RESEARCH ARTICLE

Inhibition of TNFα-induced interleukin-6 gene expression by barley (*Hordeum vulgare*) ethanol extract in BV-2 microglia

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Abstract

Background Inflammation in the central nervous system is closely associated with pathological neurodegenerative diseases as well as psychiatric disorders. Prolonged activation of microglia can produce many inflammatory mediators, which may result in pathological neurotoxic side effects. Interleukin (IL)-6 serves as a hallmark of the injured brain.

Objective Whole grains are known to contain many bioactive components. However, little information is available about anti-neuroinflammatory effects of grains in the CNS. This study aims to investigate the effect of *Hordeum vulgare* ethanol extract (HVE) on the suppression of IL-6 expression in BV2 microglia.

Methods Inhibitory effects of HVE on IL-6 expression were analyzed by immunoblot anaysis, immunofluoresce microscopic analysis, reverse transcription-polymerase chain reaction, and luciferase promoter reporter assay.

Results HVE inhibited TNFα-induced phosphorylation of IKKα/β, IκB, and p65/RelA NF-κB. TNFα-induced IL-6 mRNA expression and promoter activity were reduced by HVE. Point mutation of NF-κB-binding site within the IL-6 gene promoter abolished TNFα-induced reporter activity, whereas exogenous expression of p65 NF-κB enhanced IL-6 promoter activity.

Conclusion NF-κB-binding site within the IL-6 promoter region is a HVE target element involved in the inhibition of TNFα-induced IL-6 gene transcription. HVE inhibits TNFα-induced IL-6 expression via suppression of NF-κB signaling in BV2 microglial cells.

Keywords *Hordeum vulgare* · Interleukin-6 · Microglia · Neuroinflammation · NF-κB · TNFα

Introduction

Tissue damage or pathogenic challenges to the central nervous system (CNS) may cause neuroinflammation. A growing body of evidence postulates a role for long-term release of pro-inflammatory cytokines in the pathogenesis of neurodegenerative diseases such as Alzheimer's, amyotrophic lateral sclerosis, Parkinson's, and Huntington's diseases (Eikelenboom et al. 2002; Kim et al. 2016; Nimmo and Vink 2009), as well as psychiatric disorders such as schizophrenia and depression (Dobos et al. 2010; Goldsmith et al. 2016; Minghetti 2005; Singhal and Baune 2017). Microglia are resident mononuclear phagocytes derived from the monocyte/macrophage lineage and are distributed throughout the brain and spinal cord (Frost and Schafer 2016). They are rapidly activated by the presence of pathogens and damaged cellular debris and thus provide the first line of defense by mediating innate immune responses in the CNS (Rivest 2009). Microglia are also involved in neuroprotective functions by releasing various neurotrophic factors (Frost and Schafer 2016); however, prolonged activation of microglia can produce a variety of inflammatory mediators, which may result in pathological neurotoxic side effects (Becher et al. 2017; Singhal and Baune 2017). Accordingly, the modulation of neuroinflammatory cytokine production in microglia

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may serve to prevent or attenuate the progression of inflammation-mediated neurodegenerative diseases (Peña-Altamira et al. 2016).

Cereal grains are a rich source of a large variety of bioactive compounds including phytochemicals such as phenolics, carotenoids, vitamins, γ-oryzanol, dietary fiber, and β-glucan (Okarter and Liu 2010; Singh and Sharma 2017). Epidemiological studies have reported that whole grain consumption is closely associated with reduced risk of cardiovascular disease, type II diabetes, obesity, and some cancers including colorectal, pancreatic, and breast cancers, as well as inflammatory diseases (Okarter and Liu 2010; Singh and Sharma 2017). However, little information is available on the effects of cereal grains on anti-neuroinflammatory effects in the CNS.

In this study, the anti-inflammatory potential of various grain ethanol extracts was assessed and it was found that *Hordeum vulgare* ethanol extract (HVE) inhibits tumor necrosis factor alpha (TNFα)-induced interleukin (IL)-6 gene promoter activity via suppression of NF-κB signaling in BV2 microglial cells.

Materials and methods

Materials

Tumor necrosis factor alpha (TNFα) was obtained from PeproTech (Rocky Hill, NJ, USA). Antibodies to phospho-p65/RelA NF-κB (Ser563), phospho-IKKα/β (Ser176/180), and phospho-IκBα (Ser32) were purchased from Cell Signaling Technology (Beverly, MA, USA), while antibodies against β-tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488- and Alexa Fluor 555-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA).

Preparation of ethanol extracts from cereal grains

Cereal grains, including Adlay grain (*Coix lacrymajobi*), Hog millet grain (*Panicum miliaceum*), Corn (*Zea mays*), Glutinous foxtail millet grain (*Setaria italica*), Sorghum grain (*Sorghum bicolor*), Barley grain (*Hordeum vulgare*), Oat grain (*Avena sativa*), Wheat grain (*Triticum aestivum*), Buckwheat (*Fagopyrum esculentum*), Rye grain (*Secale cereale*), were dried in the shade and then ground with a grinder. The ground cereal flour was then immersed in ethanol (1 L/kg) for 3 days. After filtering, grain extracts were dried with a rotatory evaporator and lyophilized in a vacuum concentrator. The lyophilized cereal extracts were then dissolved in dimethyl sulfoxide (DMSO).

Cell culture

BV2 rat microglial cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (CellGro/Corning, Manassas, VA, USA) in a humidified 5% CO_2 atmosphere at 37 °C.

Immunoblotting

Immunoblot analysis was performed as described previously (Shin et al. 2017). Chemiluminescence was visualized on X-ray film using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Protein band intensities were quantified using ImageJ version 1.52a software (National Institute of Health, Bethesda, MD, USA), and expressed as a relative ratio to GAPDH.

Cytotoxicity assay

The cytotoxicity of HVE was examined using a methylthiazole tetrazolium-based Cell Count Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, exponentially growing BV2 microglial cells were treated with HVE (0, 25, 50, 100, or 200 μg/mL) for 24 and 48 h, after which the CCK-8 solution was added to the cultures for an additional hour. The resulting absorbance at 450 nm was measured with an Emax Endpoint ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Nuclear localization of NF-κB

The localization of NF-κB was examined as described previously (Jeon et al. 2018). Briefly, BV2 microglial cells seeded on coverslips were pretreated with 100 μg/mL HVE for 30 min before stimulation with 20 ng/mL TNFα for 10 min. Cells were incubated with antibodies against β-tubulin (1:200) and phospho-p65 NF-κB (S536; 1:100) for 18 h, after which Alexa Fluor 488- (green signal for β-tubulin) and Alexa Fluor 555-conjugated (red signal for phospho-p65) secondary antibodies were added for an additional 50 min. Fluorescence images were captured with an EVOSf1® fluorescence microscope (Advance Microscopy Group, Bothell, WA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (Q-PCR)

