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Kolaviron, a natural flavonoid from the seeds of *Garcinia kola*, reduces LPS-induced inflammation in macrophages by combined inhibition of IL-6 secretion, and inflammatory transcription factors, ERK1/2, NF-κB, p38, Akt, p-c-JUN and JNK

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ABSTRACT

Background: Kolaviron (Kol-v), an important component of *Garcinia kola* seed has a variety of biologic activities, including anti-inflammatory properties.
Methods: We tested the ability of Kol-v to block signalling pathways implicated in lipopolysaccharide (LPS)-induced inflammatory gene expression in RAW 264.7 macrophage cell line.
Results: When macrophages pre-treated with Kol-v (15 and 25 μM) were activated with LPS, phosphorylation of p38 and p-c-JUN but not IκBα degradation and phosphorylation of NF-κB (p65), ERK1/2, and IκBα were blocked. Furthermore, Kol-v suppressed LPS-induced increase in the expression of IL-18 gene and LPS-induced decrease in the mRNA expression of IP-10 but it had no effect on the LPS-induced decrease in the gene expression levels of IL-1α, IL-33, IL-1β, and IFNβ1-1. When macrophages pre-treated with Kol-v (50 and 100 μM) were activated with LPS, phosphorylation of Akt, ERK1/2, IκBα, and NFκB (p65) but not that of CREB was blocked by Kol-v. The protective effect of Kol-v on the LPS-induced phosphorylation of the mitogen activated protein kinase (MAPK) family member JNK was only observed at 100 μM. At all concentrations of Kol-v (0–100 μM) tested in this study, there was no effect of Kol-v on LPS-induced secretion of the pro-inflammatory cytokine TNF-α but a concentration dependent inhibition of Kol-v on IL-6 secretion was observed.
Conclusion: Kol-v interferes with LPS signalling by reducing the activation of several inflammatory transcription factors and that its inhibitory action on IL-6 secretion correlates with inhibition of ERK1/2, p38, Akt, p-c-JUN and JNK signalling pathways.
General significance: The anti-inflammatory potential of Kol-v via inhibition of IL-6 secretion in RAW macrophage was established in this study.

1. Introduction

Inflammation is a defence response of living tissues to injurious stimulus. The inflammatory response involves the activation of several immune cells such as monocytes/macrophages which secrete a series of pro-inflammatory mediators such as enzymes, cytokines, chemokines as well as signalling proteins at the site of infected tissues and cells. Dysregulation of the inflammatory immune responses can lead to a variety of diseases, such as cancer, atherosclerosis, rheumatoid arthritis, neurological diseases, diabetes, pulmonary disorders, and allergies [1]. The favourite model used to study induced inflammation both in vitro and in vivo is the stimulation of macrophages by lipopolysaccharide obtained from Gram-negative bacteria [2–4]. Binding of lipopolysaccharide (LPS) to its cognate receptors such as CD14 and Toll-like receptors [5] in mammalian cell membrane activates several signalling cascades driving the expression of pro-inflammatory cytokines e.g. tumour necrosis factor-α, interleukin-1, and interleukin-6 [6]. Furthermore, excessive inflammatory responses could lead to decreased expression of anti-inflammatory cytokines [7]. Thus, the imbalance between pro-inflammatory and anti-inflammatory cytokines may contribute to the pathogenesis of autoimmune diseases [8] which are usually characterized by chronic inflammation. Various therapeutic strategies, including the use of bioactive agents to block the production of inflammatory mediators in macrophages may be useful for various inflammatory diseases [5,9,10].

Macrophages are an important source of pro-inflammatory cytokines and a number of transcription factors and cellular signalling pathways have been suggested to contribute to the LPS-induced expressions of pro-inflammatory genes in this cell type [2,11]. Following stimulation with LPS or cytokines, NF-κB is activated via the activation of IκB-kinase complex which then phosphorylates IκB on Ser 32 and Ser 36, resulting

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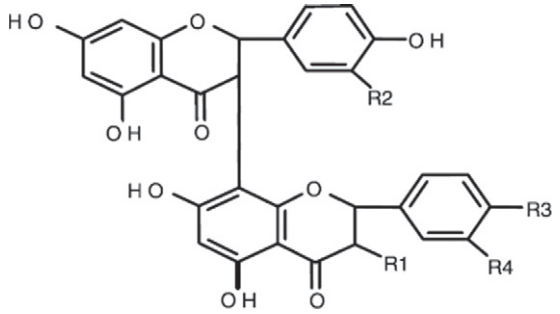
in its ubiquitination and subsequent proteasomal degradation as well as the release of NF-κB, which then translocates into the nucleus to activate the transcription of pro-inflammatory target genes [12]. Another group of transcription factors that also translocate to the nucleus and stimulate the transcription of inflammatory genes in response to LPS activation is the AP-1 proteins [9]. AP-1 transcription factors are homo- or hetero-dimeric protein complex that consists of different Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, Fra-1, Fra-2, and FosB) subfamilies [13]. Other downstream targets of LPS-induced inflammatory cascades in macrophages are the mitogen-activated protein kinase (MAPK) family members such as p38, c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2) [14]. Upon stimulation with LPS, MAP kinases in turn activate multiple downstream events, including production of the transcription factor AP-1 via the c-Jun N-terminal kinase, JNK [11]. The activation of the different MAPK signalling cascades is believed to control different steps in the pro-inflammatory cytokine production process [5].

