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## Adjudin attenuates lipopolysaccharide (LPS)- and ischemia-induced microglial activation

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### Abstract

Neuroinflammation caused by microglial activation plays a key role in ischemia, neurodegeneration and many other CNS diseases. In this study, we found that Adjudin, a potential non-hormonal male contraceptive, exhibits additional function to reduce the production of proinflammatory mediators. Adjudin significantly inhibited LPS-induced IL-6 release and IL-6, IL-1 $\beta$ , TNF- $\alpha$  expression in BV2 microglial cells. Furthermore, Adjudin exhibited anti-inflammatory properties by suppression of NF- $\kappa$ B p65 nuclear translocation and DNA binding activity as well as ERK MAPK phosphorylation. To determine the *in vivo* effect of Adjudin, we used a permanent middle cerebral artery occlusion (pMCAO) mouse model and found that Adjudin could reduce ischemia-induced CD11b expression, a marker of microglial activation. Furthermore, Adjudin treatment attenuated brain edema and neurological deficits after ischemia but did not reduce infarct volume. Thus, our data suggest that Adjudin may be useful for mitigating neuroinflammation.

### Keywords

Adjudin; Microglial activation; Cytokine; NF- $\kappa$ B; Brain edema

## 1. Introduction

Microglial cells are the resident macrophages of the central nervous system (CNS) and have been proposed to play a role in CNS immunity, development and repair (Streit, 2001; Town et al., 2005; Walton et al., 2006). Once activated, microglia produce multiple proinflammatory mediators, including cytokines, chemokines, reactive oxygen species

(ROS), nitric oxide (NO) and prostaglandin E2 (PGE2), which are responsible for neurotoxic effect under pathological conditions (Garden and Moller, 2006). Chronic inflammation caused by microglial activation has close relationship with Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Gonzalez-Scarano and Baltuch, 1999; Imamura et al., 2003; Xiang et al., 2006), while inflammatory responses by microglia exacerbate brain injury after ischemia (Yenari et al., 2010). Thus, inhibition of microglial activation would be a potential therapeutic approach to treatment of various CNS diseases.

Adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, formerly called AF-2364, performs potent reversible anti-spermatogenic activity by disrupting the adherens junction of germ cells to Sertoli cells (Mok et al., 2011). Previous results have shown that many indazole derivatives are non-steroidal anti-inflammatory drugs (NSAID) by inhibition of prostaglandin E2 (PGE2) synthesis, nitric oxide (NO) production, cytokine and chemokine release (Foster et al., 1990; Sironi et al., 1996; Bhatia et al., 2005; Pan et al., 2005). Recent studies indicate that the anti-inflammatory effect of indazole derivatives is associated with the inhibition of NF- $\kappa$ B activation (Sommers et al., 2009; Oh et al., 2010).

Since Adjudin is a small molecular derivative of indazole and has no apparent side effects in treated animals (Mok et al., 2011), in this study we aimed to investigate whether Adjudin could attenuate microglial activation and the possible mechanisms.

## 2. Materials and methods

### 2.1. Reagents and animals

LPS and DMSO were purchased from Sigma Aldrich (St. Louis, MO, USA). Adjudin was provided by The Mary M. Wohlford Laboratory, Population Council, New York. Mice were purchased from Shanghai SLAC Laboratory Animal Corporation (Shanghai, China). Animal procedures for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

### 2.2. Cell culture

The BV2 microglia cell line was provided by Institute of Neurology, Ruijin Hospital (Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle Medium (Thermo Scientific) supplemented with 5% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO) at 37 °C in a humidified incubator under 5% CO<sub>2</sub>. The absolute concentrations of penicillin/streptomycin were 100 units·ml<sup>-1</sup>/100 µg·ml<sup>-1</sup>.

### 2.3. Lactate dehydrogenase (LDH) assay

BV2 cells were lysed for 20 min in a lysing buffer containing 0.04% Triton-X, 2 mM HEPES, 0.2 mM dithiothreitol, 0.01% bovine serum albumin, and 0.1% phenol red, pH 7.5. Cell lysates (50 µl) were mixed with 150 µl 500 mM potassium phosphate buffer (pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate, and the A340 nm change was monitored over 90 s using a microplate reader (Synergy2, BioTek).

#### 2.4. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-6 in medium from cultured BV2 cells was measured by platinum ELISA Kit (eBioscience) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader (Synergy2, BioTek). Protein concentrations were determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The IL-6 concentration in the medium was calculated using an IL-6 standard, and normalized against the protein of the samples.

#### 2.5. Real-time PCR

Total RNA was isolated from BV2 cells by using Trizol Reagent (Invitrogen, CA, USA) and reverse-transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa). Quantitative real-time PCR was performed on ABI 7900HT by using SYBR Premix Ex Taq (TaKaRa) and the following primers: IL-6 (sense 5'-tagtccttctacccaattcc-3' and anti-sense 5'-ttggccttagccactccttc-3'); IL-1 $\beta$  (sense 5'-gcaactgtt cctgaactcaact-3' and anti-sense 5'-atctttggggcgtaact-3'); TNF- $\alpha$  (sense 5'-ccctcacactcagatcatcttct-3' and anti-sense 5'-gctacgacgtgg gctacag-3'); GAPDH (sense 5'-aggctcggtggaacggatttg-3' and anti-sense 5'-tgtagaccatgtagttgaggtca-3'). Data were analyzed by using the comparative threshold cycle (Ct) method, and results were expressed as fold difference normalized to GAPDH.

