HSP60 mediates the neuroprotective effects of curcumin by suppressing microglial activation

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Abstract. Curcumin has anti-inflammatory and antioxidant properties and has been widely used to treat or prevent neurodegenerative diseases. However, the mechanisms underlying the neuroprotective effects of curcumin are not well known. In the present study, the effect of curcumin on lipopolysaccharide (LPS)-stimulated BV2 mouse microglia cells was investigated using enzyme-linked immunosorbent assays of the culture medium and western blotting of cell lysates. The results showed that curcumin significantly inhibited the LPS-induced expression and release of heat shock protein 60 (HSP60) in the BV2 cells. The level of heat shock factor (HSF)-1 was upregulated in LPS-activated BV2 microglia, indicating that the increased expression of HSP60 was driven by HSF-1 activation. However, the increased HSF-1 level was downregulated by curcumin. Extracellular HSP60 is a ligand of Toll-like receptor 4 (TLR-4), and the level of the latter was increased in the LPS-activated BV2 microglia and inhibited by curcumin. The activation of TLR-4 is known to be associated with the activation of myeloid differentiation primary response 88 (MyD88) and nuclear factor (NF)-κB, with the subsequent production of proinflammatory and neurotoxic factors. In the present study, curcumin demonstrated marked suppression of the LPS-induced expression of MyD88, NF-κB, caspase-3, inducible nitric oxide synthase, tumor necrosis factor-α, interleukin (IL)-1β and IL-6 in the microglia. These results indicate that curcumin may exert its neuroprotective and anti-inflammatory effects by inhibiting microglial activation through the HSP60/TLR-4/MyD88/NF-κB signaling wpathway. Therefore, curcumin may be useful for the treatment of neurodegenerative diseases that are associated with microglial activation.

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Introduction

A number of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease (AD) and multiple sclerosis are associated with neuroinflammation reactions involving the activation of microglia (1). Microglia cells are the resident macrophages in the central nervous system (CNS), serving as the innate immune system of the CNS (2). Exposure of microglia to lipopolysaccharide (LPS) causes them to become activated, and they contribute to innate and adaptive immune responses by producing pro-inflammatory mediators, including nuclear factor (NF)-κB, caspase-3 and heat shock protein 60 (HSP60) (3-6). Extracellular HSP60 has been shown to increase the quantities of pro-inflammatory factors produced in microglia, causing neuronal death (7,8). Thus, the inhibition of HSP60 production is an effective option for use in the treatment of neurodegenerative disorders.

Curcumin has been the subject of extensive studies, which have revealed a wide range of biological activities, including anti-oxidant, anti-inflammatory, anti-infection and anticarcinogenic activities (9-12). In H_2O_2-treated BV2 microglia and glaucoma models, curcumin was shown to improve cell viability and decrease the intracellular levels of reactive oxygen species and apoptosis significantly, indicating that curcumin afforded neuroprotective effects via the inhibition of oxidative damage (13). Curcumin has also been demonstrated to have a protective effect against HIV-1 gp120-mediated neurotoxicity, mediated via the reduction of microglial inflammation (14). In comparison with other microglial inhibitors, curcumin has several advantages, including its ability to penetrate the blood brain barrier, and few side effects (15). Thus, it may be considered as a new potential therapeutic option for neuroprotection.

Thus far, despite a small number of studies indicating that curcumin is able to attenuate inflammation caused by microglial activation and promote neuronal survival, the mechanisms underlying the modulatory effects of curcumin on microglial activation remain largely unknown. Previous studies have demonstrated that HSP60 is released extracellularly in cardiac

myocytes from rat models of heart failure and induced apoptosis through binding to Toll-like receptor 4 (TLR-4) (16,17). Curcumin has been found to inhibit the TLR-4 signaling pathway in certain injury models (18-20). However, it remains unknown whether curcumin can exert its neuroprotective effects by inhibiting the HSP60/TLR-4 pathway. In the present study, the aim was to investigate whether HSP60 is involved in the neuroprotective effects of curcumin in LPS-induced inflammatory injury in BV2 microglia cells.