RT-PCR and Q-PCR were carried out as described previously (Shin et al. 2016). RT-PCR primers were synthesized by Macrogen (Seoul, Korea): GAPDH forward, 5′-ACCCACTCCTCCACCTTTG-3′; GAPDH reverse, 5′-CCCAGCAAGAGCACAAGAG-3′; IL-6 forward, 5′-TTCCATCCAGTTGCCTTCTTGG-3′; IL-6 reverse, 5′-TTCTGCAAGTGCATCATCG-3′. The amplified products were subjected to 1% agarose gel electrophoresis. For quantitative real-time PCR, the TaqMan-iQ supermix kit (Bio-Rad) was used with the Bio-Rad iCycler iQ thermal cycler according to the manufacturer's instructions. The sequences of Q-PCR primers were: IL-6 TaqMan probe, 5′-FAM-TTGCCTTCTTGGGACTGATGCT-BHQ-3′; GAPDH TaqMan probe, 5′-Yakima Yellow TM-GTATCT CTCTGAAGGACTCTGG-BHQ-1-3′. Expression values were normalized to GAPDH mRNA using the software provided by the manufacturer.

Construction and mutagenesis of IL-6 promoter reporters

Amplification of the 5′-flanking region of the IL-6 gene was performed by PCR using high-fidelity Labopass™ *SP-Taq* DNA polymerase (COSMO Genetech Co., Seoul, Korea) and mouse genomic DNA (Promega, Madison, WI, USA). The transcription start site was numbered +1. PCR products were obtained with forward primer 5′-GAG GTCCTTCTTCGATATCTT-3′ (−956/−936) and reverse primer 5′-TGGGCTCCAGAGCAGAAT-3′ (+30/+47) of the IL-6 gene and were subcloned into the pGL4.17_basic vector (Promega), yielding pIL6-Luc(−956/+47). A serial deletion construct was generated by PCR using forward primers 5′-CATCTGTAGATCCTTACAGAC-3′ (forward, −469/−449), and 5′-AGCACACTTTCCCCTTCC-3′ (forward, −182/−175) with (+30/+47) reverse primer, yielding pIL6-Luc(−469/+47), and pIL6-Luc(−182/+47), respectively. A point mutation for the NF-κB-binding sequence (GTGGGATTTTCCCAT to GTGGGATTTTAGAC) at −46/−32 in the pIL6-Luc(−182/+47) was generated using a site-directed mutagenesis kit (Engynomics, Daejeon, Korea), yielding pIL6-Luc(−182/+47)mtNFκB. All resulting constructs were verified by DNA sequencing and restriction enzyme digestion.

IL-6 promoter reporter assay

BV2 microglial cells seeded onto 12-well plates were transfected with 0.5 μg of the IL-6 promoter constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, different concentrations of the mammalian expression vector for p65 NF-κB (pCMV/p65) were also included. Luciferase reporter activities were measured with a Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

NF-κB-dependent transcriptional activity

NF-κB-dependent transcriptional activity was measured using a *cis*-acting luciferase reporter assay system as described previously (Jeon et al. 2018). BV2 cells cultured in 12-well plates were transfected with 0.1 μg of the 5×NF-κB-Luc reporter containing five repeats of NF-κB binding sites. Relative luciferase activities were measured with a Centro LB960 luminometer (Berthold Technologies).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical significance was analyzed by one-way ANOVA followed by Sidak's or Dunnett's multiple comparisons test using GraphPad Prism version 7.02 (GraphPad Software Inc., La Jolla, CA, USA). Differences with a *P*-value of less than 0.05 were considered statistically significant.

Results

Screening of cereal grains with NF-κB inhibitory activity

Chronic inflammatory response in the CNS has been implicated in neurodegenerative diseases and psychiatric disorders (Kirch 1993). TNFα is a major inflammatory cytokine and produces many pro-inflammatory cytokines through the activation of transcription factor NF-κB (Balkwill 2009). To screen cereal grains inhibiting NF-κB in the CNS, ethanolic extracts of various cereal grains were prepared and their effects on TNFα-induced phosphorylation of p65/RelA NF-κB were assessed in BV2 microglial cells. Among ten grain extracts, barley (*Hordeum vulgare*) ethanol extract (HVE; grain #6) significantly ($p < 0.0001$ by Sidak's multiple comparisons test) reduced TNFα-induced p65/RelA NF-κB phosphorylation on serine-536 residue in BV2 microglial cells (Fig. 1a). Also, HVE had no cytotoxic effects at doses of up to 200 μg/mL for 48 h exposure (Fig. 1b). These data suggest that HVE down-regulates TNFα-induced NF-κB activation with no cytotoxic effects.

H. vulgare ethanol extract (HVE) inhibits TNFα-induced IκB kinase (IKK) activation

Upon stimulation, IκB kinase (IKK) complex leads to IκB degradation, thereby allowing activation of the NF-κB

