

Ellagic Acid Protects against Activation of Microglia by Inhibiting MAPKs and NF- κ B Signalling

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ABSTRACT

Context: One of the hallmarks of neurodegenerative diseases is dysregulation of microglia resulting in neuroinflammation. Neuroinflammation is related to multiple neurodegenerative disorders, including stroke and Alzheimer's disease. Ellagic acid (EA) possesses antiapoptotic activity, anti-inflammatory, and antioxidant properties. It is most commonly found in fruits and nuts. EA has been linked to neuronal protection particularly through its anti-inflammatory and antioxidant actions. For example, EA inhibits enzyme activity which prevents the onset of Alzheimer's disease. **Aim:** In this study, the underlying protective mechanisms of EA in LPS-activated BV2 cells were investigated. **Settings and Design:** EA prepared in multiple concentrations was introduced for 24 hrs as pre-treatment in BV2 microglial cells. Lipopolysaccharide (LPS) at $1 \mu\text{g mL}^{-1}$ was used to stimulate BV2 microglial cells after the EA pre-treatment for an additional 24 hrs. After 24 hrs of stimulation, the cell lysate was harvested for bioassays. **Materials and Methods:** This study investigated the effects of EA on the viability of BV2 microglial cells using MTT assay. After LPS stimulation, the cell lysate was collected to measure nitric oxide (NO) levels using Griess assay kit and ELISA was used to measure TNF α levels. Western blotting was used to test protein levels of iNOS, MyD88, MAPKs and NF- κ B. **Statistical analysis used:** Data are provided as means and standard errors of $n = 3$. A one-way analysis of variance was used to test the statistical significance of between group comparisons. The statistical significance of $p < 0.05$ was set. **Results:** EA decreased the levels of TNF α and NO production in BV2 microglial cells induced by LPS. EA also reduced inducible nitric oxide synthase (iNOS) expression, and attenuated MyD88 and NF- κ B expression. EA decreased the phosphorylation of p38, ERK and JNK and repressed the activation of CD11b and CD40 in a concentration-dependent manner. **Conclusion:** EA has a putative role in preventing neuroinflammation but its application in prevention of neurodegenerative diseases requires further investigations.

Key words: Neuroinflammation, Anti-inflammation, Ellagic acid, Microglia, NF- κ B, MAPKs.

Key Messages: The study indicated that ellagic acid may exert its anti-neuroinflammatory effects by inhibiting the development of MyD88, MAPKs and NF- κ B signalling molecules.

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INTRODUCTION

Altered inflammatory responses may ensue excessive damage to affected brain cells and will potentially lead to cell death and neurodegeneration.¹ Microglia dysregulation has been linked to multiple neurodegenerative diseases such as dementia,² amyotrophic lateral sclerosis,³ Parkinson's disease and Alzheimer's disease.⁴ As a central nervous system (CNS) resident cell, microglia control the central nervous tissue immune homeostasis.⁵ Microglia has been implicated in the role of both



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causative and exacerbation in many neurodegenerative diseases and over-activation of microglia contributes to neuroinflammation.⁶ Chronically activated microglia due to brain injury, infections and inflammation, initiates a rapid inflammatory response that activates the development and release of massive amounts of pro-inflammatory cytokines (TNF α , IL-1 β) and neurotoxic mediators (NO, COX-2), while increasing neurodegeneration and neuronal death.⁷

There is an increasing awareness that inflammation is involved in the initial outcome of neurodegenerative disorders and in fact, inflammation-mediated neurodegeneration and microglia have also been implicated in other diseases and conditions such as stroke,⁸ hypoxia⁹ and neuropathic pain.¹⁰ Therefore, neuroinflammation has attracted attention for neuroprotective therapies in neurodegenerative disorders, particularly through inhibition of microglial activation.¹¹ Bacterial component lipopolysaccharide (LPS) causes microglia to secrete various inflammatory molecules including tumour necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , nitric oxide (NO) and inducible nitric oxide synthase (iNOS) to promote inflammatory reactions.¹² The inhibition of these mediators may thus also be a beneficial approach to neuroinflammatory prevention.