Materials and methods

Chemicals. Curcumin and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab181602) and NF-κB (ab31481) were from Abcam (Cambridge, MA, USA). Anti-HSP60 (API-SPA-901) and anti-heat shock factor 1 (HSF-1; ADI-SPA-806) antibodies were from Stressgen (Enzo Life Sciences, Farmingdale, NY, USA). Anti-caspase-3 (cat. no. 9665), anti-inducible nitric oxide synthase (iNOS; cat. no. 2977), TLR-4 (cat. no. 2219) and anti-myeloid differentiation primary response 88 (MyD88; cat. no. 4283) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The proteinase inhibitor cocktails were from Merck Chemicals (Kenilworth, NJ, USA). Interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits were from eBioscience, Inc. (San Diego, CA, USA). Bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) kits were from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc.). All additional chemicals were purchased from ZSGB-Bio (Shanghai, China), unless otherwise stated.

Microglial culture. Mouse BV2 microglia cells (Shanghai Honsun Biological Technology Co., Ltd., Shanghai, China) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37˚C in a humidified incubator under an atmosphere of 95% O_2 and 5% CO_2. Curcumin was dissolved in phosphate-buffered saline (PBS). Following incubation of the cells with LPS (1 μg/ml) for 30 min (LPS group), the LPS + CCM group cells were treated with the indicated concentrations of curcumin for 24 h. Control group cells were treated with an equivalent quantity of dimethyl sulfoxide as a solvent control.

Cell viability assay. A CCK-8 kit was used to evaluate cell viability (BestBio, Shanghai, China). Cells in 100 μl solution at $5x10^4$-$1x10^5$ cells/well were seeded into 96-well microtiter plates and treated with 1-20 μg/ml curcumin. After 24 h, CCK-8 solution was added to each well for 2 h, according to the manufacturer's instructions. Absorbance was determined at 450 nm using a microplate reader.

ELISA. The culture medium was analyzed to detect the levels of IL-6, IL-1β, HSP60 and TNF-α using ELISA kits according to the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader.

Western blot analysis. Cells were washed with PBS three times and lysed with radioimmunoprecipitation assay buffer. The protein concentration was determined with a BCA kit according to the manufacturer's protocol. Equal quantities of protein (10 μg) were loaded and run on sodium dodecyl sulfate/polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% dried milk and incubated with primary antibodies against GAPDH (1:1,000), NF-κB (1:1,000), HSP60 (1:1,000), HSF-1 (1:1,000), caspase-3 (1:2,000), iNOS(1:200), MyD88 (1:1,000) and TLR-4 (1:1,000), in Tris-buffered saline and Tween 20 (TBST) overnight at 4˚C. After being rinsed in milk-TBST, blots were incubated with horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit (ZB-2301; 1:5,000) and anti-mouse IgG (ZB-2305; 1:5,000; ZSGB-BIO, Beijing, China). The target proteins were detected using an ECL detection system and X-ray films. The blotting results were semiquantified using Quantity One software, version 4.6.9 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical differences were determined using one-way analysis of variance testing. $P<0.05$ was considered to indicate a statistically significant difference. Data in the text and figures are presented as mean ± standard error of the mean; n represents the number of experiments.

Results

Curcumin promotes the viability of BV2 microglia. To determine whether curcumin affects the viability of LPS-stimulated BV2 cells, a CCK-8 assay was performed. The results demonstrated that treatment of microglia with 1-20 μg/ml curcumin for ≤24 h significantly increased the viability of LPS-stimulated BV2 cells, compared with that cells treated with LPS alone (data not shown). Treatment of cells with 5 μg/ml curcumin showed the maximal viability among all the concentrations tested; thus 5 μg/ml was chosen for the following experiments.