Fig. 1 Screening of cereal grains for inhibitory effects on NF-κB phosphorylation. **a** BV2 cells were pre-treated with 100 μg/mL of each cereal grain ethanol extract before stimulation with 20 ng/mL TNFα. Total cell lysates were immunoblotted with phospho-specific antibody against p65/RelA (Ser536) NF-κB. GAPDH was used as an internal control. Bottom graphs show the relative band intensities of phosphorylated p65/RelA normalized to GAPDH level. Data are represented as means ± SD. *$P = 0.0128$; ***$P < 0.0001$; *NS*, not significant ($n = 3$), by Sidak's multiple comparisons test. 1, Adlay grain (*Coix lacrymajobi*); 2, Hog millet grain (*Panicum miliaceum*); 3, Corn (*Zea mays*); 4, Glutinous foxtail millet grain (*Setaria italica*); 5, Sorghum grain (*Sorghum bicolor*); 6, Barley grain (*Hordeum vulgare*); 7, Oat grain (*Avena sativa*); 8, Wheat grain (*Triticum aestivum*); 9, Buckwheat (*Fagopyrum esculentum*); 10, Rye grain (*Secale cereale*). **b** BV2 cells were treated with 0, 25, 50, 100, or 200 μg/mL HVE for 24 h, and cell viability was measured using a Cell Counting Kit-8. Data are represented as mean ± SD ($n = 3$). *NS*, not significant
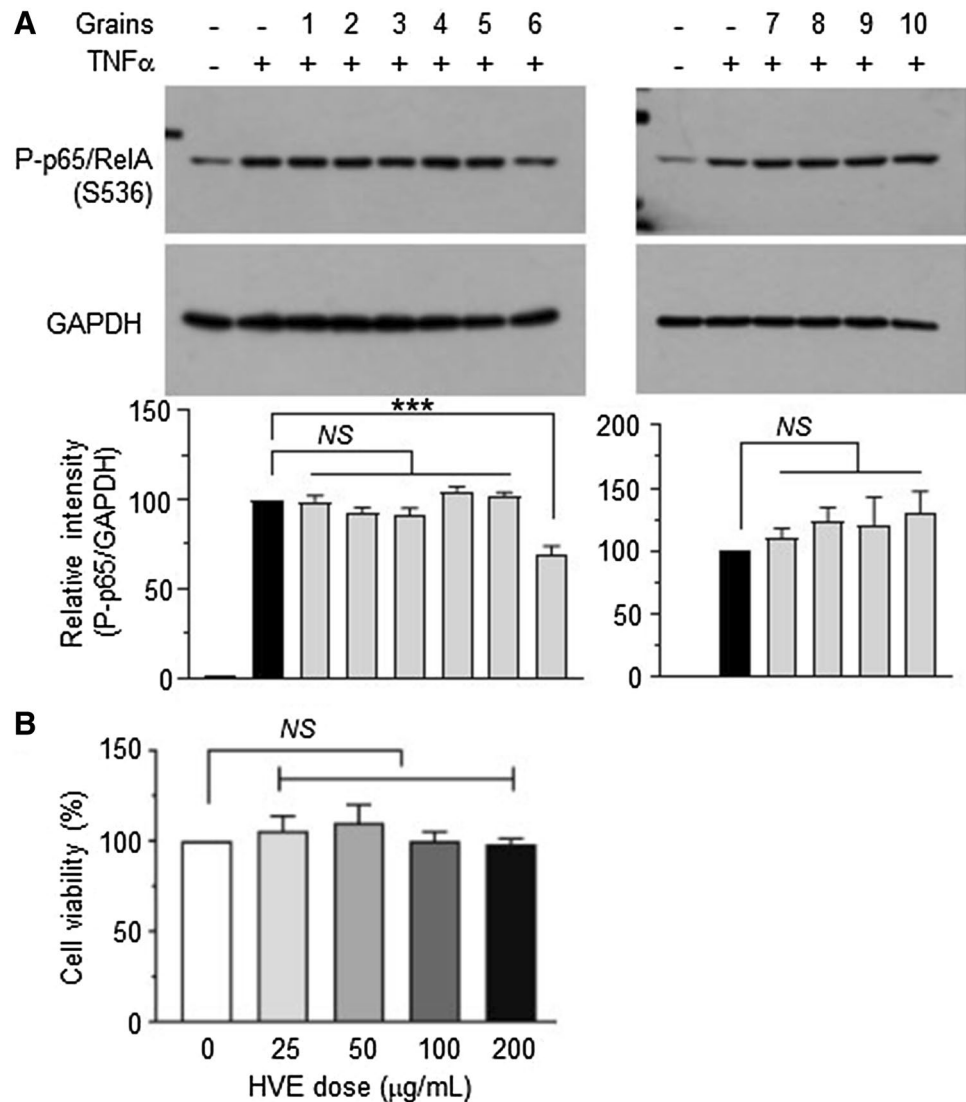
(Hoesel and Schmid 2013). To further determine the inhibitory effect of HVE on TNFα-induced p65/RelA phosphorylation, it was investigated whether HVE can modulate IKK or IκB in BV2 microglial cells. Upon TNFα stimulation, phosphorylation of IKKα/β (Serine-176/180) and IκBα (Serine-32) rapidly peaked within 10 min, after which the phosphorylation levels gradually decreased in BV2 cells (Fig. 2a). To investigate whether HVE inhibits IKK activity, we measured TNFα-induced phosphorylation of IKKα/β and IκBα in BV2 cells pretreated with HVE. As shown in Fig. 2b, pre-exposure to HVE significantly abrogated the TNFα-induced phosphorylation of IKKα/β on serine-176/180 and its downstream effector IκBα on serine-32 in a dose-dependent manner ($p < 0.0001$ by Dunnett's multiple comparisons test). These data suggest that the inhibitory effect of HVE on TNFα-induced NF-κB activity is due to the suppression of IKK-mediated IκB phosphorylation in BV2 microglial cells.

H. vulgare ethanol extract (HVE) inhibits TNFα-induced NF-κB accumulation in the nucleus and NF-κB-mediated transcriptional activity