Experimental and epidemiological research is currently focused on the inhibitory impact of plant polyphenols on many peripheral inflammations including gastrointestinal diseases. Polyphenols can be used as additional anti-inflammatory agents or to complement non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of neuroinflammation. Ellagic acid (Figure 1) is a polyphenol typically found in berry and pomegranate fruits, vegetables and nuts.¹³ Various *in vivo* and *in vitro* studies have also shown that ellagic acid possesses pharmacological properties including anti-coagulant,¹⁴ anti-inflammatory,¹³⁻¹⁵ anti-haemorrhagic¹⁶ and anti-tumour effects.¹⁷ In this study, the neuroprotective effect of ellagic acid on LPS-mediated inflammatory response in murine BV2 microglial cells was investigated by determining the inhibition of NF- κ B and MAPK signalling molecules.

MATERIALS AND METHODS

Cell culture

The immortalized murine BV2 microglial cells were a gift from Associate Professor Dr. Sharmili Vidyadaran of the Immunology Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Cells were maintained in Dulbecco's modified Eagle's medium

high glucose (Gibco, Grand Island, NY) complemented by foetal bovine serum (10%; Nacalai, Japan), penicillin (100 U mL⁻¹) and streptomycin 100 μ g mL⁻¹) at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Confluent cultures were passaged by Accutase™ (Nacalai, Japan). Cell density was determined according to respective culture plates: 2.5 x 10⁴ cells/cm² on 96-well, 5 x 10⁴ cells/cm² on 24-well (NO measurement and ELISA) and 1 x 10⁶ cells/cm² on 24-well plates (flow cytometry and Western blot).

Preparation of EA

The lyophilised EA was purchased from Cayman Chemical, USA. EA powder was first weighed and dissolved in 100% DMSO (stock 1), sonicated for 10 mins and kept in -80°C. A second stock from stock 1 (now labelled as stock 2) was diluted in PBS, sonicated and kept in -20°C. Upon addition to the cells, stock 2 was dissolved in the culture medium accordingly to get the final concentration in the cell cultures.

Preparation of LPS

LPS was purchased from Sigma-Aldrich, St Louise, MT (strain from *Escherichia coli* 026: B6). A 1 μ g mL⁻¹ of lyophilised LPS was weighed and dissolved in PBS, sonicated and kept in -20°C. Upon addition to cells, the LPS stock solution was dissolved in the culture medium accordingly to get the final concentration in the cell cultures.

Treatment of cells

Cells were pre-treated with EA (0.5, 1, 5 and 10 μ M) for 24 hrs, then stimulated with LPS for another 24 hrs. Cell supernatant/lysate was collected after 24 hrs.

Cell viability assay

After cell treatment, culture media was discarded and MTT (0.5 mg mL⁻¹; Nacalai, Japan) was applied for 2 hrs incubated at 37°C. After discarding the cell supernatant, dimethyl sulfoxide (Sigma-Aldrich) was added to the wells. The developed crystals were mixed to dissolve and the plates were read on an automated absorbance reader, NanoQuant (Tecan Trading AG, Switzerland), using a 570 nm and a reference of 630 nm wavelength.

Nitrite measurement

Secreted NO amounts were measured using the Griess reagent (Sigma-Aldrich). Supernatants of the treated cells were mixed with Griess reagent (of equal volume) in a 96-well plate and allowed to stand at room temperature for 10 min. Nitrite concentrations were determined following instructions by manufacturer at a wavelength of 540 nm.

Enzyme-linked immunosorbent assay

Secreted TNF- α levels were measured in the culture medium by commercial ELISA kits (R&D systems, UK) according to the manufacturer's instructions.

Immunophenotyping analysis

CD11b and CD40 marker expressions in BV2 microglial cells induced by LPS were analysed by flow cytometry as described by manufacturer's protocol. The supernatants were discarded after treatment and the cells were washed with warm PBS. Cells were detached using Accutase™ and centrifuged at 300 x g for 5 min at 4°C. After centrifugation, cell pellets were rinsed twice with ice-cold PBS and resuspended (10^4 cells) before being distributed in micro-centrifuge tubes at 100 μ L each. Blocking was done using 1% BSA in PBS and incubated at room temperature for 10 min. Subsequently, CD11b (FITC-stained) and CD40 (APC-stained) and their isotype control antibodies were added to respective tubes and incubated in the dark for 20 min on ice. The cells were rinsed with ice-cold PBS and centrifuged twice for 10 min. On the final wash, 0.5 mL PBS was added to the cell pellets and kept on ice in the dark. A minimum of 10,000 events of the gated population was acquired from forward and side scatter plots. Dead cells or debris were identified based on lower forward and side scatter signals and eliminated during FACS analysis. Data were analysed using FACS Diva software v6 (BD FACS Canto II, USA).