Curcumin inhibits HSP60 expression and release in LPS-stimulated BV2 microglia. The expression level and release of HSP60 in activated BV2 cells were tested. The western blotting results showed that HSP60 expression was significantly increased following LPS treatment and curcumin significantly inhibited this increase ($P<0.05$; Fig. 1A). HSF-1 has been found to bind with the heat shock element on the HSP60 promoter to regulate HSP60 gene expression (21). Therefore, the HSF-1 level was determined, and the results revealed that it was upregulated by LPS and downregulated by curcumin, indicating that HSP60 expression was promoted by HSF-1 (Fig. 1B). HSP60 has been reported to undergo extracellular translocation when stressed to cause injury (17). ELISA results indicated that HSP60 was released when BV2 was activated and the increased extracellular HSP60 was suppressed by curcumin (Fig. 1C).

Curcumin inhibits the expression of proteins associated with the TLR-4/MyD88/NF-κB signaling pathway. The effects of curcumin on TLR-4, MyD88, NF-κB and caspase-3 expression in LPS-stimulated BV2 microglia were investigated. Curcumin strongly inhibited TLR-4 and MyD88 expression

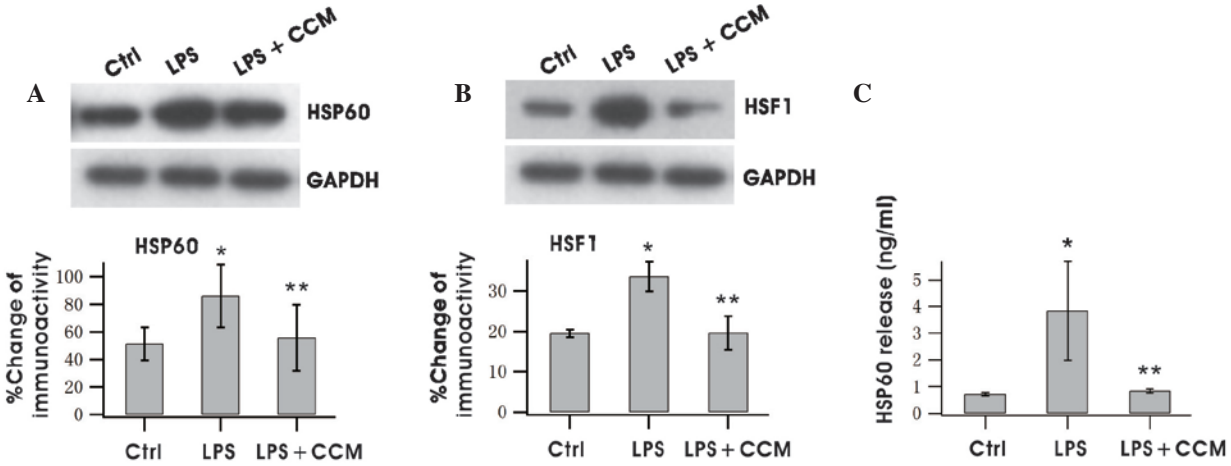

Figure 1. CCM inhibited the LPS-induced increase in the expression and release of HSP60 and HSF-1 expression in BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by incubation with 5 μg/ml CCM for 24 h. The lysates were probed by immunoblotting with antibodies against (A) HSP60 and GAPDH and (B) HSF-1 and GAPDH. The bar graphs shows ratios of the signal intensity of HSP60 or HSF-1 to GAPDH. (C) Extracellular levels of HSP60 were detected by ELISA. Results are the mean ± standard error of three independent experiments performed in triplicate. *P<0.05 vs. the Ctrl group; **P<0.05 vs. the LPS group. HSP60, heat shock protein 60; HSF, heat shock factor; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; Ctrl, control; CCM, curcumin.