#### 2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were prepared by using Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. DNA-protein interactions were measured by LightShift<sup>®</sup> Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA) as follows: nuclear extracts (10  $\mu$ g) were incubated with biotin-labeled NF- $\kappa$ B probe (5'-atgtg ggatttcccatgag-3') at room temperature for 20 min. Protein-DNA complexes were resolved by 5% polyacrylamide gel, transferred to Nylon Membranes (Millipore, CA, USA), and crosslinked with 312 nm UV-light bulbs. The membranes were incubated with Streptavidin-HRP for 20 min and biotin-labeled DNA signals were visualized using ECL detection system.

#### 2.7. Western blotting

Immunoblotting was carried out as previously described (Xia et al., 2007). Cells were lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail and 2 mM PMSF. 20  $\mu$ g of total protein was separated by 10% SDS-PAGE and then transferred to 0.45  $\mu$ m Nitrocellulose Membrane (Millipore, CA, USA). The membranes were incubated with primary antibodies at 4 °C overnight and then hybridized with appropriate HRP-conjugated secondary antibody (1:5000 dilution, Epitomics, Zhejiang Province, China) at room temperature for 1 h. Protein signals were visualized using ECL detection system. The primary antibodies used were as follows: ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK (1:1000 dilution, Cell Signaling Technology, Danvers, USA); NF- $\kappa$ B p65 (1:2000 dilution, Epitomics); Lamin A/C,  $\beta$ -actin (1:1000 dilution, Santa Cruz Biotechnology, CA, USA).

## 2.8. Permanent middle cerebral artery occlusion (pMCAO) model

pMCAO model in mice was performed as previously described (Yang et al., 1994). In brief, adult male ICR mice (25–30 g) were anesthetized by intraperitoneal administration of ketamine (100 mg/kg) plus xylazine (10 mg/kg). Then animal surgery was performed as follows: a 6–0 nylon suture was inserted from the left external carotid artery (ECA) into the internal carotid artery (ICA) and reached to the circle of Willis to occlude the origin of the middle cerebral artery (MCA). Successful occlusion was characterized as a reduction of cerebral blood flow (CBF) down to 10% of baseline. Sham-operated mice underwent the same procedure without suture insertion. For the animal experiments, mice were intraperitoneally administrated with 50 mg/kg Adjudin (100 mg/ml DMSO stock dissolved in corn oil at a dilution of 1:10) or vehicle (DMSO dissolved in corn oil at a dilution of 1:10) 2 h before pMCAO.

## 2.9. Immunohistological staining

Brain cryosections (20  $\mu\text{m}$ ) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum and incubated with anti-CD11b primary antibody (1:100 dilution, BD Biosciences). After washing, sections were incubated with Alexa-488-conjugated secondary antibody (1:200 dilution, Invitrogen, CA, USA) and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (1:500 dilution, Beyotime Institute of Biotechnology, China). Confocal microscopic images were acquired using a confocal laser-scanning microscope (Leica TCS SP5 II, Germany).

## 2.10. Measurement of infarct volume

Brain cryosections (20  $\mu\text{m}$ ) were immersed in 0.1% cresyl violet for 30 min and then rinsed in distilled water for 10 min. The infarction area in each section was calculated using the ImageJ analysis software by the following formula: contralateral hemisphere area ( $\text{mm}^2$ )–ipsilateral undamaged area ( $\text{mm}^2$ ). The infarction areas of each slice were separately summed and multiplied by the interval thickness to obtain infarct volumes.

## 2.11. Measurement of brain edema and brain water content

Brain edema was calculated as ipsilateral volume/contralateral volume. Brain tissue samples were weighed before and after dehydration in an oven at 100 °C for 24 h. Tissue water content (%) was calculated as [(wet weight–dry weight)/wet weight] $\times$ 100.

## 2.12. Behavioral assessment

Modified neurologic severity scores (mNSS): mNSS is a composite of motor, reflex and balance tests. Total neurological score was calculated as the sum of scores on limb flexion (range: 0–3), walking gait (range: 0–3), beam balance (range: 0–6) and reflexes absence (range: 0–2). Therefore, neurologic function was graded on a scale of 0 to 14 (normal score 0; maximal deficit score 14) as previously described (Chen et al., 2005).