Western blotting analysis

After cell treatment, cells were washed with PBS. Cells were dissolved with RIPA buffer + protease inhibitor cocktail. Total protein was measured using Bradford assay kit (Bio-Rad). 20 μ g of total protein was fractionated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Blocking of membranes was carried out for 1 hr with skimmed milk (1%) in TBST, then immunoblotted with conjugated antibodies against MyD88, NF- κ B p65, phospho-p38 (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p44/42 (Erk1/2) (Thr202/Tyr204) [PVDF membrane; diluted 1:1000 in BSA (5% w/v), TBS (1X), Tween-20 (1%)], iNOS [NC membrane; dilution 1:700 in BSA (5% w/v), TBS (1X), Tween-20 (0.1%)], β -actin [dilution 1:3000 in BSA (5% w/v), TBS (1X), Tween-20 (0.1%)] and IgG (anti-rabbit)-HRP [1:3000 dilution in skim milk (5% w/v), TBS (1X), Tween-20 (0.1%)]. ECL substrate kit, Western Bright™ enhanced chemiluminescent (Advansta Corporation, San Jose, CA, USA) was used to visualize the immunoblotted protein

bands complying with manufacturer's instructions. Protein expression was measured by using Image Lab™ software v5.2.1 (Gel Doc™ XR+, Bio-Rad, CA, USA). All antibodies were from Cell Signalling Technology, MA, USA.

Statistical Analysis

Data were expressed as mean and standard error of mean (SEM) obtained from repeated experiments. All experiments were repeated thrice with $n = 3$. Group differences were statistically assessed by one-way analysis of variance (ANOVA) and Dunnett's test was applied as a *post hoc* test. Statistical significant were considered if $p < 0.05$. Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Cell viability

To show that EA was not toxic to BV2 microglial cells, MTT was applied to measure the cell viability following treatment with EA and LPS. Figure 1a depicts a no toxic effect of EA at concentrations of 0.5 to 10 μ M.

Ellagic acid attenuated NO production and iNOS protein expression

iNOS is an inflammation-related enzyme needed in NO production. It was found that EA significantly ($P < 0.001$) attenuated NO development dose-dependently in LPS-induced BV2 cells (Figure 1b). Furthermore, using Western blot, the study found that EA (5 and 10 μ M) significantly reduced iNOS in comparison to LPS alone (Figure 1c).

Ellagic acid inhibited LPS-induced production of TNF α

TNF α is a pro-inflammatory cytokine. The study investigated the effects of LPS stimulation on the activation of BV2 cells and whether EA affected the production of TNF α . Figure 2 shows that EA inhibited TNF α production dose-dependently in LPS-stimulated BV2 cells as determined by ELISA.

Ellagic acid inhibited NF- κ B signalling pathway via MyD88

Activation of MyD88 and NF- κ B are involved in controlling inflammatory response and modulate the development of pro-inflammatory cytokines.¹⁸ The investigation into the effects of EA on NF- κ B inflammatory pathway via MyD88 in LPS-stimulated BV2 microglial cells revealed in Figure 3 where LPS-induced NF- κ B p65 via MyD88 was significantly ($p < 0.001$) decreased by EA in a concentration dependent manner as determined by Western blot.