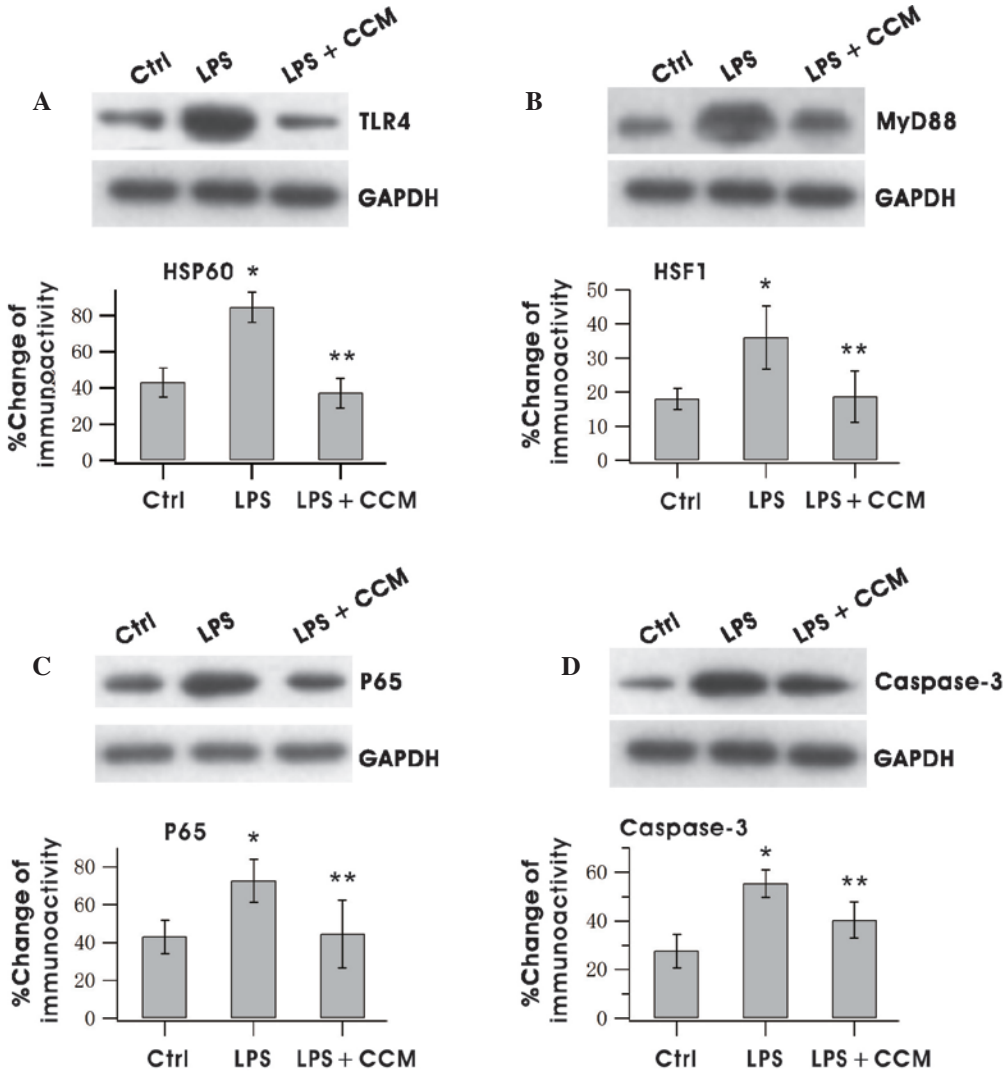

Figure 2. CCM inhibited the increased expression of (A) TLR-4, (B) MyD88, (C) NF-κB (p65) and (D) caspase-3 in LPS-stimulated BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by incubation with 5 μg/ml CCM for 24 h. The lysates were probed by immunoblotting with antibodies against TLR-4, MyD88, NF-κB and caspase-3 and GAPDH individually. Results are the mean ± standard error. Each experiment was derived from at least three independent cultures. *P<0.05 vs. the Ctrl group; **P<0.05 vs. the LPS group. TLR-4, Toll-like receptor 4; MyD88, myeloid differentiation primary response 88; NF, nuclear factor; LPS, lipopolysaccharide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ctrl, control; CCM, curcumin.