Garcinia kola Heckel is a valued edible nut in parts of Nigeria, West and Central Africa. In Nigeria, the seed is commonly referred to as 'bitter kola' because of its bitter astringent taste, and it is readily presented to visitors as a sign of peace [15]. The Nigerian names are Akilu in Ibo language; Orogbo in Yoruba language; and Mijin goro in Hausa language. Extractives of the plant have been used as part of several preparations in the African Traditional Medicine for the treatment of bronchitis, throat infections, liver diseases, oedema, diabetes, asthma, common cold and cough [16]. *G. kola* is safe taken with or without other foods and may be clinically useful in patients with knee osteoarthritis [17]. Major constituents of *G. kola* extract responsible for the pharmacological activity include flavonoids (biflavonoid), xanthenes and benzophenones [17]. Kolaviron (Fig. 1), a major flavonoid derived from *G. kola* extract [18] has been shown to posses anti-hepatotoxic [19], antioxidant and anti-apoptotic effects [20]. Both crude extracts of *G. kola* and kolaviron have shown the capacity to attenuate gastrointestinal inflammatory disorders [16], and the anti-inflammatory effects of kolaviron have been demonstrated against carrageenan-induced paw oedema in rats [21] and in LPS-stimulated macrophages [16]. However there is no information on the molecular targets of kolaviron in the inflammatory cascades, the cellular signalling pathways involved in cytokine production and the underlying mechanisms of action. The aim of the present study was to identify the molecular targets for kolaviron and correlate inhibition of specific signalling molecules with the inhibitory action on pro-inflammatory cytokine (IL-6, TNF-α) secretion. This could provide a molecular basis for the therapeutic use of kolaviron against various inflammatory diseases.



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Fig. 1. The chemical structure of kolaviron.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysacharride from *Escherichia coli* (0127:B8) was purchased from Sigma-Aldrich, Chemie, GmbH, Taufkirchen, Germany. Kolaviron was isolated from *G. kola* seeds according to published procedure [18]. The extract (which consists of biflavanones GB-1, GB-2, and kolaflavanone) was dissolved in a culture-grade DMSO (Sigma-Aldrich, St. Louis, MO, U.S.A.) (final concentration < 0.03%) in a complete medium. Reagents for cell culture including DMEM-high glucose, supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin were purchased from PAA, Laboratories (GmBH, Pasching, Austria). Cell culture plates and wares were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, U.S.A.). Antibodies against phospho-Akt (rabbit mAb), phospho-ERK1/2 and total ERK1/2 antibodies (rabbit mAb), phospho-JNK, phospho-NF-κB (rabbit mAb), phospho-IκBα (rabbit mAb), phospho-p38 (rabbit mAb), phospho-CREB (mAb), phospho-c-JUN (rabbit mAb), IκBα (rabbit mAb) and β-actin (mouse mAb) were obtained from Cell Signaling Technology. The peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Oligonucleotide primers were purchased from Qiagen (Hilden, Germany). RNeasy mini kit was obtained from Qiagen Corporation (Hilden, Germany). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.) unless otherwise indicated.

2.2. Cell culture and treatment

RAW264.7 murine macrophages were from the American Type Culture Collection and were grown in DMEM-high glucose supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a humidified incubator with 5% CO_2. The medium was replaced every 2 days. Cells were harvested after removing the medium, followed by a washing step with Dulbecco's PBS without Ca^{2+} and Mg^{2+}. All experiments were repeated at least twice to confirm the reproducibility of the results. Cells were allowed to grow until it reaches 90–95% confluence, and then it was washed with phosphate-buffered saline and the culture medium was replaced. To evaluate the effects of Kol-v on mRNA expression, the RAW 264.7 cells were pre-treated with Kol-v (15 and 25 μM) for 2 h followed by 1 h of stimulation with LPS (100 ng/mL). To evaluate the effects of Kol-v on protein expression, the cells were pre-treated with higher Kol-v concentrations (50 and 100 μM) or lower Kol-v concentrations (15 and 25 μM) for 24 h followed by LPS (100 ng/mL) stimulation for 2 h prior to the completion of the 24 h. After 24 h, supernatants were collected and stored at −80 °C. Working stock solutions of LPS (1 mg/mL) and Kol-v (500 μM) were prepared fresh in DMSO. The concentration of DMSO in the mixture did not exceed 0.03%. After the 3 h or 24 h incubation period, the detached cells were discarded and the attached cells were collected and processed directly for RNA or protein extraction respectively. A viability test was done on all cells of the different experimental groups using the Trypan Blue Exclusion Test [20]. Viability averaged 90–95%, always with values close to 90%.

2.3. RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated from cells using the RNeasy mini kit. Each RNA preparation was subjected to DNase I (Invitrogen) digestion to remove possible contamination of genomic DNA. For reverse transcription, 2.5 μg of total RNA was transcribed for 1 h at 42 °C in a 40 μL reaction using 2000 U (200 U/μL) of SuperScript™ Reverse Transcriptase (Invitrogen, Germany). Quantitative real-time RT-PCR was performed in an iCycler RT-PCR system (Bio-Rad, Munich, Germany) using the iQ™ SYBR® Green PCR kit (Bio-Rad, Munich, Germany). The PCR amplification condition for each primer set (Table 1) includes initial

Table 1
Primers used in quantitative real-time RT-PCR experiment in this study.

Gene	Primer sequence	Annealing temperature	Product size (bp)	Accession number
IL-1α	F5′-ccgggtggtggtgtcagcaa-3′ R5′-gctgtgaggtgctgatctggg-3′	61.8 °C	148	NM_017019
IL-1β	F5′-tgcctcgtgctgtctgaccca-3′ R5′-aggcccaaggccacagggat-3′	61.8 °C	137	NM_031512
IP-10	F5′-gaagcaccatgaacccaagt-3′ R5′-catggaagtcgatgcaggt-3′	60 °C	117	NM_139089
IL-33	F5′-tcaggcagagccctggtc-3′ R5′-ggtgaggccagaacggag-3′	60 °C	102	NM_001014166
IL-18	F5′-ggctgccatgtcagaaga-3′ R5′-ctcctcgaacacaggcgg-3′	60 °C	201	NM_019165
IFNβ1-1	F5′-tgctgtgcttctccaccact-3′ R5′-tccgtcctgtagctgaggtt-3′	60 °C	133	NM_019127
β-microglobulin	F5′-ccgtgatctttctggtgctt-3′ R5′-aagttgggcttcccattctc-3′	60 °C	109	NM_012512
β-actin	F5′-atggtgggtatgggtcagaa-3′ R5′-gggtcatcttttcacggttg-3′	60 °C	232	NM_031144

denaturation for 1 cycle (95 °C for 8 min), 45 cycles of denaturation (95 °C for 20 s), annealing and extension (72 °C for 30 s). IL-1α, IP-10, IL-33, IL-1β, IFNβ1-1, and IL-18 primers were purchased from Qiagen (Hilden, Germany) and PCR amplification conditions were followed according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The relative quantification of PCR products was determined by the comparative Ct method. The target gene expression was normalized by the non-regulated reference gene, β-actin or β-microglobulin in Kol-v and/or LPS treated samples. Data were presented as relative expression $= 2^{\Delta Ct\ Treatment\ -\ \Delta Ct\ Control}$, $\Delta Ct = Ct_{target\ gene} - Ct\beta\text{-}actin$ (where Ct = threshold cycle).