Activated NF-κB in the cytoplasm immediately translocates to the nucleus. To determine the effect of HVE on the nuclear translocation of NF-κB, we examined the nuclear phospho-form of NF-κB using an immunofluorescence microscope. As expected, substantial levels of p65/RelA phosphorylated on serine-536 accumulated in the nucleus upon TNFα stimulation; however, pre-treatment with HVE inhibited this accumulation (Fig. 3a). Next, the potential effects of HVE on NF-κB-dependent transcription were examined. BV2 microglial cells were transfected with the 5×NF-κB *cis*-acting luciferase reporter construct containing five repeats of NF-κB-binding sites and luciferase reporter activity was measured. As shown in Fig. 3b, treatment with TNFα resulted in a ~ fivefold increase in luciferase activity,
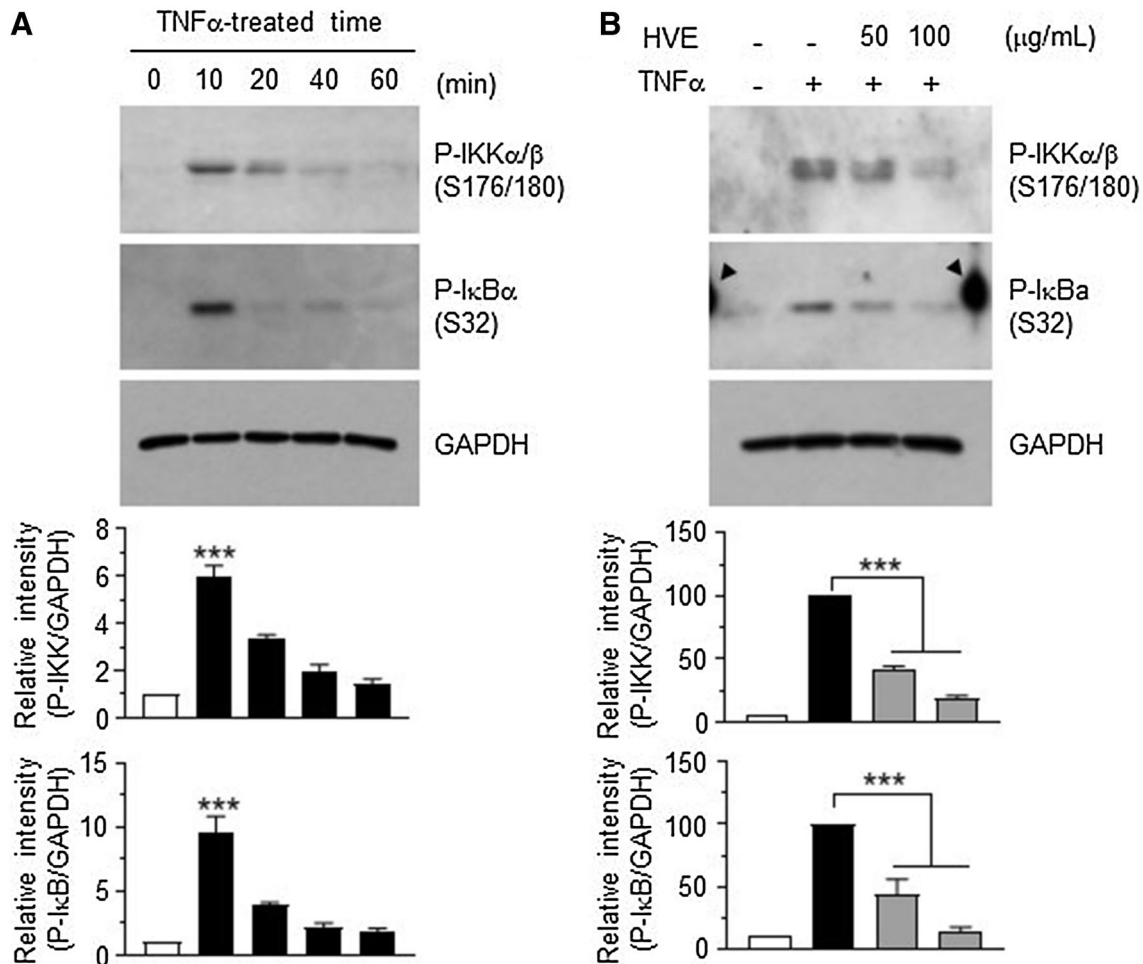
Fig. 2 Effect of HVE on the inhibition of TNFα-induced IKK phosphorylation. **a** BV2 cells were treated with 20 ng/mL TNFα for different time periods. **b** BV2 cells were treated with 50 or 100 μg/mL HVE, followed by treatment with 10 ng/mL TNFα for 10 min. Total cell lysates were immunoblotted with phospho-specific antibodies against IKKα/β (Ser176/180) and IκBα (Ser32). GAPDH was used as an internal control. Bottom graphs show the relative band intensities of phosphorylated IKK and IκB normalized to GAPDH level. Data are represented as means ± SD ($n = 3$). ***$P < 0.0001$; by Sidak's multiple comparisons test (**a**) and Dunnett's multiple comparisons test (**b**). Arrow heads indicate non-specific spots (**b**)

while in the presence of HVE, TNFα-induced reporter activity was significantly ($P < 0.001$) reduced in an HVE dose-dependent manner. These data suggest that HVE inhibits TNFα-induced NF-κB-dependent transcriptional activity through the inhibition of IKK activity.

H. vulgare ethanol extract (HVE) inhibits IL-6 mRNA expression in BV2 microglial cells

IL-6 is a multifunctional cytokine that plays an essential role in the regulation of B-cell maturation, hematopoiesis, inflammation, and hepatic acute-phase response (Garbers et al. 2015; Tanaka et al. 2014) and serves as a hallmark of the injured brain (Campbell et al. 2014; Yang et al. 2013). Given that NF-κB regulates the IL-6 and IL-1β gene expression in various cell types (Tak and Firestein 2001),

we tested the effect of HVE on IL-6 and IL-1β expression in BV2 microglial cells. RT-PCR experiments revealed that TNFα stimulation led to the up-regulation of both IL-6 and IL-1β mRNA expression within 3 h in BV2 microglial cells (Fig. 4a). Under these experimental conditions, TNFα-induced IL-6 and IL-1β mRNA expression was substantially decreased in the presence of HVE (Fig. 4b). To confirm the relative changes in mRNA expression levels, we performed quantitative real-time PCR (Q-PCR) analysis. The result shows that an increase in IL-6 transcripts was detectable as early as 3 h after TNFα treatment inconsistent with the RT-PCR data (Fig. 4c). TNFα-induced IL-6 mRNA expression was furthermore confirmed to be significantly ($P < 0.001$) reduced in the presence of HVE (Fig. 4d). These data demonstrate that HVE inhibits TNFα-induced IL-6 and IL-1β expression at the mRNA level in BV2 microglial cells.
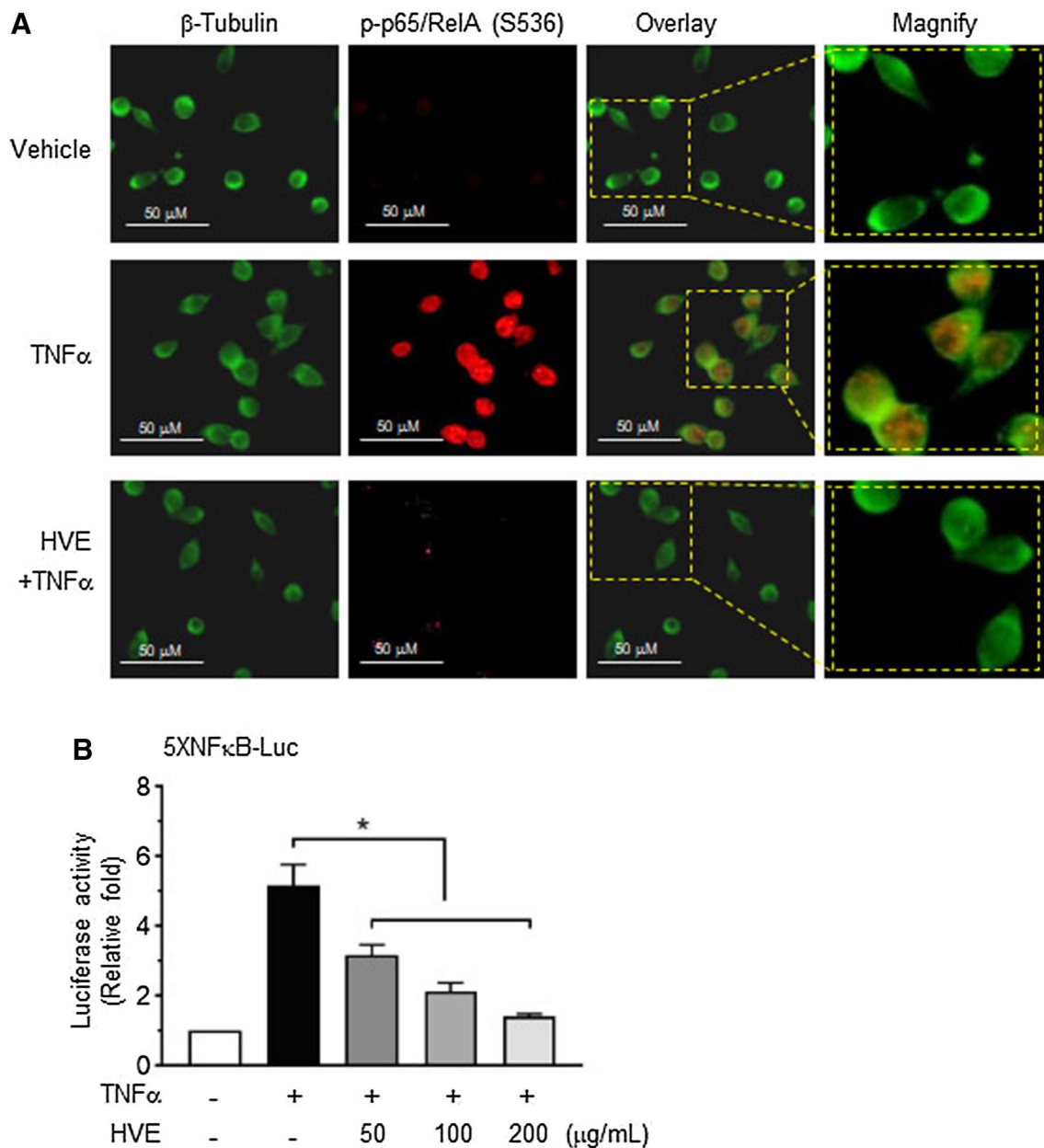
Fig. 3 Effect of HVE on NF-κB-dependent transcriptional activity. **a** BV2 cells cultured on coverslips were treated with 100 μg/mL HVE or vehicle (DMSO) for 30 min, followed by stimulation with 20 ng/mL TNFα. After 10 min, the cells were fixed and incubated with primary antibody against β-tubulin or phospho-p65 (Ser536). After 18 h, secondary antibodies, Alexa Fluor 488-conjugated (*green signal*) and Alexa Fluor 555-conjugated (*red signal*), were added for an additional 50 min. Magnified images are shown on the right. Scale bar, 50 μm. **b** BV2 cells were transfected with 0.1 μg of the $5 \times$ NFκB-Luc plasmid, along with 50 ng pRL-null plasmid to normalize transfection efficiency. At 48 h post-transfection, cells were treated with either vehicle (DMSO) or with various concentrations of HVE (50, 100, or 200 μg/mL) for 30 min, followed by 20 ng/mL TNFα treatment. After 8 h, luciferase activity was measured. Data are represented as means ± SD ($n = 3$). *P values were measured by Sidak's multiple comparisons test. (Color figure online)