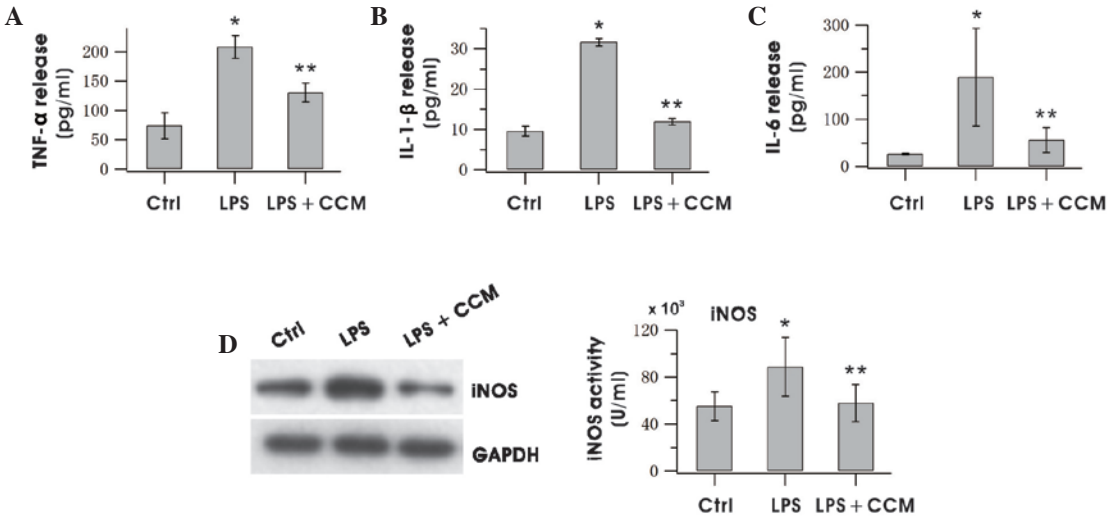

Figure 3. CCM decreased the release of (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) iNOS in LPS-stimulated BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by incubation with 5 μg/ml CCM for 24 h. The expression level of iNOS was assayed by western blotting of BV2 cells and levels of TNF-α, IL-1β and IL-6 in the culture medium were measured using ELISA. The results are the mean ± standard error of three separate experiments performed in triplicate. *P<0.05 vs. the Ctrl; **P<0.05 vs. the LPS group. TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; Ctrl, control; CCM, curcumin.

(Fig. 2A and B). NF-κB regulates the expression of numerous proinflammatory factors. Therefore, the NF-κB level was measured following curcumin treatment and it was found that levels of the p65 subunit of NF-κB increased following LPS treatment, but the LPS-induced increase was markedly attenuated by curcumin (Fig. 2C). Caspase-3 is an important protein of the NF-κB signaling pathway (22). Inhibition of caspase-3 prevents neuronal loss induced by activated microglia in brain diseases (23). Thus, the effects of curcumin on caspase-3 expression were evaluated, and it was observed that caspase-3 expression was suppressed following treatment with curcumin (Fig. 2D).

Curcumin inhibits the production of proinflammatory factors. Whether curcumin could reduce the release of the proinflammatory factors TNF-α, IL-1β and IL-6 in LPS-stimulated BV2 cells was investigated by ELISA. As shown in Fig. 3, treatment of BV2 cells with curcumin for 24 h resulted in marked increases of the aforementioned factors in the culture media as compared with the control. The expression of iNOS decreased notably after curcumin treatment (Fig. 3D). These results indicated that curcumin effectively suppressed the production of neurotoxic factors in over-activated microglia.

Discussion

The present study demonstrates that treatment with 5 μg/ml curcumin effectively inhibits microglial activation. Curcumin reduced the expression of TLR-4, MyD88, NF-κB, caspase-3, HSP60, HSF-1 and iNOS in LPS-activated microglia and effectively suppressed the release of HSP60 and the proinflammatory cytokines TNF-α, IL-6 and IL-1β in LPS-stimulated microglia. To the best of our knowledge, the present study is the first to report that curcumin potentially attenuates microglia activation via the modulation of acute neuroinflammation mediated by the HSP60/TLR-4/MyD88/NF-κB signaling pathway.