2.4. Western blot detection of isolated proteins

Western blot procedure for the detection of target protein was performed as described by the manufacturer of target Abs (Cell Signaling Technology, Massachusetts, U.S.A.). In brief, cells were washed with PBS and thereafter lysed by 2× SDS sample buffer (10% SDS, 100% glycerol, 0.5 M Tris–HCl, pH 6.8, 12.5% β-mercaptoethanol, 5% bromophenol blue) supplemented with 1× protease and phosphatase inhibitors (Sigma-Aldrich). Immediately, cells were scraped off the plate and transferred to a microcentrifuge tube and then kept on ice. The cells were sonicated for 10 s for complete cell lysis and microcentrifuged for 1–2 min. Lysates were then heated to 95–100 °C for 10 min and cooled on ice. 25 μL of each sample was fractionated in 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-ECL (0.2 μm); GE Healthcare). Membranes were blocked with 5% non-fat dry milk for 1 h in TBS (20 mM Tris–HCl (pH 7.6) and 150 mM NaCl) containing 0.1% Tween 20 (v/v) (TBS/Tween) before incubation with antibodies against IκBα (dilution 1/500), phospho-IκBα (1/1000), phospho-p38 (1/500), phospho-c-JUN (1/500), phospho-CREB (dilution 1/500), phospho-JNK (dilution 1/1000), phospho-p65 (dilution 1/500), phospho-Akt (dilution 1/1000), ERK1/2 (dilution 1/500), and phospho-ERK1/2 (1/500) in 5% non-fat milk overnight at 4 °C. Anti-actin antibody was used as loading control. For detection of bound primary Abs, membranes were treated with a peroxidase-conjugated secondary Ab at room temperature for 1 h. After three washes with TBS/Tween, bands were visualized using ECL (GE Healthcare).

2.5. Cytokine measurement

RAW 264.7 cells were pre-treated with Kol-v for 24 h and then stimulated with LPS (100 ng/mL) 2 h prior to the completion of the 24 h. Supernatants were collected and analyzed for production of TNF-α (e-Bioscience, Frankfurt, Germany) and IL-6 (DuoSet, R&D Systems, Wiesbaden, Germany) by ELISA in accordance to the manufacturer's instructions.

2.6. Statistical analyses

Data are presented as mean ± S.D. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. All analyses were undertaken using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). For comparison, differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of Kol-v on LPS-induced changes in the expression of IL-1α, IP-10, IL-33, IL-1β, IFNβ1-1, and IL-18 in RAW 264.7 macrophages

To exclude the possibility that the cytotoxicity of Kol-v might contribute to its anti-inflammatory effects, only concentrations of Kol-v up to 100 μM were used at 24 h in the present study. Kol-v did not exhibit any cytotoxic effect at concentration up to 100 μM at 24 h with the Trypan Blue Exclusion assay (Fig. 2). Therefore, we used Kol-v at the concentration of 0–100 μM within 24 h of incubation in all the experiments reported here. To examine the protective effect of Kol-v on the expression of the inflammatory genes, cells were pre-treated with Kol-v (15, 25 μM) for 2 h and stimulated with LPS (100 ng/mL, 1 h), and then mRNA levels of IL-1α, IP-10, IL-33, IL-1β, IFNβ1-1, IL-18 were measured by quantitative real-time RT-PCR. As shown in Fig. 3,

