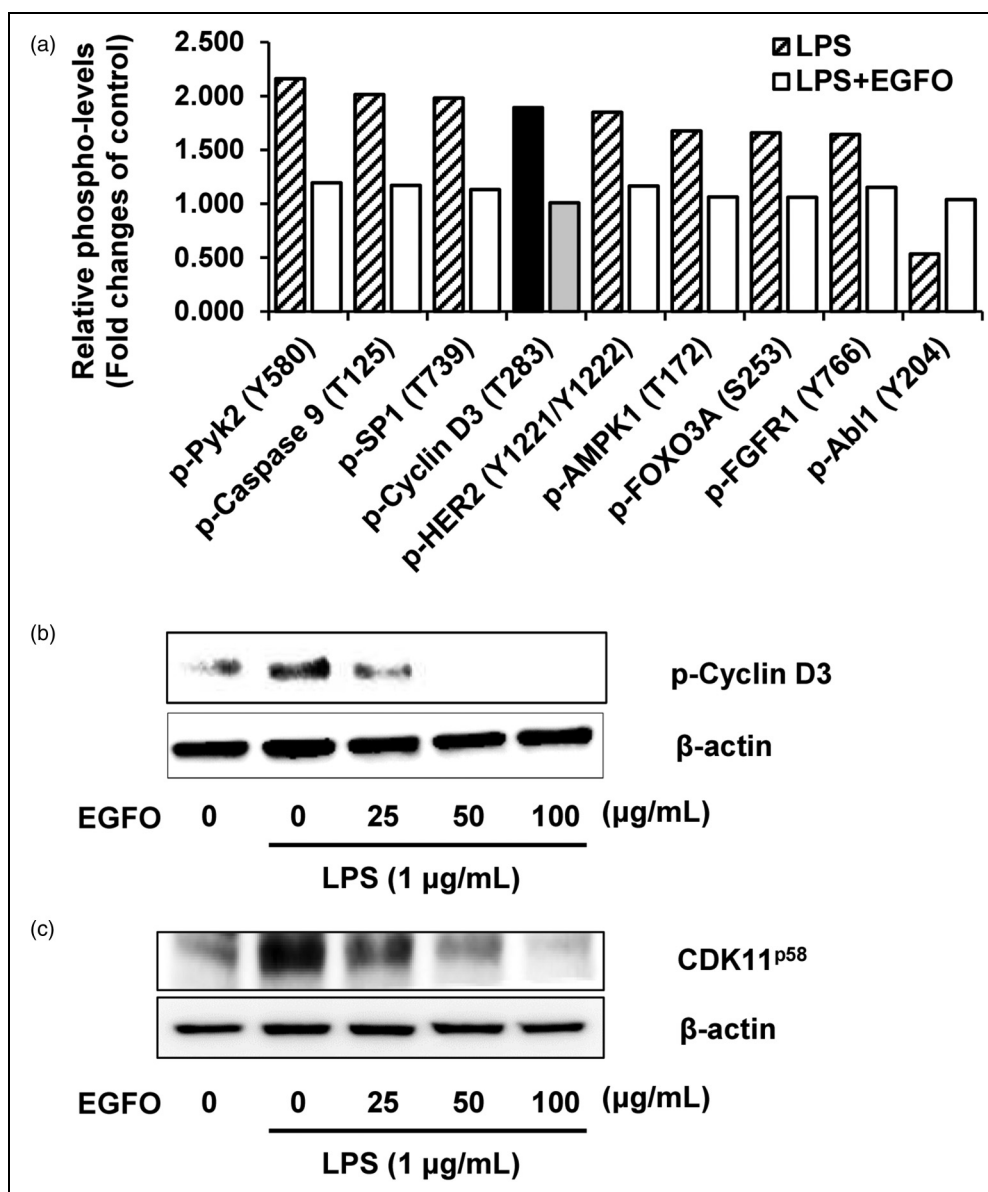


Figure 5. Inhibitory effects of EGFO on CDK11/cyclin D3 in LPS-stimulated BV-2 microglia. Cells were pretreated with EGFO extract 2 h before LPS stimulation (1 μg/mL) and maintained for 24 h. (A) An antibody microarray analysis was performed using phospho-specific antibody microarray slides (Full Moon BioSystems). The ratio of phosphorylation was calculated and is presented as fold changes of the indicated phospho-proteins normalized to total protein expression. (B and C) Western blotting was performed to determine the expression of cyclin D3 (B) and CDK11^{p58} (C). β-Actin was used as an internal control.

Abbreviations: EGFO, *Elaeagnus glabra* f. *oxyphylla*; LPS, lipopolysaccharide; CDK11^{p58}, cyclin-dependent kinase 11^{p58}.

we investigated the effects of EGFO on the phosphorylation of MAPKs in BV-2 cells. The phosphorylation of p38 MAPK and JNK was markedly increased in the LPS-treated cells compared with the untreated control. In contrast, cotreatment with the EGFO extract and LPS significantly attenuated the phosphorylation levels of p38 and JNK in a dose-dependent manner compared with the LPS-treated cells ($P < .01$ at 25 μg/mL; $P < .001$ at 50 and 100 μg/mL for phospho-p38; $P < .01$ at 50 μg/mL; $P < .001$ at 25 and 100 μg/mL for phospho-JNK, Figure 4A and B, and

Supplemental Figure 1). However, EGFO had no significant effect on extracellular signal-regulated kinase (ERK) phosphorylation in BV-2 cells (Figure 4A and B, and Supplemental Figure 1).