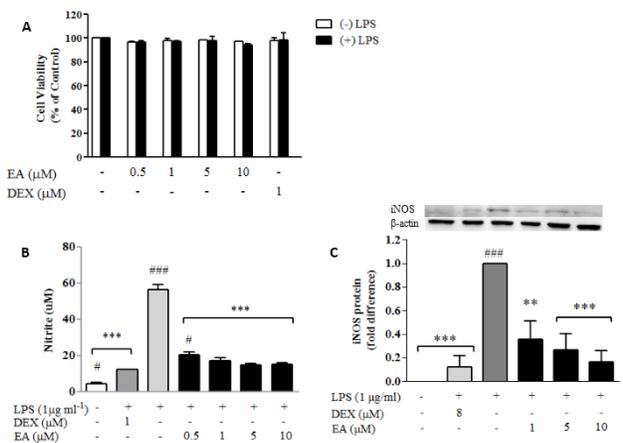


Figure 1: Effects of ellagic acid (EA) on cell viability, production of NO and iNOS protein expression on LPS-induced BV2 cells. Cultures were pre-treated with various doses of EA for 24 hr and then stimulated with LPS (1 µg mL⁻¹) for 24 hr. (a) MTT assay. (b) Griess reaction. (c) Western blot. Blots are representative blots. The relative ratio of (c) iNOS/βactin is shown. Data represent mean ± SEM of three independent experiment (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs dexamethasone (DEX) #: $p < 0.05$; ##: $p < 0.01$ and ###: $p < 0.001$).

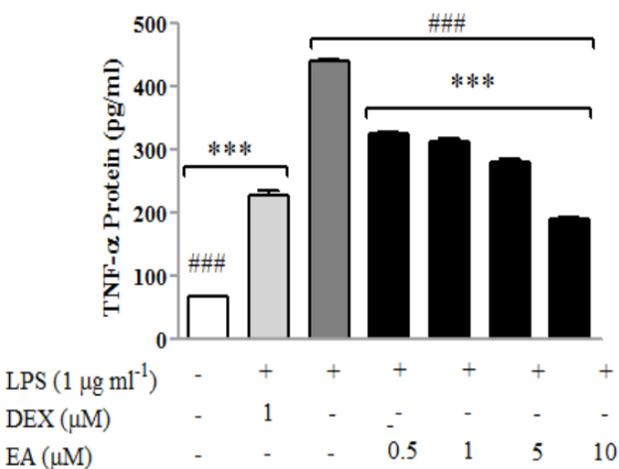


Figure 2: Effect of ellagic acid (EA) on the production of TNFα by LPS activated BV2 cells. Cultures were pre-treated with EA for 24 hr, followed by LPS stimulation for 24 hrs. The TNFα expression in the culture supernatant was analysed using ELISA. The expression of TNFα in the cell culture supernatant was analysed using ELISA. Data represented mean ± SEM of three independent experiments. One-way ANOVA and Dunnett's multiple comparison's test were used to analysed data (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs dexamethasone (DEX) #: $p < 0.05$; ##: $p < 0.01$ and ###: $p < 0.001$).

Ellagic acid suppresses the phosphorylation of MAPKs

MAPKs (P38 MAPK, JNK and ERK) phosphorylate various range transcription factors which modulate and regulate inflammatory response by producing pro-inflammatory mediators and cytokines.¹⁹ Figure

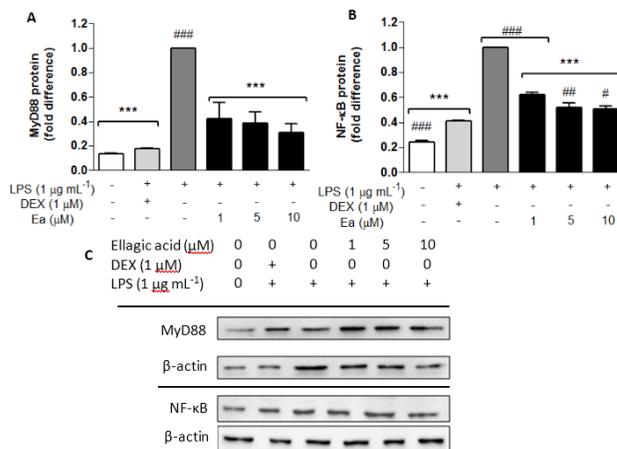


Figure 3: Effect of ellagic acid (EA) on NF-κB via MyD88 signalling pathway by LPS-activated BV2 cells. Cultures were pre-treated with EA for 24 hr, followed by LPS (1 µg mL⁻¹) for 24 hr. a, b Quantification was performed on three independent experiments and are presented as mean ± SEM. c Western blot analysis of MyD88 and NF-κB. Blots are representative of a and b. One-way ANOVA and Dunnett's multiple comparison's test were used to analysed data (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs dexamethasone (DEX) #: $p < 0.05$; ##: $p < 0.01$ and ###: $p < 0.001$).