## 2.13. Statistical analysis

All data are presented as mean  $\pm$  SEM. Data were assessed by one-way ANOVA, followed by Tukey post hoc test. P values less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Effect of Adjuvin on viability of BV2 microglia

To study whether Adjuvin could serve as a neuroinflammation modulator, we used BV2 microglial cell line as a model. We first evaluated its cytotoxicity to BV2 cells after 24-h incubation with different concentrations of Adjuvin. As shown in Fig. 1, cell viability following treatment with 10  $\mu$ M and 30  $\mu$ M Adjuvin was not significantly different from the control in the absence or presence of LPS. However, exposure of BV2 cells to 60  $\mu$ M Adjuvin led to about 40% decrease in cell survival.

#### 3.2. Adjuvin reduces LPS-induced cytokine production in BV2 microglia

BV2 cells were pretreated with Adjuvin (0, 10, 30, 60  $\mu$ M) for 1 h and then stimulated with 200 ng/ml LPS for a 24-h incubation period. As anticipated, LPS significantly enhanced IL-6 release in the culture media, while pretreatment with Adjuvin reduced IL-6 production by about 40% (Fig. 2A). We further determined whether Adjuvin could regulate the expression of several cytokines important in neuroinflammation. LPS incubation for 6 h caused a remarkable increase in mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which could be suppressed by Adjuvin in a dose-dependent manner (Fig. 2B–D).

#### 3.3. Adjuvin inhibits LPS-induced NF- $\kappa$ B activation in BV2 microglia

NF- $\kappa$ B has been shown to be one of the most important modulators for multiple proinflammatory cytokines, so we determined whether Adjuvin could affect NF- $\kappa$ B signaling pathway. BV2 cells were pretreated with 30  $\mu$ M Adjuvin for 1 h and then stimulated with 200 ng/ml LPS for a 2-h incubation period. As shown in Fig. 3A, stimulation with LPS caused strong NF- $\kappa$ B DNA binding activity, which was markedly inhibited by Adjuvin. The binding specificity was verified by incubating nuclear extracts from LPS-stimulated BV-2 cells with excess unlabeled specific competitor or nonspecific oligonucleotide probe. In addition, Adjuvin also affected LPS-induced NF- $\kappa$ B p65 translocation from cytoplasm to nucleus. As shown in Fig. 3B, significant levels of NF- $\kappa$ B p65 were localized to the nucleus after LPS treatment, while Adjuvin partially reversed this nuclear translocation.

#### 3.4. Adjuvin decreases LPS-induced phosphorylation of ERK in BV2 microglia

We then assessed whether the suppressive effect of Adjuvin on proinflammatory mediators occurred via MAPK signaling pathway. BV2 cells were pretreated for 1 h with indicated concentrations of Adjuvin (0, 10, 30  $\mu$ M) and then stimulated with 200 ng/ml LPS for 1 h. As anticipated, stimulation with LPS resulted in rapid activation of ERK, p38, and JNK MAPKs. However, pretreatment with 10  $\mu$ M and 30  $\mu$ M Adjuvin only reduced ERK phosphorylation but not p38 or JNK phosphorylation (Fig. 4).

#### 3.5. Adjuvin attenuates ischemia-induced microglial activation

To further determine the *in vivo* effect of Adjuvin on microglial activation, we used a mouse pMCAO model. The result showed that CD11b expression—an indicator for microglial activation—was remarkably upregulated at 24 h after pMCAO, while *i.p.* administration of

Adjudin (50 mg/kg b.w.) 2 h before ischemia reduced CD11b expression in both cerebral cortex (Fig. 5A and C) and striatum (Fig. 5B and D). These findings suggest that Adjudin could attenuate pMCAO-induced microglial activation.

### 3.6. Adjudin reduces brain edema and neurological deficits after pMCAO

Next we investigated whether a decrease in microglial activation is related to the neuroprotective effect against ischemia. Adjudin administration did not reduce brain infarct volume (Fig. 6A and B) or the number of degenerating neurons (Supplementary Fig. 1) after ischemia. However, brain edema (Fig. 6C) and brain water content (Fig. 6D) of the ipsilateral hemisphere were smaller in the Adjudin-treated group than those in the control group at 24 h after pMCAO. Furthermore, neurological deficit improvement was observed with Adjudin treatment compared to vehicle at 72 h after pMCAO (Fig. 6E).

## 4. Discussion

In this study, we showed that Adjudin reduced IL-6 release and TNF- $\alpha$ , IL-1 $\beta$ , IL-6 mRNA expression in LPS-stimulated BV2 microglial cells. The anti-inflammatory property of Adjudin was associated with the inhibition of NF- $\kappa$ B activation and ERK MAPK phosphorylation. Using a well-recognized pMCAO model, we observed that Adjudin suppressed ischemia-induced microglial activation indicated by reduction of CD11b staining. Furthermore, Adjudin ameliorated brain edema and neurological deficits after ischemia but did not reduce infarct volume or the number of degenerating neurons.