The NF-κB-binding site within the IL-6 gene promoter is a target response element for *H. vulgare* ethanol extract (HVE)

It has been demonstrated that NF-κB-binding element within the IL-6 gene promoter region is considered a potential target for TNFα-induced IL-6 promoter activation in U937 human monocyte cells (Libermann and Baltimore 1990). To explore the possibility that NF-κB-binding site within the IL-6 gene is the target element for HVE in microglia, we generated a serial deletion construct of the IL-6 gene promoter (−956/+47, −469/+47, and −182/+47) containing NF-κB-binding site in

Fig. 4 Inhibitory effect of HVE on TNFα-induced IL-6 mRNA expression. **a, c** BV2 cells were treated with 10 ng/mL TNFα for various time periods. **b, d** BV2 cells were treated with 50 or 100 µg/mL HVE for 30 min after which they were stimulated with 10 µg/mL TNFα. After 3 h, total RNA was isolated and IL-6 and IL-1β mRNA levels were determined by RT-PCR (**a, b**). IL-6 mRNA levels were quantitated by Q-PCR (**c, d**). GAPDH mRNA was used as an internal control. *P*-values were determined using Dunnett's multiple comparisons test (*n*=3). *NS*, not significant; *0.03; **P=0.07; ***P<0.001 versus vehicle-treated control
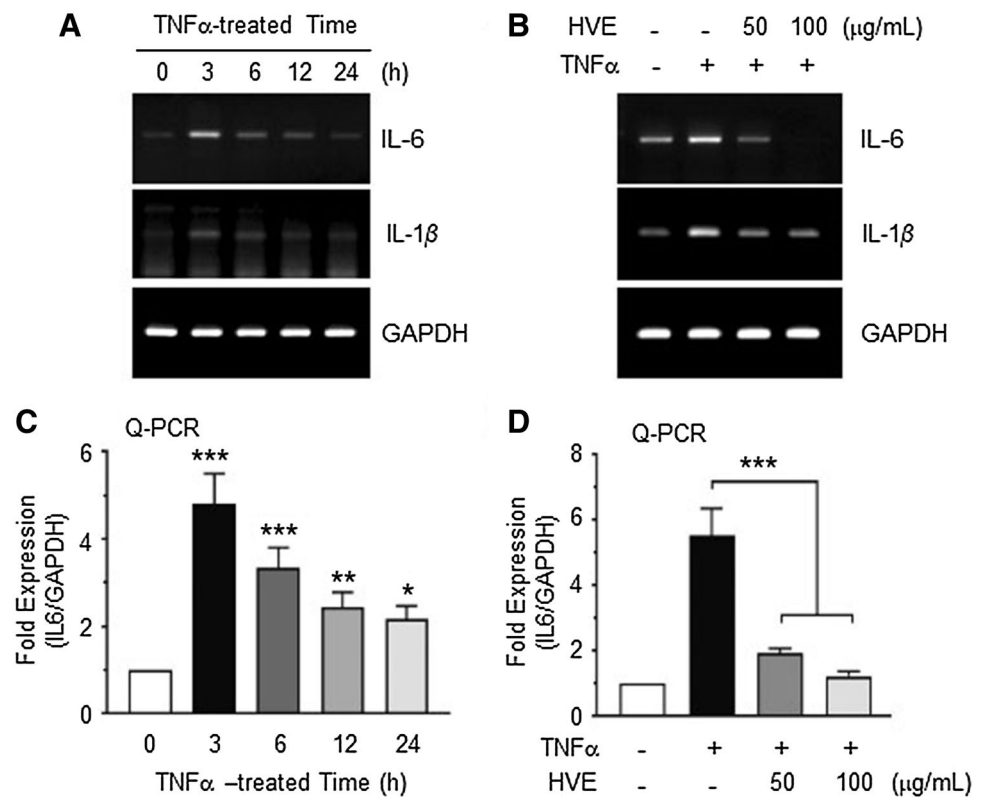
a luciferase-based reporter plasmid. BV2 cells were transfected with reporter plasmids and treated with TNFα in the absence or presence of HVE. The shortest construct (−182/+47) containing only NF-κB-binding site still retained TNFα-stimulated reporter activity, which was significantly ($p < 0.001$) reduced in the presence of HVE (Fig. 5a), suggesting that NF-κB-binding element is involved in the inhibitory response to HVE.