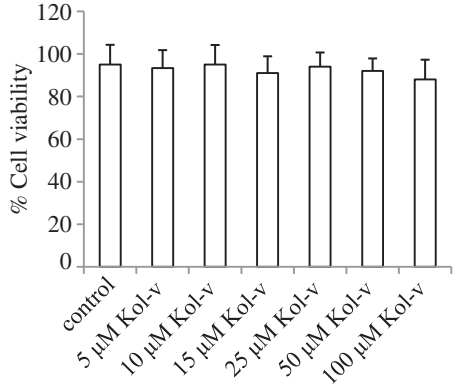
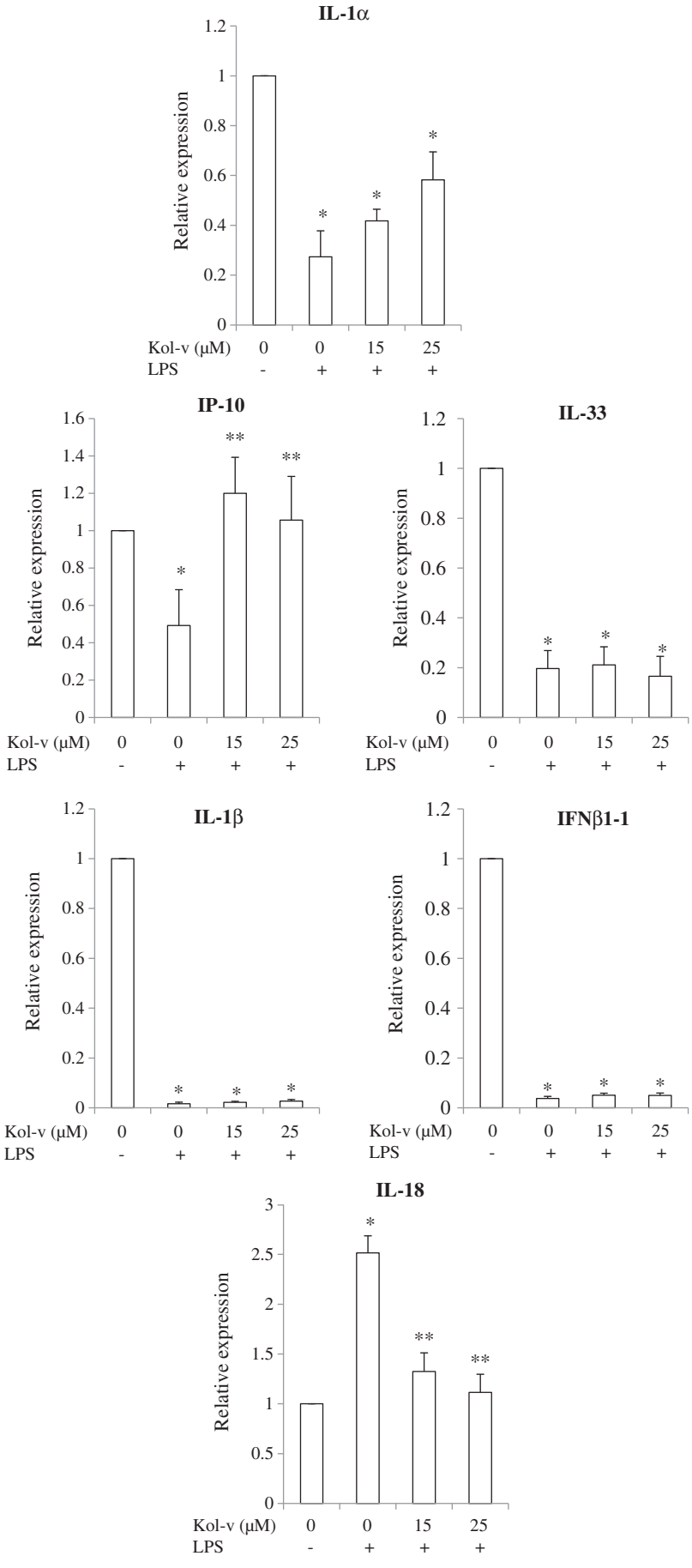


Fig. 2. Effects of Kol-v on RAW 264.7 macrophage viability. Cells were treated with Kol-v (0 μM to 100 μM) for 24 h. Cell viability was determined by the Trypan Blue Exclusion assay. Data are the mean ± S.D. of three independent experiments.


IL-1α
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Kol-v (μM) 0 0 15 25
LPS - + + +
IP-10
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Kol-v (μM) 0 0 15 25
LPS - + + +
IL-33
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Kol-v (μM) 0 0 15 25
LPS - + + +
IL-1β
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Kol-v (μM) 0 0 15 25
LPS - + + +
IFNβ1-1
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
Kol-v (μM) 0 0 15 25
LPS - + + +
IL-18
Relative expression
3
2.5
2
1.5
1
0.5
0
Kol-v (μM) 0 0 15 25
LPS - + + +

Kol-v significantly up-regulated mRNA expression of IP-10 and suppressed mRNA expression of IL-18 in LPS-stimulated RAW 264.7 cells. Kol-v pre-treatment had no effect on the LPS-induced decrease in the mRNA expressions of IL-1α, IL-1β, IL-33, and IFNβ1-1 (Fig. 3).

3.2. Effects of Kol-v on LPS-induced MAPK phosphorylation in RAW 264.7 macrophages

Previous studies have reported that the activation of MAPKs is crucial for LPS-induced NF-κB and AP-1 and the subsequent activation of pro-inflammatory mediators in macrophages [14]. To investigate whether the inhibition of inflammatory mediator secretion by Kol-v is mediated through the MAPK pathway, we examined the effect of Kol-v on LPS-induced phosphorylation of ERK1/2, JNK, and p38 MAPK by Western blot analysis using phospho-specific antibodies. As shown in Fig. 4A–F, Kol-v (15 and 25 μM) pre-treatment resulted in the inhibition of LPS-induced phosphorylation of p38, whereas it did not affect ERK1/2 and JNK phosphorylation and also the total level of ERK1/2. At higher Kol-v concentrations (50 and 100 μM), the pre-treatment resulted in the blockade of LPS-induced phosphorylation of ERK1/2 and p38, and only the 100 μM Kol-v could inhibit LPS-induced JNK phosphorylation. Taken together, these results suggest that Kol-v suppresses LPS-induced expressions of inflammatory mediators (IL-6 secretion and IL-18 mRNA expression) by inhibiting phosphorylation of JNK, p38, and ERK1/2 in RAW 264.7 macrophages and that different concentrations of Kol-v would be required to target each signalling molecule.

3.3. Effects of Kol-v on LPS-induced NF-κB phosphorylation in RAW 264.7 macrophages

NF-κB is one of the major transcription factors that are activated during the inflammatory response to LPS. The activation of NF-κB mediates the transcription of numerous pro-inflammatory mediators [22–24]. To determine if Kol-v has the ability to block the activation of NF-κB, macrophages were pre-treated with Kol-v (15 and 25 or 50 and 100 μM) for 22 h and then followed with LPS (100 ng/mL) challenge for 2 h. Such pre-treatment resulted in the suppression of LPS-induced phosphorylation of NF-κB (p65) at higher Kol-v concentrations (50 and 100 μM) but not at lower concentrations of Kol-v (15 and 25 μM). Moreover, Kol-v (15 and 25 μM) did not inhibit LPS-induced degradation of IκBα, and neither did it block LPS-induced phosphorylation of IκBα. However, pre-treatment with the higher Kol-v concentrations (50 and 100 μM) resulted in the blockade of LPS-induced IκBα phosphorylation in a concentration dependent manner (Fig. 5A–E).