Effects of EGFO Extract on the Cyclin D3/ Cyclin-Dependent Kinase 11 Pathway in BV-2 Microglia

To investigate the molecular mechanisms underlying the regulation of neuroinflammation by EGFO, we performed a

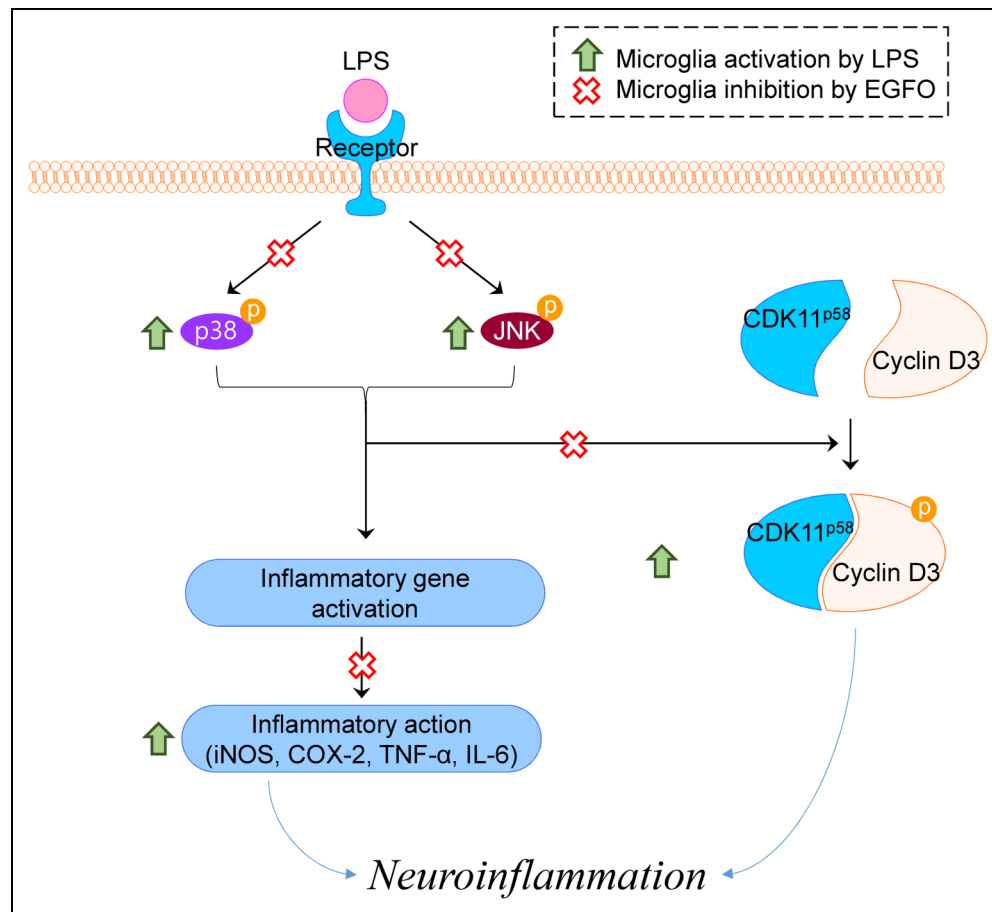


Figure 6. Schematic diagram of the neuroinflammatory modulation afforded by the *Elaeagnus glabra* f. *oxyphylla* (EGFO) extract in activated microglia.

phospho-protein antibody microarray analysis. As shown in Figure 5A, significant up- or downregulation of phospho-proteins was observed in the cells treated with EGFO and LPS compared with the LPS treatment alone. Of the total 1318 phosphorylation-specific proteins, EGFO treatment upregulated eight phospho-proteins and downregulated one phospho-protein in the inflammatory response induced by LPS in BV-2 cells. Of these 13 phospho-proteins, we focused on the changes in phospho-cyclin D3 induced by EGFO in LPS-stimulated BV-2 cells. Cyclin D3 is a cell-cycle-regulatory protein at the G1/S phase.³² It is of interest that cyclin D3 promotes microglial activation through an interaction with CDK11^{p58}.¹⁴ The effects of EGFO on cyclin D3 phosphorylation were validated using western blotting in LPS-treated BV-2 cells. LPS stimulation enhanced the phospho-cyclin D3 levels compared with the untreated control. Consistent with the results of the antibody microarray, EGFO treatment suppressed phospho-cyclin D3 in a dose-dependent manner (Figure 5B). As mentioned above, cyclin D3 exerts its function by coupling with CDK11^{p58} during the inflammatory regulation of microglia. Our data revealed that the expression of

CDK11^{p58} was markedly increased by LPS stimulation, while it was suppressed by the concurrent treatment with EGFO and LPS in BV-2 microglia (Figure 5C).