To further verify that the NF-κB-binding site within the IL-6 gene promoter is an HVE target element, site-directed mutagenesis was carried out. Point mutation of the core sequence of the NF-κB-binding sites (CCC to CAC) within the −182/+47 construct significantly ($P < 0.001$) abolished TNFα inducibility (Fig. 5b). Furthermore, forced expression of p65/RelA NF-κB resulted in the stimulation of the −182/+47 reporter in a plasmid concentration-dependent manner (Fig. 5c), implicating the *trans*-acting potential of NF-κB. Collectively, these data demonstrate that the NF-κB-binding site at −46/−32 in the IL-6 promoter region is an HVE target element involved in the inhibition of TNFα-induced IL-6 gene transcription in microglial cells.

Discussion

Previous studies have reported that *H. vulgare* contains various health-beneficial components including vitamin E and phenolic compounds (Quinde-Axtell and Baik 2006), and exerts anti-oxidative, antiproliferative, and anti-inflammatory properties (Choi et al. 2013; Madhujith and Shahidi 2007). However, little is known about the anti-neuroinflammatory action of *H. vulgare* in microglial cells. In the present study, we demonstrate that *H. vulgare* ethanol extract (HVE) suppressed TNFα-induced IL-6 expression. We also found that HVE targets IKK/NF-κB responsible for transactivation of the IL-6 gene promoter in BV2 microglial cells.

NF-κB is a member of the Rel family of transcription factors consisting of p65/RelA, RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). The inactive form of NF-κB complex exists in the cytoplasm, bound with inhibitory proteins called inhibitor of IκB. When TNFα stimulates cells, NF-κB activation occurs via phosphorylation of IκB by IKK complex (IKKα, IKKβ, and IKKγ/NEMO), leading to IκB degradation bound to NF-κB, resulting in the translocation of NF-κB to the nucleus (Hoesel and Schmid 2013). NF-κB is expressed in almost all cell types in the body and regulates cell proliferation, cell survival, inflammation, and immune responses (Barnes and Karin 1997; Chen and Greene 2004; Li and Verma 2002). In the CNS, the most common NF-κB complex is p65/RelA and p50/NF-κB1 heterodimer, expressed in neurons, glial cells, and oligodendrocytes (Mattson and Camandola 2001; O'Neill and Kaltschmidt 1997; Shih et al. 2015). In neurons, NF-κB activation promotes neuronal survival through up-regulation of survival-related genes including inhibitors of apoptosis

A

B

C pIL6-Luc(-182/+47)
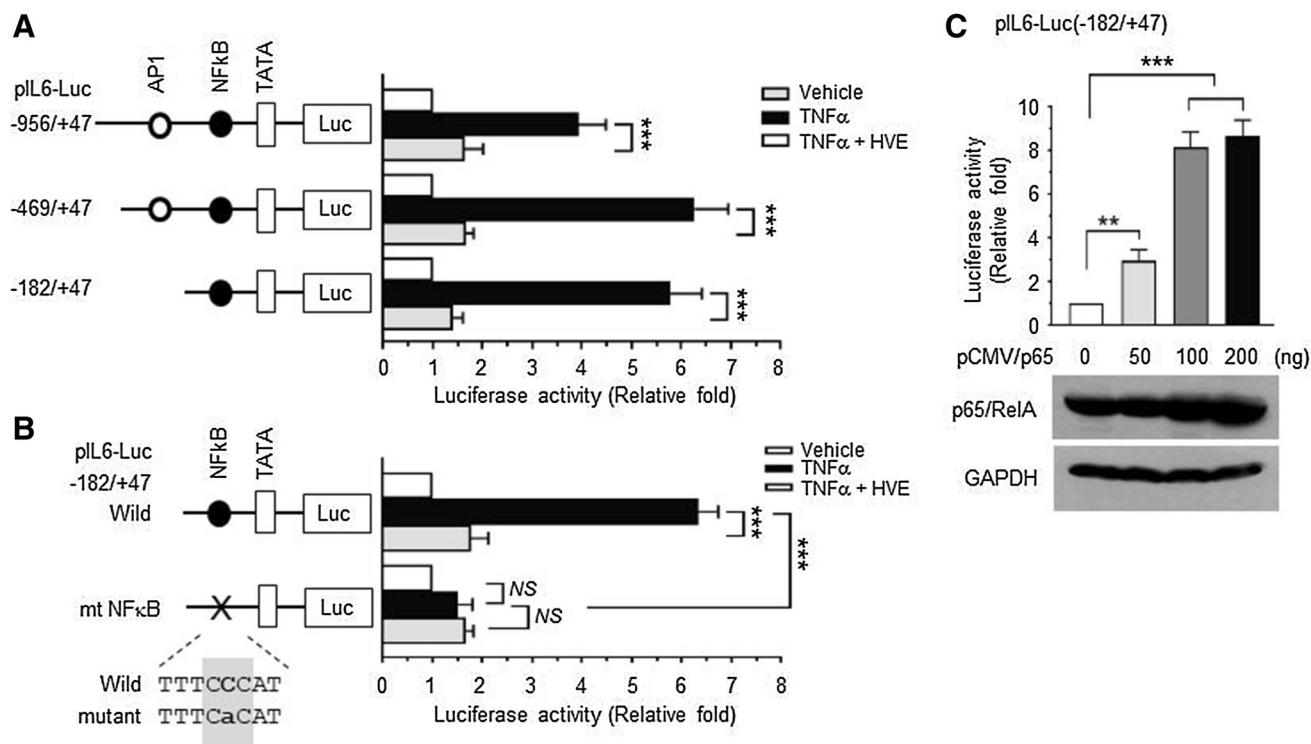
Fig. 5 The role of NF-κB in TNFα-induced IL-6 gene promoter activation. **a, b** BV2 cells were transfected with 0.1 μg of serial 5′-deletion constructs (**a**) or NF-κB mutated construct (**b**) of the IL-6 promoter. After 48 h, cells were treated with 10 ng/mL TNFα in the absence or presence of 100 μg/mL HVE. After 12 h, luciferase activities were measured. Data represent means ± S.D (n = 3; *NS*, not significant; ***$P < 0.001$ by Sidak's multiple comparisons test). **c** BV2

cells were co-transfected with 0.1 μg of − 182/+ 47 construct and different concentrations of p65 NF-κB expression plasmid (pCMV/p65). After 48 h, cells were collected and p65 NF-κB protein levels and luciferase activities were measured. Data represent means ± SD (n = 3; **$P = 0.007$; ***$P < 0.001$ versus empty-vector transfection by Dunnett's multiple comparison test)

proteins (IAPs), BCL-2, and superoxide dismutase (Bhakar et al. 2002). In contrast, NF-κB in microglial cells is activated by various inflammatory conditions such as microbial infection, physical tissue damage, and physiological stresses. Microglial NF-κB plays a critical role in the production of large amounts of pro-inflammatory cytokines including TNFα, IL-6, and IL-1β (Shih et al. 2015), indicating that the prolonged activation of microglial NF-κB may cause chronic neuroinflammatory effects. Accordingly, proper modulation of NF-κB activity in microglial cells is essential for effective prevention or treatment strategies for inflammation-related neurodegenerative diseases.