Microglial cells are brain macrophages which serve important functions in many CNS diseases (Gonzalez-Scarano and Baltuch, 1999; Imamura et al., 2003; Xiang et al., 2006; Yenari et al., 2010). Several stimulants such as Amyloid-beta (A $\beta$ ), cytokines, LPS or IFN- $\gamma$  can activate microglial cells (Hanisch and Kettenmann, 2007), and among them LPS is the most common stimulant used in in vitro studies (Qin et al., 2005). Microglial activation is complicated according to different concentrations or duration time of stimuli. Microglia after prolonged (72 h) exposure to LPS showed potentially neuroprotective effect compared with the acute (24 h) LPS stimulation (Cacci et al., 2008). Microglia activated by low level of IFN- $\gamma$  seemed to promote neurogenesis despite the deleterious effect of IFN- $\gamma$  reported previously (Butovsky et al., 2006). In the present study, our experimental model is stimulation with 200 ng/ml LPS for 24 h, which induced acute inflammatory responses in microglia.

NF- $\kappa$ B is an important upstream modulator of multiple inflammatory mediators and enzymes, including IL-1 $\beta$ , IL-6, TNF- $\alpha$ , i-NOS and COX-2 (Griscavage et al., 1996; Ghosh et al., 1998; Jobin et al., 1998). Previous studies have shown that NF- $\kappa$ B plays a pivotal role in inflammatory responses and neurotoxic effects by glial cells (Akama et al., 1998; Wilms et al., 2003). In the resting state, NF- $\kappa$ B remains in the cytoplasm as a heterotrimer complex consisting of p50, p65 and I $\kappa$ B sub-units. Upon activation, phosphorylation and degradation of I $\kappa$ B lead to p50/p65 nuclear translocation, then binding to DNA and regulating gene transcription (Karin and Ben-Neriah, 2000). In the present study, we found that down-regulation of inflammatory mediators by Adjudin resulted from the inhibition of NF- $\kappa$ B

signaling pathway at two levels: first, Adjudin prevented NF- $\kappa$ B p65 translocation from cytoplasm to nuclei and second, Adjudin decreased NF- $\kappa$ B DNA binding activity.

The MAPK family comprises ERK1/2, JNK, p38, and ERK5/BMK1 (Kyriakis and Avruch, 2001). Diverse stimuli, such as Amyloid-beta ( $A\beta$ ), alpha-synuclein, LPS and IFN- $\gamma$ , are able to activate MAPK signaling pathways in microglia (Koistinaho and Koistinaho, 2002; Klegeris et al., 2008). Our data showed that Adjudin could reduce ERK phosphorylation but not p38 or JNK phosphorylation. It is noteworthy that although Adjudin significantly inhibited NF- $\kappa$ B activation, the suppressive effect on cytokine production by Adjudin is merely 50%. There remains the possibility that Adjudin might not influence another inflammation-related transcription factor activator protein-1 (AP-1), which could be supported by our result that Adjudin did not inhibit JNK phosphorylation, since JNK is a key regulator of AP-1 by phosphorylating c-Jun subunit of AP-1 and thereby enhancing its transcriptional activity (Shaulian and Karin, 2002).

CD11b is a  $\beta$ -integrin marker of microglia and the increased CD11b expression corresponds to microglial activation in various CNS diseases (Gonzalez-Scarano and Baltuch, 1999). In our study, pretreatment with 50 mg/kg Adjudin reduced CD11b positive staining in both cerebral cortex and striatum at 24 h after pMCAO, which provided evidence for suppressive effect of Adjudin on microglial activation in vivo. Next we investigated whether a decrease in microglial activation is correlated to the neuroprotective effect against ischemia. Results showed that Adjudin reduced brain edema after ischemia but did not affect infarct volume or the number of degenerating neurons. Brain swelling is correlated to neurological deficits at later time points (Manley et al., 2000), which agrees with our findings that behavioral improvement was observed by Adjudin treatment at 72 h after ischemia. Brain edema includes two major types. The cytotoxic edema represents a shift of water from the extracellular to the intracellular compartments and does not contribute to a net increase in brain volume. However, the vasogenic edema aggravates brain swelling through a shift of water from the intravascular to the extravascular compartments (Ayata and Ropper, 2002). After ischemia, blood-brain barrier (BBB) destruction leads to increased paracellular permeability, directly contributing to cerebral vasogenic edema and hemorrhagic transformation (Sandoval and Witt, 2008). Matrix metalloproteinases (MMPs) are matrix-degrading enzymes which become markedly upregulated in response to ischemic injury. Increased MMP activity results in tight junction protein degradation, causing damage to the BBB (Cunningham et al., 2005). Numerous evidence indicates that activated microglia can be a major source of secreted MMPs following stroke (del Zoppo et al., 2007), thus Adjudin might protect the brain edema partially by inhibiting microglia-mediated BBB destruction. To further assess the neuroprotective effect of Adjudin on ischemia, different regimens of Adjudin (post treatment or multiple doses), longer observation window and the choice of tMCAO model shall be considered.