3.4. Effects of Kol-v on LPS-induced Akt, AP-1, and CREB phosphorylation in RAW 264.7 macrophages

Along with NF-κB, the transcription factors AP-1, CREB, and Akt are also known to be involved in the transcriptional regulation of inflammatory responses [5,25,26]. Therefore, we examined the effect of Kol-v (15 and 25 μM) on LPS-induced activation of AP-1 such as phosphorylation of c-JUN. As shown in Fig. 6, pre-treatment with Kol-v inhibited LPS-induced phosphorylation of c-JUN. In addition, Kol-v (50 and 100 μM) pre-treatment resulted in the blockade of LPS-induced Akt but not CREB phosphorylation (Fig. 6A–C).

3.5. Effects of Kol-v on LPS-induced TNF-α and IL-6 secretion in RAW 264.7 macrophages

The levels of pro-inflammatory cytokines, such as TNF-α and IL-6 are increased during inflammatory and immune responses and so they play an important role during the development of inflammatory diseases. We further investigated the effect of Kol-v on production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. Macrophages were pre-treated with Kol v (10–100 μM) for 24 h and stimulated with LPS (100 ng/mL) for 2 h before the completion of the 24 h. The culture supernatants were analyzed for the levels of cytokines by ELISA. Kol-v significantly decreased LPS-induced secretion of IL-6 but not that of TNF-α in a concentration dependent manner (Fig. 7A and B).

4. Discussion

Persistent inflammatory response leads to overproduction of inflammatory mediators that are associated with various pathological diseases including cancers [1]. Thus, modulation of pro-inflammatory cytokines in the macrophages is considered one of the strategies to develop therapeutic compounds against several inflammatory diseases. In this study, we showed that Kol-v (10–100 μM) exerts its anti-inflammatory effects by suppressing LPS-induced secretion of IL-6 but not that of TNF-α in the murine macrophages. In addition, Kol-v (15 and 25 μM) modulated the LPS-induced changes in the mRNA expressions of IP-10 and IL-18 but not on the expression level of other inflammatory genes such as IL-33, IL-1β, and IFNβ1-1. The down-regulation of IL-33, IL-1β, and IFNβ1-1 by LPS and which was not abrogated by Kol-v pre-treatment could be due to the impairment of specific signalling pathway [27] and in response to a coordinated attempt by macrophages to limit the severity of inflammatory injury [28]. Although there was an increased tendency for the recovery in the gene expression level of IL-1α in the Kol-v pre-treated macrophages stimulated with LPS, the expression level was still lower compared to the control level. Thus, LPS-induced IL-6 secretion but not LPS-induced TNF-α secretion is involved in the anti-inflammatory effect of Kol-v in LPS-stimulated RAW macrophages.

The protective effects of Kol-v against inflammatory immune responses were mediated by blocking NF-κB–Akt–AP-1–p38/ERK1/2-JNK MAPK signalling pathways. However different concentrations of Kol-v were required to inhibit specific inflammatory signalling pathways. For example, the AP-1 pathway was more responsive to the anti-inflammatory effects of Kol-v at lower concentrations whereas higher concentrations of Kol-v were required to inhibit the LPS-induced activation of MAPK and NF-κB signalling pathways. Therefore, multiple signalling pathways are involved in the anti-inflammatory effects of Kol-v in a macrophage model of inflammatory injury. Furthermore, all concentrations of Kol-v tested in this study were ineffective in blocking LPS-induced TNF-α production. Inflammatory responses are regulated by several transcriptional factors such as NF-κB and AP-1. Upon stimulation, NF-κB and AP-1 translocate into the nucleus, where they mediate the expression of many pro-inflammatory genes. Signalling pathways resulting to the activation of NF-κB and AP-1 are involved in the production of the cytokines IL-6 and TNF-α in LPS-stimulated macrophages [9]. Because Kol-v pre-treatment significantly attenuated LPS-induced secretion of IL-6 but not LPS-induced secretion of TNF-α, it could suggest that the secretion of TNF-α in our experimental model was not dependent upon the NF-κB and AP-1 signalling pathways.

Fig. 3. Effects of Kol-v on LPS-induced expressions of inflammatory mediators in RAW 264.7 macrophage cells. Following pre-treatment with Kol-v (15 and 25 μM) for 2 h, cells were treated with LPS (100 ng/mL) for 1 h. Controls were not treated with LPS or Kol-v. Lysates were prepared from control, LPS (100 ng/mL) alone, or LPS plus Kol-v (15, 25 μM). Total RNA was prepared for quantitative real-time RT-PCR of IL-1α, IP-10, IL-33, IL-1β, IFNβ1-1, and IL-18 from RAW 264.7 macrophages. β-Actin was used as an internal control to normalize the data except for IL-18 wherein β-microglobulin was used. Data are presented as the mean ± S.D. of three experiments. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$ vs. the control group; **$p < 0.05$ vs. the LPS-stimulated group.