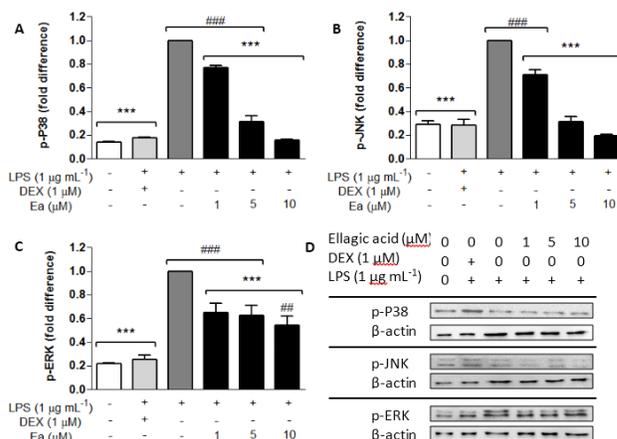


Figure 4: Effects of ellagic acid (EA) on MAPK signalling pathway by LPS-activated BV2 cells. Cells were incubated with various concentration of EA for 24 hr and then treated with LPS (1 µg mL⁻¹) for 24 hr. a, b, c Quantitation was performed on three independent experiments and are presented as mean ± SEM. One-way ANOVA and Dunnett's multiple comparison's test were used to analysed data. EA *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs dexamethasone (DEX) #: $p < 0.05$; ##: $p < 0.01$ and ###: $p < 0.001$. d Western blot analysis of phospho-P38, phospho-JNK and phospho-ERK. Blots are representative of a, b and c.

4 indicates a large increase in P38 MAPK, ERK and JNK phosphorylation following stimulation by LPS. Pre-treatment with EA significantly reduced ($p < 0.001$) phosphorylation of P38 MAPK, ERK and JNK at 1 µM, 5 µM and 10 µM concentrations (Figure 4a, b, c, respectively).

Ellagic acid modulates microglial inflammatory phenotype

Upon LPS stimulation, a characteristic of inflammatory of microglial phenotype was seen on the cell surface where costimulatory molecules (CD40) and key adhesion (CD11b) were expressed. LPS-induced BV2 microglial cells significantly increased surface expression of CD40 and CD11b (Figure 5a and b). Pre-treatment with EA at various concentrations (1, 5 and 10 μM) significantly reduced ($p < 0.001$) CD11b by 50% (Figure 5a) which was seen across all three concentrations, whereas the reduction of CD40 was also significant ($p < 0.001$) at EA concentrations of 5 μM and 10 μM .

DISCUSSION AND CONCLUSION

Polyphenol compounds such as ellagic acid (EA) are found abundantly in nuts and fruits such as raspberries and pomegranates.¹³ To the best of our knowledge, this is the first evidence indicating the protective effects of EA in suppressing LPS-stimulated acute neuroinflammation in murine BV2 microglia.

Pre-treatment of EA (0.5 – 10 $\mu\text{g mL}^{-1}$) significantly diminished neuroinflammation induced by LPS in a dose-

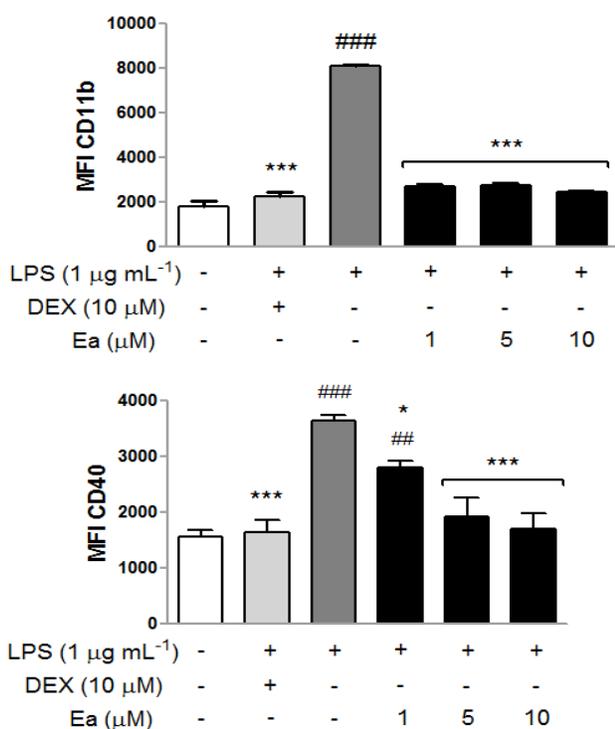


Figure 5: Ellagic acid (EA) modulates inflammatory cell surface marker expression in LPS-treated BV2 microglia. Flow cytometric analysis of EA-treated BV2 cells following LPS activation. a and b quantitation of CD11b and CD40 expression by median fluorescence intensity (MFI) from three independent experiments. Data are expressed as mean \pm SEM where EA (* $p < 0.05$) vs dexamethasone (DEX) (# $p < 0.05$).