In the in vitro study, 60  $\mu$ M Adjudin caused about 40% reduction in the cell viability of BV2 microglial cell lines. Our previous studies have shown that Adjudin could induce apoptosis in many mitotic cells (cancer cell lines and BV2 cell line) partly by targeting mitochondrial function. We presume that Adjudin has preferential effect on proliferating cells rather than normal cells. To verify this hypothesis, we treated primary mixed glial cultures with Adjudin

in the presence of LPS. As shown in Supplementary Fig. 2, cell viability of glial cultures following Adjudin treatment was not different from those in the control group at the concentration of up to 100  $\mu$ M. In the in vivo study, we chose a dose of 50 mg/kg b.w. for Adjudin in animal experiments. Previous reports have shown that two doses of 50 mg/kg b.w. Adjudin (one dose per week) could induce reversible infertility in adult rats without fatality, body weight reduction or apparent side effects (Cheng et al., 2001). Histological analyses showed that cell adhesion in other organs, such as kidney, liver, brain, heart and prostate, was not affected by Adjudin (Grima et al., 2001) and toxicity studies confirmed that Adjudin has no genotoxicity or mutagenesis in acute dose even at 2000 mg/kg b.w. in rats and mice (Mruk et al., 2006). Furthermore, the serum testosterone, FSH, and LH levels of the Adjudin-treated animals had no detectable change compared with the control animals, indicating that the hypothalamic–pituitary–testicular axis is not disrupted (Cheng et al., 2001). Thus, Adjudin administration at a dose of 50 mg/kg b.w. appears to be safe in animal treatment.

In summary, this study has shown that Adjudin could inhibit microglial activation in both in vitro and in vivo models, and the anti-inflammatory property of Adjudin was due to the inhibition of NF- $\kappa$ B activation and ERK MAPK phosphorylation. Therefore, these data suggest potential applications of Adjudin for mitigating neuroinflammation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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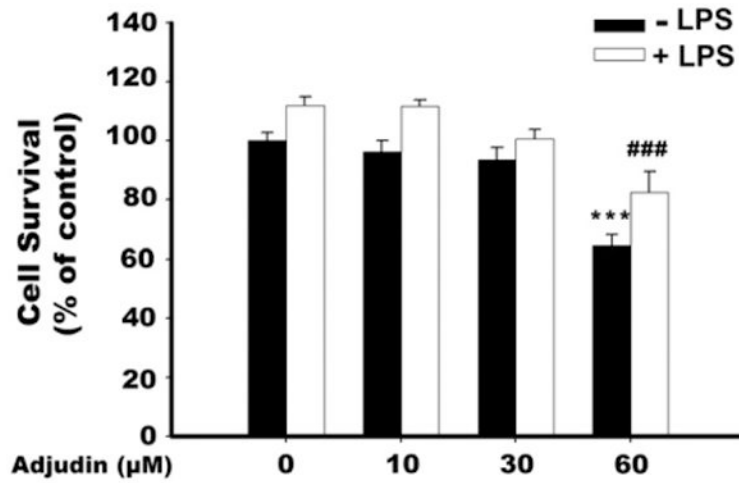
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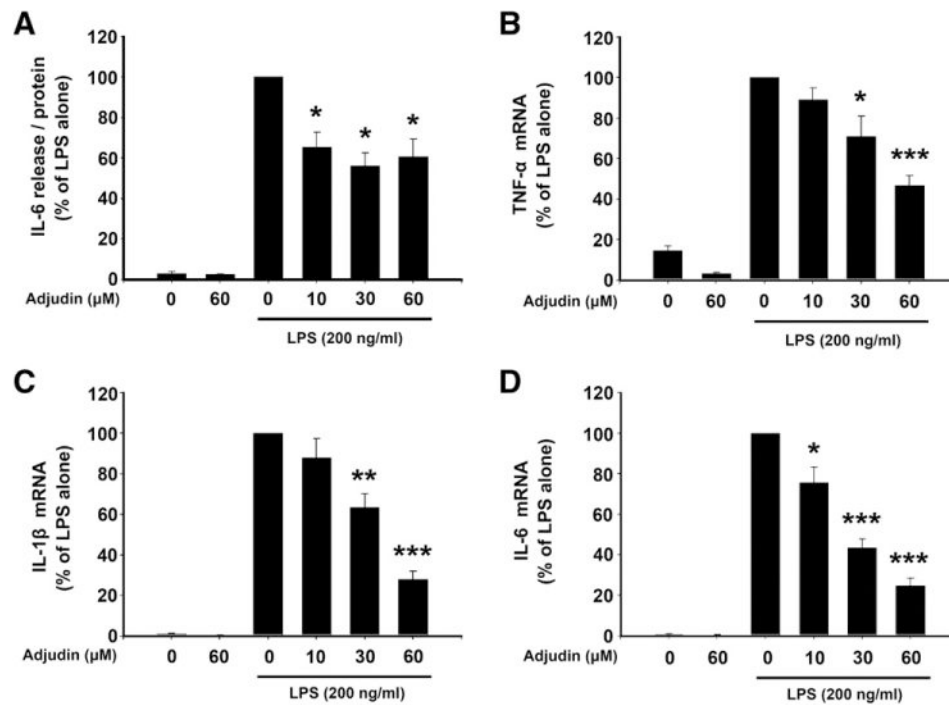