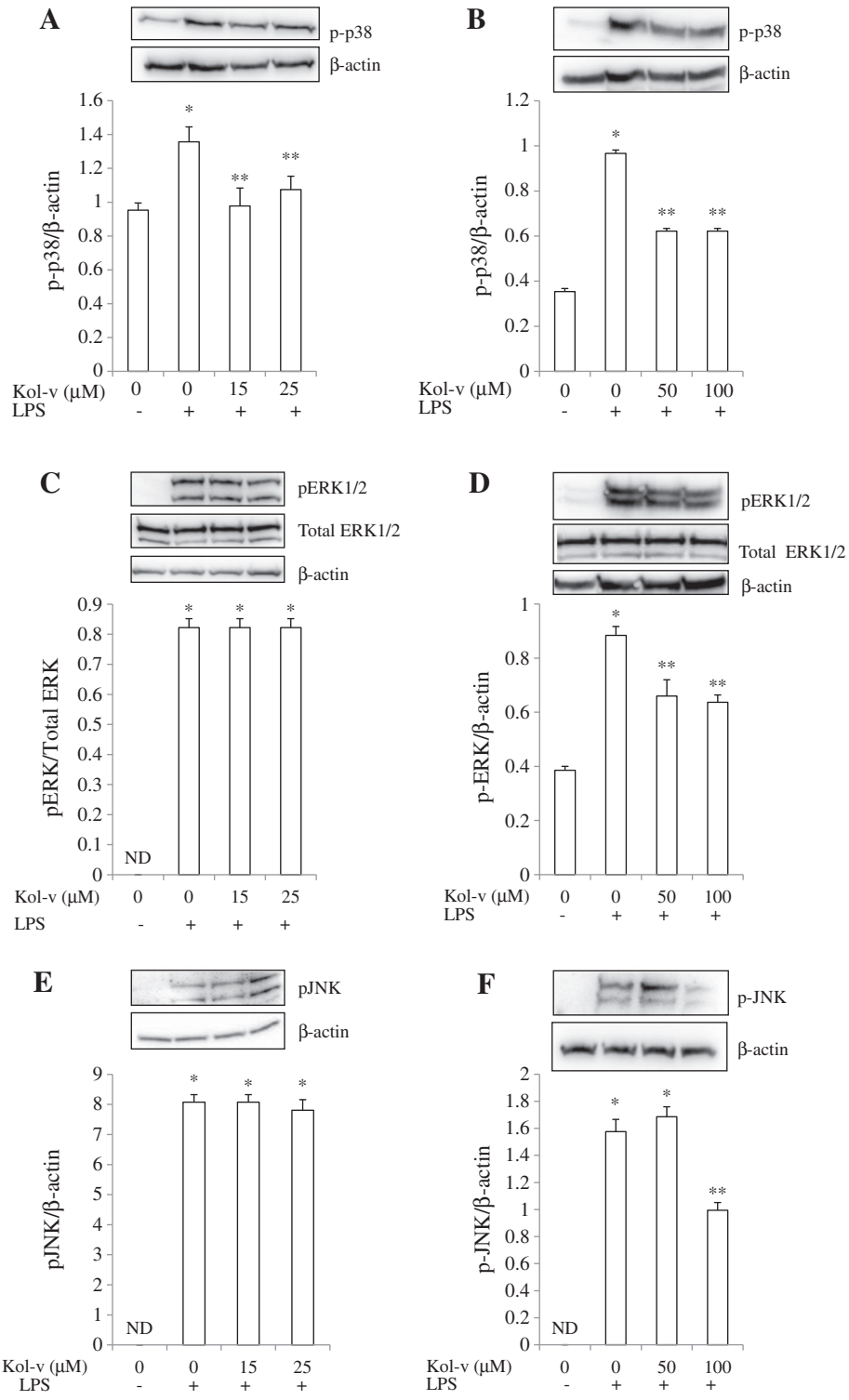


Fig. 4. Effects of Kol-v on LPS-induced p38, ERK1/2, and JNK phosphorylation in RAW 264.7 macrophage cells. Following pre-treatment with Kol-v (15 and 25 μM or 50 and 100 μM) for 24 h, cells were treated with LPS (100 ng/mL) for 2 h prior to the completion of the 24 h culture period. Lysates were prepared, and Western blotting was performed using specific phospho-p38, phospho-ERK1/2, and phospho-JNK antibodies. β actin was used as an internal control. Data are presented as the mean ± S.D. of three experiments. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$ vs. the control group; **$p < 0.05$ vs. the LPS-stimulated group.

We further demonstrated that Kol-v inhibited the phosphorylation of both NF-κB and c-JUN with the inhibition of c-JUN achieved at much lower Kol-v concentration than NF-κB. In addition, Kol-v suppressed LPS-induced phosphorylation of IκBα. Activation of NF-κB requires that IκBα be phosphorylated, which results in targeted degradation of IκBα. The dissociation of IκBα causes the translocation of NF-κB from the cytoplasm to the nucleus where it binds and triggers the inflammatory gene expression [6]. Previous reports indicated that Kol-v inhibits the production of nitric oxide, TNF-α and prostaglandin-E2 in LPS-activated macrophages [16]. These divergent results on TNF-α might be due to the different experimental models employed in our study and those of Olaleye et al. [16]. In the study by Olaleye