TNFα is a major inflammatory cytokine produced by various types of immune cells including macrophages and lymphocytes as well as from non-immune cells such as smooth muscle cells and epithelial cells (Balkwill 2009). In the CNS, TNFα is primarily produced by microglia, and its autocrine activation of microglia facilitates the production of various inflammatory mediators including IL-1β, IL-6, and nitric oxide (NO) via NF-κB activation (Kuno et al. 2005). Therefore, we attempted to analyze the impact of HVE on the inhibition of TNFα-induced IκB and NF-κB

phosphorylation. Our results clearly show that HVE reduced TNFα-induced phosphorylation of IκBα on Ser-32 and phosphorylation of p65/RelA on Ser-536 in BV2 microglial cells. Moreover, HVE reduced TNFα-induced transcriptional activity of NF-κB, as revealed by a *cis*-acting reporter assay system. The previous study has reported that p65/RelA phosphorylation at Ser-536 is associated with nuclear translocation and transactivation potential (Sakurai et al. 1999). We also confirmed by fluorescence microscopy that HVE inhibition of TNFα-induced NF-κB phosphorylation at Ser-536 attenuated NF-κB nuclear translocation. Furthermore, HVE also reduced the expression of IL-6, a downstream target of NF-κB. Therefore, inhibition of NF-κB, through the inhibition of IKK, provides a novel mechanism for HVE-induced modulation of neuroinflammatory responses.

In the CNS, IL-6 acts as a major inflammatory mediator and regards IL-6 as a hallmark of the injured brain (Campbell et al. 2014; Yang et al. 2013). Accordingly, blocking IL-6 could be considered a strategy for the prevention of various neuroinflammatory disorders (Rothaug et al. 2016). We sought to clarify whether HVE inhibition of NF-kB is functionally related to the inhibition of TNF-induced IL-6

gene expression. The 5′-flanking region of the IL-6 gene contains multiple response elements, including multiple response element (MRE), NF-κB, C/EBPβ, cAMP response element-binding protein (CREB), and AP-1 (Baccam et al. 2003; Dendorfer et al. 1994). Of these, the NF-κB-binding element is known to be necessary for TNFα-induced IL-6 promoter activation in U937 human monocyte cells (Libermann and Baltimore 1990). We also observed that disruption of the NFκBbinding site in the IL-6 gene promoter by site-directed mutagenesis significantly reduced TNFα inducibility of IL-6 gene promoter activity. Furthermore, forced expression of p65/RelA NF-κB significantly increased IL-6 gene promoter activity. These results clearly show that TNFα-induced transcriptional activation of the IL-6 gene is largely dependent on NF-κB activation in BV2 microglia. In addition to neuroinflammation, many inflammatory diseases, including autoimmune arthritis, septic shock, lung fibrosis, asthma, atherosclerosis, and cancers also have high uncontrolled NF-κB activity (Karin 2006; Luo et al. 2005; Okamoto et al. 2007). Thus, inhibition of the IKK-NF-κB signaling pathway by HVE could be beneficial in preventing neuroinflammation triggered by microglia cells as well as in the prevention of various inflammatory diseases and tumorigenesis.

The findings described here are in accordance with a previous study demonstrating that methanol extract of the aerial parts of *H. vulgare* suppresses the LPS-induced production of TNFα, IL-1β, and IL-6 through the inhibition of NF-κB (Choi et al. 2013). This study further elucidates the action of HVE in the suppression of IL-6 gene expression by targeting the NF-κB-binding element in the IL-6 gene promoter region. At present, however, it is still unclear whether HVE directly inhibits IKK or inhibits IKK upstream kinases such as NF-κB-inducing kinase (NIK), mitogen-activated protein kinase kinase kinase (MAPKKK), or Akt (Jeon et al. 2018). The possibility that different pathways besides IKK contribute to HVE inhibition of NF-κB activity cannot be excluded. Several bioactive components exhibiting anti-inflammatory property have been isolated and identified in *H. vulgare* extracts, including benzeneacetic acid and its derivatives including 4-hydroxy-3-methoxybenzeneacetic acid, 4-hydroxy-3-methoxybenzeneacetic acid, and benzene-propanoic acid and its derivatives including 3-hydroxy-1-(4-hydroxy-3-) methoxyphenyl-1-propanone and polyphenolic compounds (Ferreres et al. 2009; Hernanz et al. 2001). Further studies are needed to identify the active ingredients that can pass through the blood–brain barrier (BBB) to inhibit TNFα-induced IL-6 expression in microglia in the CNS. There are no evidences that the active ingredients in the barley extracts can pass the BBB. We selected an active ingredient in the barley extracts, benzenepropanoic acid, which shows an anti-inflammatory activity (Choi et al. 2013), and tested whether it could pass through the BBB using the

Volsurf module provided by the Sybyl program (Tripos, St. Louis, MO, USA). We found that benzenepropanoic acid can pass through the BBB (Supplemental Figure S1). Based on this *in silico* experiment, it is assumed that some active ingredients in HVE could pass through the BBB.

In summary, the findings reported here demonstrate that *H. vulgare* ethanol extract (HVE) reduces TNFα-induced IL-6 expression at the mRNA level. We also show that HVE inhibits NF-κB-dependent transcriptional activity via IKK inhibition in BV2 microglial cells. Neuroinflammation is crucial for various neurodegenerative diseases such as Alzeimer's and Parkinson's diseases, as well for as psychiatric disorders such as depression and schizophrenia. Our data suggest that HVE contains bioactive compound(s) that is effective in reducing the inflammatory response induced by TNFα in the CNS.