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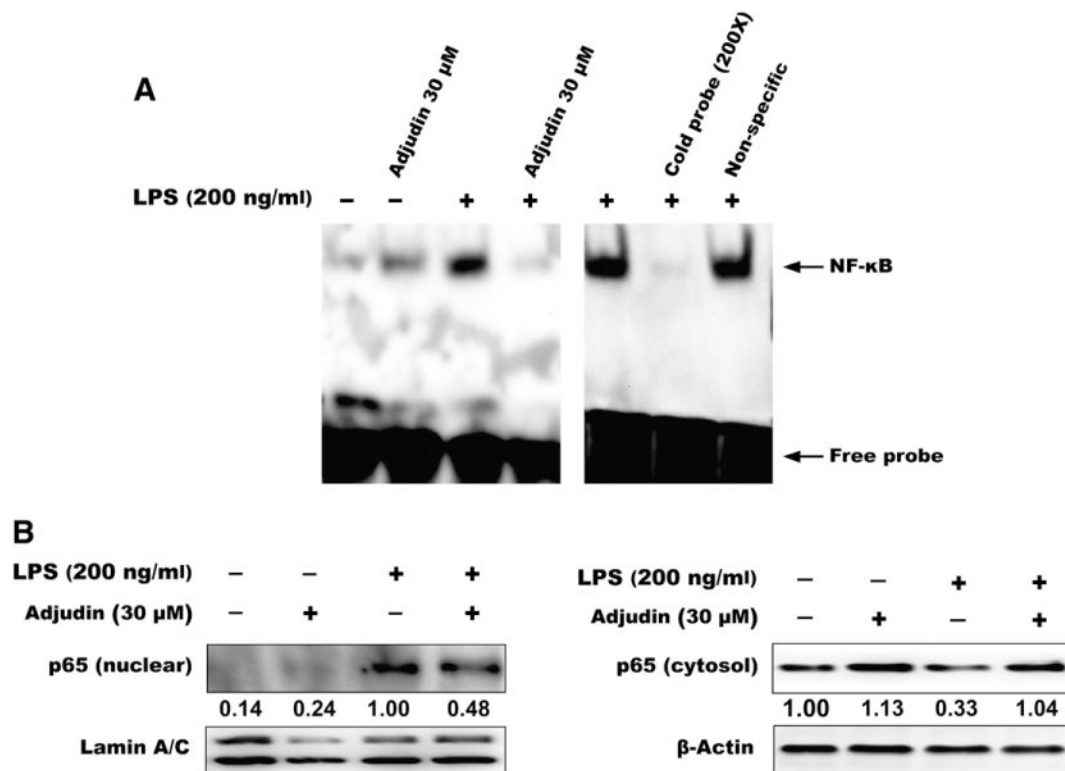
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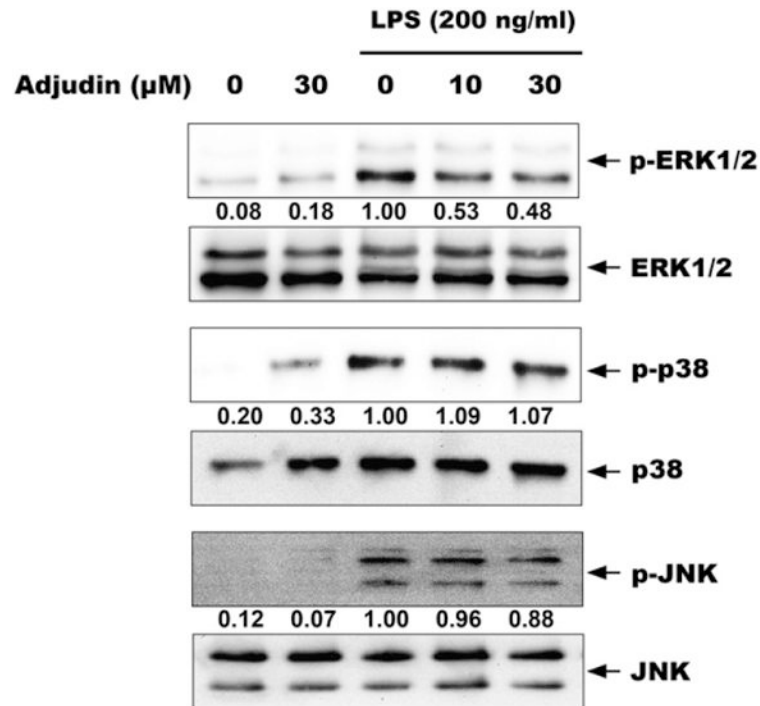
**Fig. 1.** Effect of Adjudin on the cell viability of BV2 microglia. Cells were pretreated with indicated concentrations of Adjudin for 1 h and then stimulated with 200 ng/ml LPS for a 24-h incubation period. Cell viability was assessed by LDH assay. Bars represent the mean  $\pm$ SEM from three independent experiments. \*\*\* $P < 0.001$  compared with control treatment; ### $P < 0.001$  compared with the LPS alone treatment.