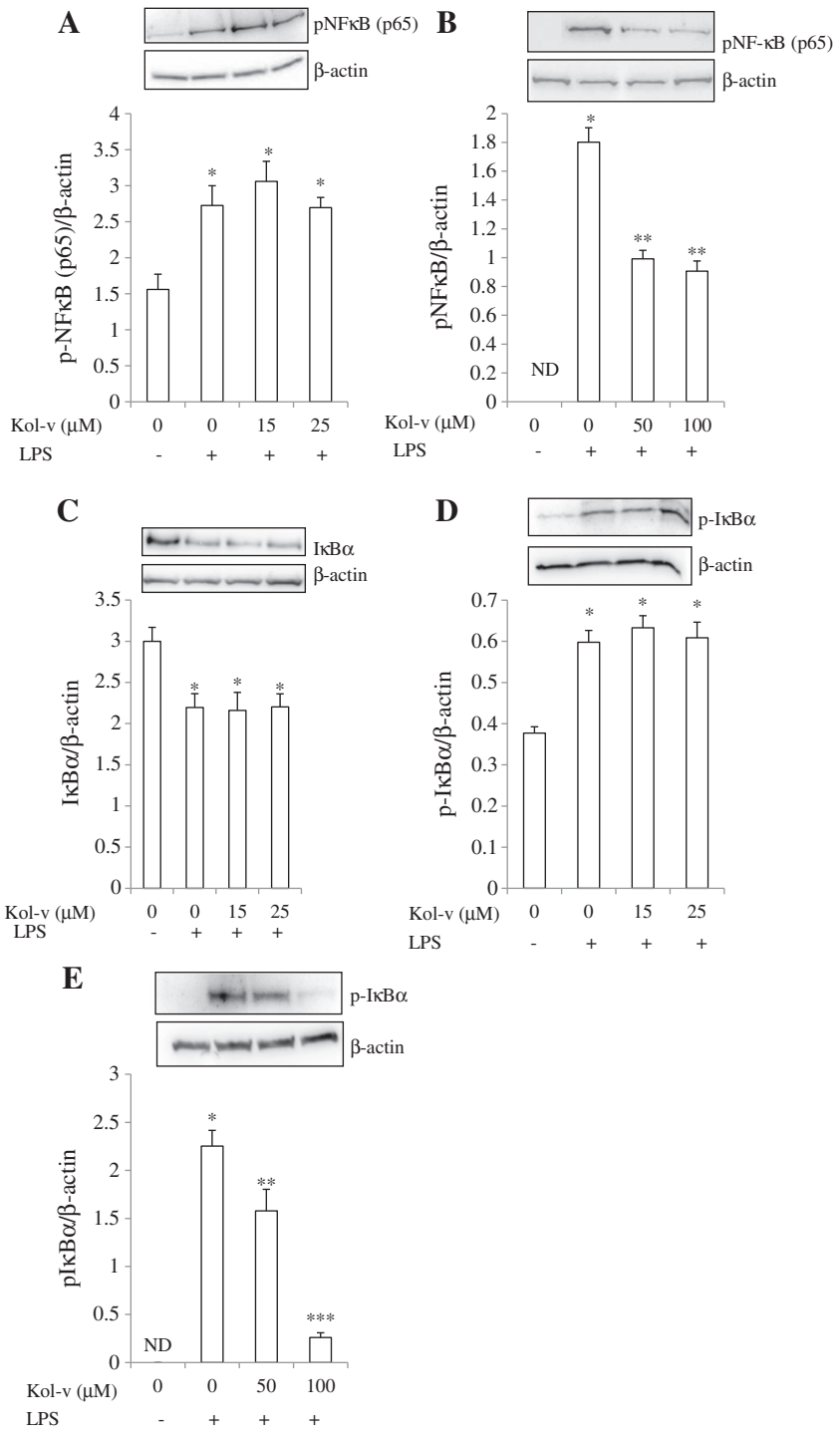


Fig. 5. Effects of Kol-v on LPS-induced NF-κB (p65) phosphorylation, IκBα degradation, and IκBα phosphorylation in RAW 264.7 macrophage cells. Following pre-treatment with Kol-v (15 and 25 μM or 50 and 100 μM) for 24 h, cells were treated with LPS (100 ng/mL) for 2 h prior to the completion of the 24 h culture period. Lysates were prepared, and Western blotting was performed using specific phospho-NF-κB (p65) antibodies. β-Actin was used as an internal control. Data are presented as the mean ± S.D. of three experiments. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$ vs. the control group; **$p < 0.05$ vs. the LPS-stimulated group; ***$p < 0.05$ vs. Kol-v (50 μM) pre-treated cells stimulated with LPS.

et al. [16], RAW macrophages were stimulated for 48 h with LPS (200 ng/mL) in the presence or absence of Kol-v, while we activated RAW macrophages with LPS (100 ng/mL) for only 2 h during the 24 h pre-treatment with Kol-v. Thus the dose of LPS used and the evaluation of cytokine release at different time points could be responsible for the divergent results [29]. Taken together, these data suggest that Kol-v, a class of biflavonoids, has anti-inflammatory effects which could be routed through the NF-κB–AP-1 signalling pathways to inhibit IL-6 production but not TNF-α secretion. Thus Kol-v may not be a potent flavonoid in inhibiting TNF-α release in murine macrophages. We further demonstrated that the LPS-induced phosphorylation of the MAPKs, ERK1/2, p38 and JNK was suppressed by Kol-v especially at higher concentrations (50 and 100 μM), and only the high Kol-v concentration (100 μM) was effective in the blockade of LPS-induced JNK phosphorylation while all the concentrations tested (15, 25, 50, and 100 μM) could suppress LPS-induced phosphorylation of p38. Some reports have

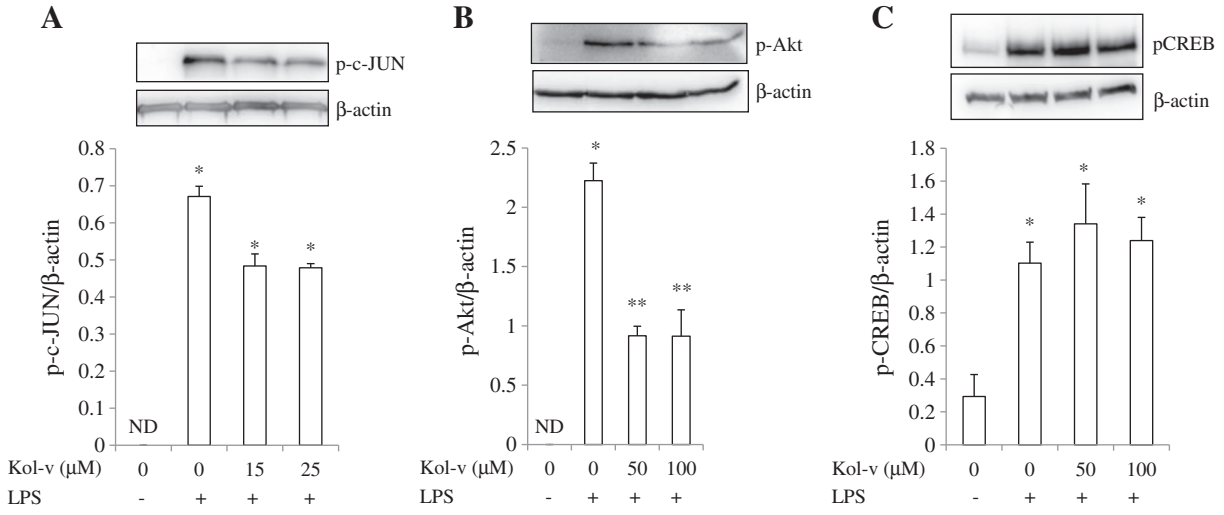


Fig. 6. Effects of Kol-v on LPS-induced c-JUN, Akt, and CREB phosphorylation in RAW 264.7 macrophage cells. Following pre-treatment with Kol-v (15 and 25 μM or 50 and 100 μM) for 24 h, cells were treated with LPS (100 ng/mL) for 2 h prior to the completion of the 24 h culture period. Lysates were prepared, and Western blotting was performed using specific phospho-c-JUN, phospho-Akt, and CREB, antibodies. β-Actin was used as an internal control. Data are presented as the mean ± S.D. of three experiments. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$ vs. the control group; **$p < 0.05$ vs. the LPS-stimulated group.