Authors' contributions SYS, YL, and YHL designed research; JC, JK, DYM, and EJ performed experiments. SYS and YHL wrote the paper.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

References

Baccam M, Woo SY, Vinson C, Bishop GA (2003) CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-kappa B, AP-1, and C/EBP. J Immunol 170:3099–3108

Balkwill F (2009) Tumour necrosis factor and cancer. Nat Rev Cancer 9:361–371

Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071

Becher B, Spath S, Goverman J (2017) Cytokine networks in neuroinflammation. Nat Rev Immunol 17:49–59

Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci 22:8466–8475

Campbell IL, Erta M, Lim SL, Frausto R, May U, Rose-John S, Scheller J, Hidalgo J (2014) Trans-signaling is a dominant mechanism for the pathogenic actions of interleukin-6 in the brain. J Neurosci 34:2503–2513

Chen LF, Greene WC (2004) Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol 5:392–401

Choi KC, Hwang JM, Bang SJ, Son YO, Kim BT, Kim DH, Lee SA, Chae M, Kim DH, Lee JC (2013) Methanol extract of the aerial parts of barley (*Hordeum vulgare*) suppresses lipopolysaccharide-induced inflammatory responses in vitro and in vivo. Pharm Biol 51:1066–1076

Dendorfer U, Oettgen P, Libermann TA (1994) Multiple regulatory elements in the interleukin-6 gene mediate induction by

prostaglandins, cyclic AMP, and lipopolysaccharide. Mol Cell Biol 14:4443–4454

Dobos N, Korf J, Luiten PG, Eisel UL (2010) Neuroinflammation in Alzheimer's disease and major depression. Biol Psychiatry 67:503–504

Eikelenboom P, Bate C, Van Gool WA, Hoozemans JJ, Rozemuller JM, Veerhuis R, Williams A (2002) Neuroinflammation in Alzheimer's disease and prion disease. Glia 40:232–239

Ferreres F, Krskova Z, Goncalves RF, Valentao P, Pereira JA, Dusek J, Martin J, Andrade PB (2009) Free water-soluble phenolics profiling in barley (Hordeum vulgare L.). J Agric Food Chem 57:2405–2409

Frost JL, Schafer DP (2016) Microglia: architects of the developing nervous system. Trends Cell Biol 26:587–597

Garbers C, Aparicio-Siegmund S, Rose-John S (2015) The IL-6/gp130/STAT3 signaling axis: recent advances towards specific inhibition. Curr Opin Immunol 34:75–82

Goldsmith DR, Rapaport MH, Miller BJ (2016) A meta-analysis of blood cytokine network alterations in psychiatric patients: comparisons between schizophrenia, bipolar disorder and depression. Mol Psychiatry 21:1696–1709

Hernanz D, Nunez V, Sancho AI, Faulds CB, Williamson G, Bartolome B, Gomez-Cordoves C (2001) Hydroxycinnamic acids and ferulic acid dehydrodimers in barley and processed barley. J Agric Food Chem 49:4884–4888

Hoesel B, Schmid JA (2013) The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer 12:86

Jeon S, Kim SH, Shin SY, Lee YH (2018) Clozapine reduces Toll-like receptor 4/NF-kappaB-mediated inflammatory responses through inhibition of calcium/calmodulin-dependent Akt activation in microglia. Prog Neuropsychopharmacol Biol Psychiatry. 81:477–487. https://doi.org/10.1016/j.pnpbp.2017.04.012

Karin M (2006) Nuclear factor-kappaB in cancer development and progression. Nature 441:431–436

Kim YK, Na KS, Myint AM, Leonard BE (2016) The role of proinflammatory cytokines in neuroinflammation, neurogenesis and the neuroendocrine system in major depression. Prog Neuropsychopharmacol Biol Psychiatry 64:277–284

Kirch DG (1993) Infection and autoimmunity as etiologic factors in schizophrenia: a review and reappraisal. Schizophr Bull 19:355–370

Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A (2005) Autocrine activation of microglia by tumor necrosis factor-alpha. J Neuroimmunol 162:89–96

Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2:725–734

Libermann TA, Baltimore D (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol 10:2327–2334

Luo JL, Kamata H, Karin M (2005) IKK/NF-kappaB signaling: balancing life and death–a new approach to cancer therapy. J Clin Invest 115:2625–2632

Madhujith T, Shahidi F (2007) Antioxidative and antiproliferative properties of selected barley (Hordeum vulgarae L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. J Agric Food Chem 55:5018–5024

Mattson MP, Camandola S (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 107:247–254

Minghetti L (2005) Role of inflammation in neurodegenerative diseases. Curr Opin Neurol 18:315–321

Nimmo AJ, Vink R (2009) Recent patents in CNS drug discovery: the management of inflammation in the central nervous system. Recent Pat CNS Drug Discov 4:86–95

O'Neill LA, Kaltschmidt C (1997) NF-kappa B: a crucial transcription factor for glial and neuronal cell function. Trends Neurosci 20:252–258

Okamoto T, Sanda T, Asamitsu K (2007) NF-kappa B signaling and carcinogenesis. Curr Pharm Des 13:447–462

Okarter N, Liu RH (2010) Health benefits of whole grain phytochemicals. Crit Rev Food Sci Nutr 50:193–208

Peña-Altamira E, Prati F, Massenzio F, Virgili M, Contestabile A, Bolognesi ML, Monti B (2016) Changing paradigm to target microglia in neurodegenerative diseases: from anti-inflammatory strategy to active immunomodulation. Expert Opin Ther Targets 20:627–640

Quinde-Axtell Z, Baik BK (2006) Phenolic compounds of barley grain and their implication in food product discoloration. J Agric Food Chem 54:9978–9984

Rivest S (2009) Regulation of innate immune responses in the brain. Nat Rev Immunol 9:429–439

Rothaug M, Becker-Pauly C, Rose-John S (2016) The role of interleukin-6 signaling in nervous tissue. Biochim Biophys Acta 1863:1218–1227

Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W (1999) IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 274:30353–30356

Shih RH, Wang CY, Yang CM (2015) NF-kappaB signaling pathways in neurological inflammation: a mini review. Front Mol Neurosci 8:77

Shin SY, Kim CG, Jung YJ, Lim Y, Lee YH (2016) The UPR inducer DPP23 inhibits the metastatic potential of MDA-MB-231 human breast cancer cells by targeting the Akt-IKK-NF-kappaB-MMP-9 axis. Sci Rep 6:34134

Shin SY, Kim HW, Jang HH, Hwang YJ, Choe JS, Lim Y, Kim JB, Lee YH (2017) Gamma-Oryzanol-rich black rice bran extract enhances the innate immune response. J Med Food 20:855–863

Singh A, Sharma S (2017) Bioactive components and functional properties of biologically activated cereal grains: a bibliographic review. Crit Rev Food Sci Nutr 57:3051–3071

Singhal G, Baune BT (2017) Microglia: an interface between the loss of neuroplasticity and depression. Front Cell Neurosci 11:270

Tak PP, Firestein GS (2001) NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107:7–11

Tanaka T, Narazaki M, Kishimoto T (2014) IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 6:a016295

Yang SH, Gangidine M, Pritts TA, Goodman MD, Lentsch AB (2013) Interleukin 6 mediates neuroinflammation and motor coordination deficits after mild traumatic brain injury and brief hypoxia in mice. Shock 40:471–475