**Fig. 2.**

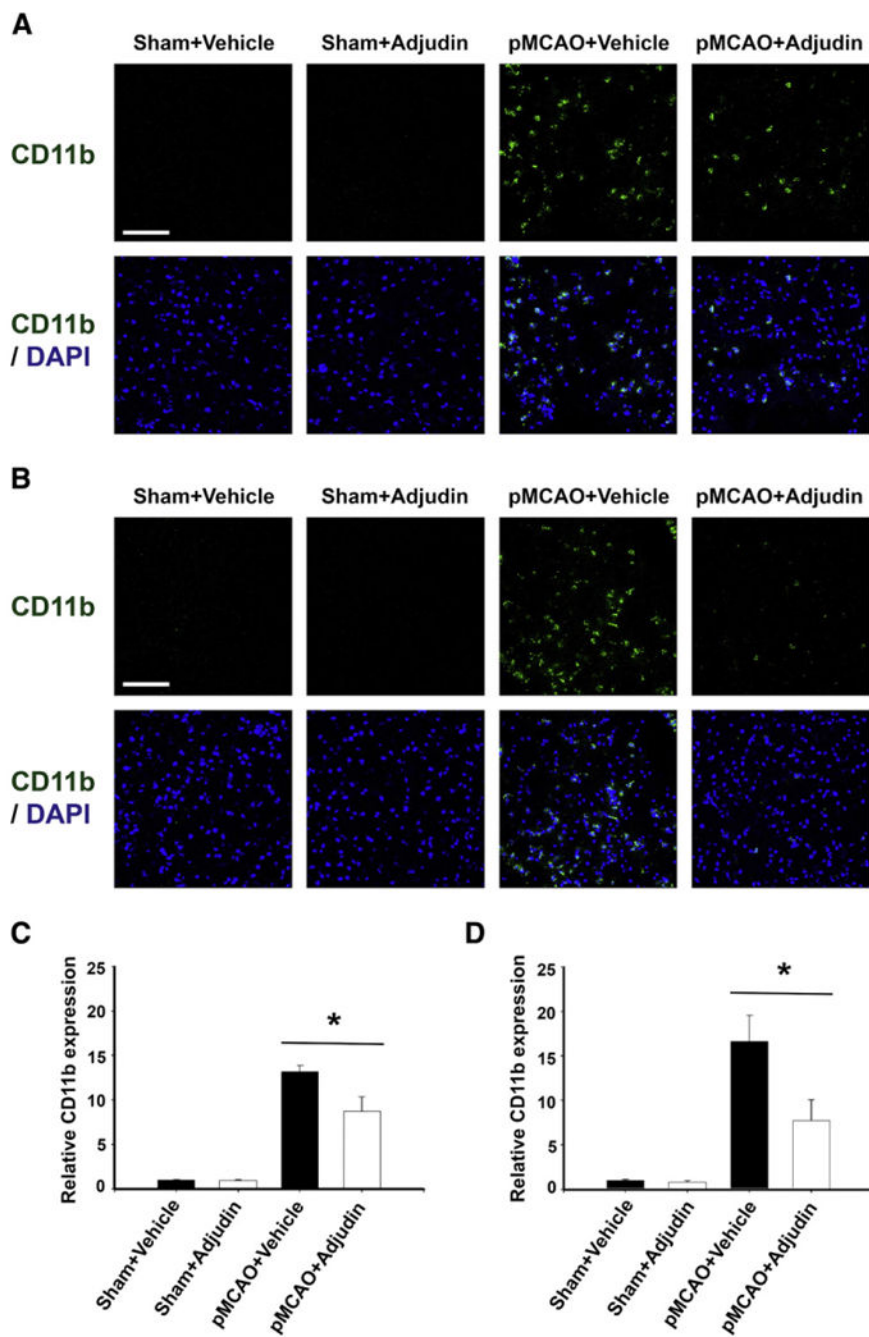
Adjudin reduces LPS-induced cytokine production in BV2 microglia. Cells were pretreated with Adjudin for 1 h and then incubated with LPS. (A) After 24 h incubation, IL-6 release in supernatants was measured by ELISA. Data were normalized against the protein of the samples. (B–D) After 6 h incubation, TNF- $\alpha$  (B), IL-1 $\beta$  (C) and IL-6 (D) mRNA expression was evaluated by qPCR. Data were normalized by GAPDH. Bars represent the mean $\pm$ SEM from four independent experiments. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001, compared with the LPS alone treatment.