When over-activated, microglia secrete a variety of proinflammatory and neurotoxic factors inducing infectious diseases, inflammation and neurodegeneration (24,25). Since microglial activation plays an important role in neurodegeneration, it has been suggested that inhibition of microglial activation, in particular control of the production of neurotoxic factors, may be an effective method for treating neurodegenerative diseases. A number of microglia-targeted pharmacotherapies, including protein kinase C inhibitors, minocycline and naloxone have been suggested to inhibit microglia and promote neuronal survival (26,27). However, the inability to cross the blood-brain barrier and their possible side-effects limit their clinical use.

Curcumin has been used as a spice or pigment by humans for centuries. Curcumin has various benefits, such as a marked safety profile with very low toxicity, numerous pharmacological activities, ability to cross the blood-brain barrier, widespread availability and low cost (15,28). In addition, curcumin is potentially useful for the prevention and treatment of various neuroinflammatory and neurodegenerative conditions of the CNS (29). Curcumin is a promising agent for protecting against AD and HIV-1-assocated neurological disorders (30,31). Studies have demonstrated that curcumin is highly pleiotropic and interacts with numerous molecular targets; it has been shown to inhibit the homodimerization of TLR-4, thereby attenuating inflammatory injury via the TLR-4 pathway (18-20). However, it remains unknown whether HSP60 is involved in the neuroprotective effects of curcumin.

HSP60 is primarily a mitochondrial protein, but upon stress or injury HSP60 is able to translocate to the plasma membrane and be released extracellularly in the failing heart (32). The present study showed that HSP60 was present in the culture medium of LPS-activated BV2 cells, which is consistent with the results in cardiac myocytes. This released HSP60 may be able to act in a paracrine or autocrine manner to activate TLR-4, for which HSP60 has been reported to be a ligand (33). Within the TLR-4 signaling pathway, the MyD88-dependent

pathway is an important activator of NF-κB. The activation of NF-κB is a key event in the inflammatory response caused by microglial activation as NF-κB activation is necessary for the transcription of various proinflammatory molecules (34,35). Caspase-3 is an important mediator of cell apoptosis (22). When caspase 3 or 7 is inhibited, LPS-stimulated microglia have been shown to be non-toxic to neighboring neurons (23). The activation of NF-κB by caspase-3 is critical in inflammation. HSP60 gene expression is regulated by the transcription factor HSF-1 binding to the HSP60 gene promoter. NF-κB has also been shown to induce the transcription of the HSP60 stress protein gene, which elicits a potent proinflammatory response in innate immune cells (32). Thus, the effects of curcumin on HSP60, TLR-4, MyD88, NF-κB and caspase-3 expression levels were investigated in the present study. The results demonstrate that curcumin treatment markedly attenuates HSP60, TLR-4, MyD88, caspase-3 and the NF-κB downstream mediator p65, suggesting that the anti-inflammatory effects of curcumin may occur via inhibition of the HSP60/TLR-4 signaling pathway.

Microglia activation is known to produce a number of proinflammatory cytokines. This was confirmed in the present study by measuring iNOS protein expression in BV2 cells and the levels of TNF-α, IL-1β and IL-6 in the culture medium of LPS-stimulated BV2 cells. The iNOS gene is under the transcriptional control of a variety of inflammatory mediators, such as cytokines and LPS (36). TNF-α is a mediator of NF-κB signaling and triggers increased HSP60 expression, which has is reversed by p65 inhibition (15). However, following curcumin treatment, the levels of TNF-α, IL-1β, IL-6 and iNOS were clearly inhibited.

This study indicates that HSP60 may participate in the neuroprotective effects of curcumin via inhibition of the NF-κB signaling pathway to prevent over-activation of microglia.

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