dependent manner. The protective capacity of EA as revealed in this study has attenuated neuroinflammation in the extend of reduction of murine microglial activity which was accompanied by a decrease in the cellular network of inflammatory signalling and a decrease in the relative mediator/pro-inflammatory cytokines.

This study was aimed to examine LPS-mediated activation of BV2 cells in relevance to the putative protective effects of EA. This work included the use of murine BV2 microglial cells and triggered an inflammatory reaction to LPS. Previous studies have shown that LPS-activated microglia secrete various pro-inflammatory mediators and cytokines.²⁰ In response to LPS stimuli, activated BV2 microglia promote the production of NO and TNF α . Such neuroinflammatory symptoms cause neuronal damage and lead to neurodegenerative diseases.²¹ There is evidence that iNOS/NO production differs from single-cell culture in astrocyte-microglial mixed culture conditions.²² Numerous observations have also been made on the possible effects of NO production in brain cells, especially in co-culture conditions such as astrocytes and microglia.²³ In this analysis, the up-regulation of iNOS by LPS stimulation has been shown to lead to the development of NO and has the potential to increase NF- κ B activation as demonstrated in a report by McAdam and co-workers.²⁴ Although our study did not correlate between iNOS/NO and NF- κ B activation, we demonstrated that EA pre-treatment decreased iNOS/NO and NF- κ B p65 subunits in BV2 microglial cells mediated by LPS. In addition, the NO produced also activated not only NF- κ B but also MAPKs.²⁵ Our investigation on the protective effects of EA was continued with determining the inflammatory factors produced in activated BV2 cells mediated by LPS. The results showed that EA pre-treatment significantly inhibited microglial activation (CD11b and CD40) and the development of TNF α stimulated by LPS. These results demonstrate the potential therapeutic effects of EA in various inflammatory reactions.

The increased expression of CD11b is an indicator of microglial activation. According to Roy and colleagues²⁶ the expression of CD11b starts after NO development and the signalling mechanisms for CD11b expression in LPS-induced microglia are not fully understood. In this analysis, EA dose-dependently decreased CD11b signals.

CD40 expression in microglial cells protects the CNS from invasion and retention of inflammatory leukocytes.²⁷ CD40 and microglial activation are involved in various CNS diseases such as multiple sclerosis and Alzheimer's disease and therefore, we

were specifically investigating the protective role of EA on CD40 expression in LPS-mediated BV2 activation. According to Qin and colleagues, CD40 expression upon LPS activation in BV2 microglial cells is an intricate process requiring endogenous production of IFN- β and STAT-1 α activation. Also, LPS induction of CD40 expression involves NF- κ B elements in the CD40 promoter.²⁸ Therefore the results of our present study are in agreement with Qin and colleagues in which LPS activated NF- κ B/p65 subunit contributing to the binding of CD40 promoter which eventually activated the CD40 expression on the surface of microglial cells.

Dysregulated activation of microglial cells can trigger a phenotype shift leading to disruption of signalling pathways and inflammatory process,¹³ chronic mediator/pro-inflammatory cytokine secretion and neuronal injury/death seen in neurodegenerative diseases.²⁹ The three inflammatory signalling pathways include MAPKs, P13K/Akt and NK- κ B.³⁰ In mammalian cells, MAPKs consist of extracellular signal protein (p42/44; ERK), the p38 MAPK and the JNK³¹ which are expressed in many tissues, including CNS.³² The activation of intracellular signalling includes the phosphorylation of these MAPKs. There is currently, insufficient knowledge on the expression of MAPK in the brain due to LPS or bacterial infection.³² Our findings showed that LPS increased the phosphorylated p38, JNK and ERK1/2 in the control group after 72 hrs and this phosphorylation was significantly reduced by EA pre-treatment in BV2 microglial cells. Limited findings have shown that phosphorylation of p38 MAPK, ERK1/2 and JNK occurred in response to LPS in cells pre-treated with corylin³³ and benfotamine,³⁴ thus MAPKs signalling may be involved in TNF α production in microglia.