Discussion

Microglia are the major immune resident cells of the central nervous system and play a key role in brain development.³³ A growing body of literature reports that exposure of microglia to various stimuli, such as bacterial endotoxins, amyloid- β , and damaged neurons, leads to the activation of cells, thereby inducing an inflammatory response in the brain^{34,35}; activated microglia induce the generation of inflammation-related factors and cytokines, such as iNOS, COX-2, TNF- α , and IL-6.^{36,37} Hence, targeting the inflammatory factors in activated microglia could be an attractive therapeutic approach for alleviating the progression of various neuronal diseases.³⁸ In their recent review, Azodi and Jacobson³⁹ suggested the importance of cytokine therapies in providing new strategies for treating neurological diseases. Therefore, it is important to evaluate the effects of new drug candidates against microglial activation by controlling inflammatory cytokine

production. In the present study, we investigated the effects of EGFO on the inflammatory response in activated microglia. Our results revealed that EGFO significantly reduced the secretion of NO, PGE₂, TNF- α , and IL-6, and effectively suppressed the mRNA expression of iNOS, COX-2, TNF- α , and IL-6 in LPS-stimulated BV-2 cells.

In addition, we examined the molecular mechanisms underlying the antineuroinflammatory actions of EGFO and found that they are associated with the p38/JNK and cyclinD3/CDK11^{p58} pathways. It is well known that the MAPK pathway is involved as a key regulator of brain inflammation.⁴⁰ In particular, microglial p38 and JNK activation contributes to the upregulation of inflammatory cytokines.⁴¹ p38 and JNK play a critical role in microglial activation by the secreted amyloid- β precursor protein.⁴² Blocking p38 using specific inhibitors attenuates several aspects of the inflammatory response, such as cytokine production, in activated microglia.^{9,43} JNK inhibitors also improve functional recovery via the suppression of neuroinflammation in microglia.^{10,44} Similar to our results, several previous studies reported the inhibitory effects of various plants on the p38 and/or JNK pathway in activated microglia.^{8,45}

Regarding the neuroinflammatory molecules, Liu et al¹³ reported a role for the interaction between CDK11^{p58} and cyclin D3 in astrocytes activated by LPS treatment. Blocking CDK11^{p58} in LPS-stimulated astrocytes induced the downregulation of p38 and JNK phosphorylation, suggesting the involvement of the p38/JNK pathway in the CDK11^{p58}-mediated inflammation of astrocytes. Shen et al¹⁴ showed that CDK11^{p58} and cyclin D3 play essential roles in LPS-induced inflammation in microglia. Our analysis of the antibody microarray consistently revealed that LPS stimulation upregulated phospho-cyclin D3 compared with the untreated control. In contrast, EGFO treatment in LPS-activated BV-2 cells reduced the phosphorylation of cyclin D3 as validated by immunoblotting. Concurrently, LPS treatment enhanced the expression of CDK11^{p58}, whereas cotreatment with EGFO and LPS reversed the effect of LPS on the expression of CDK11^{p58} in microglia. As mentioned above, EGFO significantly downregulated the phosphorylation of p38 and JNK in LPS-treated BV-2 cells. Taken together, these data suggest that the inhibitory effects of EGFO on p38 and JNK phosphorylation may be related to the cyclin D3/CDK11^{p58} pathway. Based on our and Shen's data, the cyclin D3/CDK11^{p58} pathway can be considered as a potential therapeutic target for microglia-mediated neurodegenerative disorders.

The schematic diagram presented in Figure 6 summarizes the molecular mechanisms underlying the antineuroinflammatory activity of EGFO in activated microglia. EGFO inhibits the phosphorylation of p38 and JNK and subsequently suppresses the production and expression of proinflammatory mediators and cytokines in LPS-stimulated microglia. In addition, the downregulation of the p38/JNK pathway may be associated with the interruption of the coupling of cyclin D3 to CDK11^{p58}. Further in vivo studies will be necessary to

confirm the role of cyclin D3/CDK11^{p58} in microglial activation and the antiinflammatory effects of EGFO through the regulation of the cyclin D3/CDK11^{p58} pathway in microglia.

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Authors' Note

The data used to support the findings of the current study are available from the corresponding author on reasonable request.

Author Contributions

S-JJ conceptualized the study, and H-SL and ES analyzed the data and prepared the manuscript. ES, H-SL, YJK, and B-YK executed the biological examination of experiments. YJK and J-WK prepared and analyzed *Elaeagnus glabra* f. *oxyphylla* (EGFO) extract. S-JJ interpreted the data, edited the manuscript, and supervised the study. All authors reviewed the manuscript and approved the final manuscript.

Declaration of Conflicting Interests

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Ethical Approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent

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Trial Registration

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ORCID iD

Eunjin Sohn  <https://orcid.org/0000-0002-9410-4788>

Yu Jin Kim  <https://orcid.org/0000-0001-5152-9439>

Supplemental Material

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