**Fig. 3.**

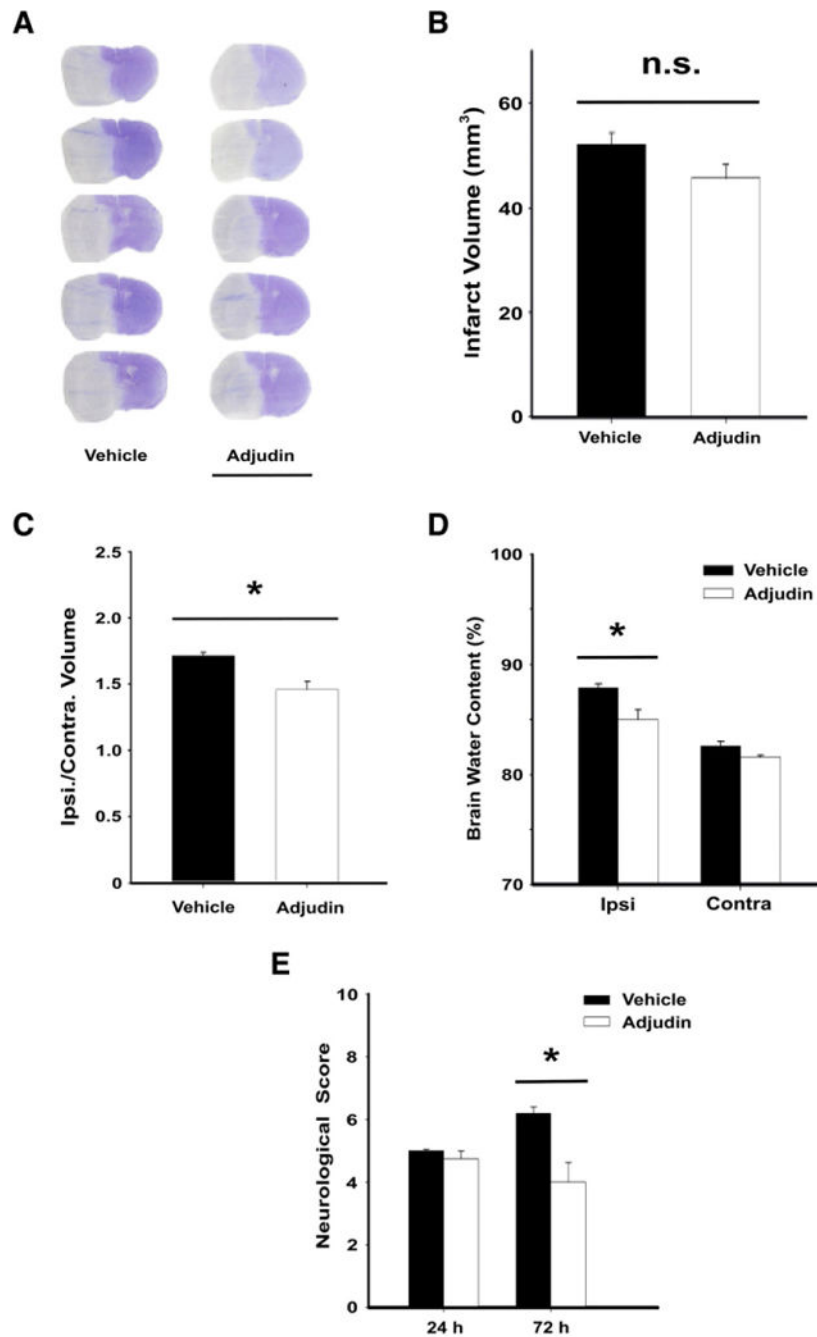
Adjudin inhibits LPS-stimulated NF- $\kappa$ B activation in BV2 microglia. Cells were pretreated with Adjudin for 1 h and then stimulated with LPS for 2 h. (A) Nuclei were extracted for NF- $\kappa$ B EMSA. (B) Nuclear and cytosol proteins were separated and analyzed using antibody against NF- $\kappa$ B p65. Lamin A/C was used as internal control for nuclear proteins and  $\beta$ -actin was used as internal control for cytosol proteins.



**Fig. 4.** Adjudin decreases LPS-induced phosphorylation of ERK in BV2 microglia. Cells were pretreated with Adjudin for 1 h and then stimulated with LPS for 1 h. Cell lysates were analyzed by immunoblotting with antibodies specific to phospho-ERK1/2 and ERK1/2 (upper panels), phospho-p38 and p38 (middle panels), and phospho-JNK and JNK (lower panels).



**Fig. 5.** Adjudin attenuates ischemia-induced microglial activation. Immunohistological staining for CD11b in the ischemic cerebral cortex (A) and striatum (B) at 24 h after pMCAO. The blue staining represented DAPI. Scale bar=100  $\mu$ m. Microglial activation in the ischemic cerebral cortex (C) and striatum (D) is quantified by the intensity of CD11b immunofluorescence. Data were mean $\pm$ SEM,  $N=4$  in each group. \* $P<0.05$ .



**Fig. 6.** Adjudin reduces brain edema and neurological deficits after pMCAO. (A) Cresyl violet staining at 24 h after ischemia. White color indicates infarction. Scale bar=1 cm. (B) Infarct volume at 24 h after ischemia. Brain edema (C) and brain water content (D) at 24 h after ischemia. (E) Neurological deficits were assessed at 24 h and 72 h after ischemia. Data were mean $\pm$ SEM,  $N=4-5$  in each group. \* $P<0.05$ .