Transcription factor NF- κ B omnipresently regulates the gene expression of a broad variety of neuropathological processes, including hypoxia, neuroinflammation and trauma.³⁵ A more important function for fully activated NF- κ B was also played by the subunit NF- κ B/p65 and our analysis showed that LPS induced NF- κ B/p65 phosphorylation after 72 hrs. Pre-treatment with EA not only decreased the NF- κ B/p65 levels but also the downstream pro-inflammatory cytokine TNF α levels. Previous study found that inhibition of MAPKs decreased the upregulation of NF- κ B/p65 following treatment with LPS, involving the downstream molecules of NF- κ B and MAPKs and there was a cross link between them.³²

This work thus identified the effects of EA in LPS-induced expression of MyD88 in BV2 cells. The adaptor protein MyD88 mediates the signals for most TLRs leading to activation of MAPKs and NF- κ B, which

results in the formation of inflammatory mediators.²⁵ The results in this study demonstrated that EA blocked MyD88 expression induced by LPS suggestive of an anti-neuroinflammatory effect of EA by modulating the interaction of MyD88 with its downstream counterparts. Consequently, these results also supported the putative role of EA in preventing neuroinflammation but its application in prevention of neurodegenerative diseases requires further investigations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

Akt: Protein kinase B; **APC:** Allophycocyanin; **BSA:** Bovine serum albumin; **CD:** Cluster of difference; **CNS:** Central nervous system; **CO₂:** Carbon dioxide; **COX-2:** Cyclooxygenase-2; **DMSO:** dimethyl sulfoxide; **EA:** Ellagic acid; **ERK:** Extracellular signal-regulated kinases; **FITC:** Fluorescein-5-isothiocyanate; **hrs:** hour; **HRP:** Horse radish peroxidase; **IL:** Interleukin; **iNOS:** Inducible nitric oxide synthase; **JNK:** C-JUN N-terminal kinases; **LPS:** Lipopolysaccharide; **MAPKs:** Mitogen-activated protein kinase; **mg mL⁻¹:** Miligram per millilitre; **min:** Minute; **mL:** Mililitre; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **MyD88:** Myeloid differentiation primary response 88; **NC:** Nitrocellulose; **NF- κ B:** Nuclear factor kappa B; **nm:** Nanometre; **NO:** Nitric oxide; **NSAIDs:** Non-steroidal anti-inflammatory drugs; **P13K:** phosphatidylinositol 3-kinase; **p38:** P38 mitogen-activated protein kinases; **PBS:** Phosphor buffer saline; **PVDF:** Polivinylidenedifluoride; **RT:** Room temperature; **TBS:** Tris buffered saline; **TBST:** tris buffered saline with tween-20; **TNF α :** Tumour necrosis factor alpha; **U mL⁻¹:** μ g mL⁻¹; **μ M:** micromolar.

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PICTORIAL ABSTRACT

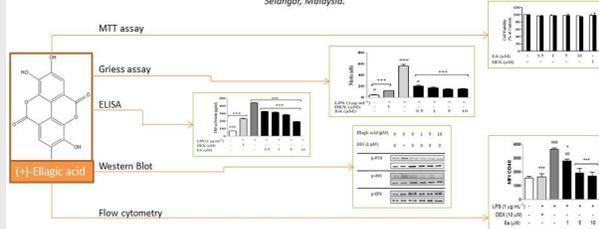
Ellagic Acid Suppresses Lipopolysaccharide-Induced Microglial Activation via Inhibition of NF- κ B and MAPKs Signalling in BV2 Cells

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SUMMARY

Dysregulated activation of microglia is one of the hallmarks of neurodegenerative diseases that results in neuroinflammation. Neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease have been associated with neuroinflammation. The present study proposed a protective role of ellagic acid (EA) against neuroinflammation studied using LPS-activated BV2 microglial cells. The results provide an insight into the mechanisms of EA in preventing activation of microglia mediated through attenuation of inflammatory signalling molecules such as MAPKs and NF- κ B. However, the putative role of EA in neuroprotection and its application in prevention of neurodegenerative diseases require further investigations.

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