demonstrated the requirement of MAPKs for NF-κB activation [9,11,30]. These MAPKs regulate NF-κB activation through IκBα/β kinase activation which induces IκB degradation [31,32]. Thus, the NF-κB and AP-1 along with MAPKs may participate in the amplifying loop of the inflammatory responses in our experimental model, and the modulations of the three-tiered cascades provide a rationale to develop therapeutic agents against inflammatory diseases. Several studies have also shown that exposure of mammalian cells including macrophages/monocytes to LPS activates all known MAPK signalling cascades [5,11,33]. It is likely that the different MAPKs play a role in up-regulation of pro-inflammatory cytokines in LPS-stimulated macrophages. Previous studies reported that MAPKs especially p38/JNK have been implicated in the regulation of inflammatory mediators, including the pro-inflammatory cytokines, which make them potential targets for anti-inflammatory therapeutics [24,34]. Since p38 activation in the present study was more responsive to the inhibitory effects of lower concentration of Kol-v than the other MAPKs that require higher concentration of Kol-v, it is assumed that the anti-inflammatory effect of Kol-v in RAW 264.7 macrophages may depend primarily on the inhibition of the p38 MAPK activation.

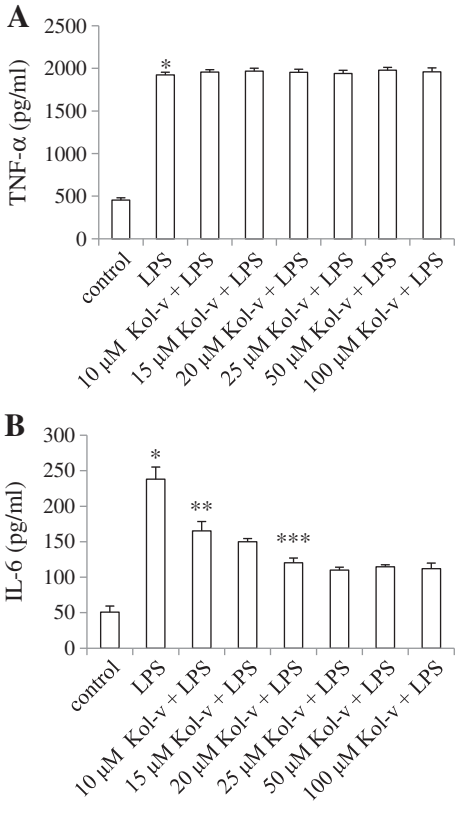


Fig. 7. Effects of Kol-v on LPS-induced production of TNF-α (A) and IL-6 (B) in RAW 264.7 macrophage cells. Following pre-treatment with Kol-v (10–100 μM) for 24 h, cells were treated with LPS (100 ng/mL) for 2 h prior to the completion of the 24 h culture period. Supernatants were collected and analyzed for TNF-α and IL-6 by ELISA. Data are presented as the mean ± S.D. of three experiments. Statistical analyses were performed by one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$ vs. the control group; **$p < 0.05$ vs. the LPS-stimulated group; ***$p < 0.05$ vs. Kol-v (15 μM) pre-treated cells stimulated with LPS.

Our study further demonstrated that Kol-v suppressed Akt phosphorylation in response to LPS stimulation of RAW 264.7 macrophages. Akt activation has been reported to stimulate cytokine production in macrophages [5,35] and other immune cells such as mast cells [36]. In agreement with our results, flavonoids are also known to inhibit LPS-induced production of pro-inflammatory cytokines such as IL-6 macrophages [35]. The suppression of Akt by Kol-v in our experimental model did not alter LPS-induced secretion of TNF-α but could suppress IL-6 production. We therefore conclude that the inhibition of NF-κB–MAPKs–AP-1–Akt-dependent IL-6 production is involved in the anti-inflammatory effects of Kol-v in this macrophage cell line. Conversely, these signalling pathways did not mediate LPS-induced TNF-α release in our experimental conditions.

We next examined the activation of another transcription factor, CREB which is known to have both pro-inflammatory and anti-inflammatory functions [26]. The activation of CREB is induced by a variety of growth factors and inflammatory signals [26]. As expected, LPS-induced CREB phosphorylation, but the activation of CREB by LPS was not suppressed by Kol-v even at Kol-v concentrations that suppressed LPS-induced phosphorylation of NF-κB–MAPKs–AP-1–Akt. Previous reports on the pro-inflammatory functions of CREB suggested that the activation CREB induces the transcription of pro-inflammatory genes such as IL-6, TNF-α and Cox-2 [26,37]. Because Kol-v was ineffective in blocking LPS-induced phosphorylation of CREB it could suggest that the CREB signalling pathway mediated LPS-induced TNF-α production in our experimental model. Thus the release of TNF-α in LPS-

stimulated macrophages pre-treated with Kol-v is because of an active CREB signalling pathway.

In conclusion, we provide evidence that Kol-v interferes with multiple intracellular targets (NF-κB–MAPKs–AP-1–Akt) but not CREB in LPS-stimulated macrophages. Thus the inhibition of these NF-κB–MAPKs–AP-1–Akt signalling pathways contributes to the inhibitory action of Kol-v on LPS-induced interleukin-6 release and the induced normalization in the gene expression levels of interferon-induced protein-10 (IP-10) and interleukin-18 (IL-18). Furthermore, as Kol-v could not suppress the LPS-induced phosphorylation of CREB, it was ineffective to inhibit the release of TNF-α and the normalization in the gene expression levels of interleukin-1α (IL-1α), interleukin-33 (IL-33), interleukin-1β (IL-1β), and interferon-β1 (IFNβ1). Further studies on the mechanisms by which Kol-v modulates the expression of pro-inflammatory mediators in experimental inflammatory models are therefore warranted.

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