

## Identification of the Bioactive Constituent and Its Mechanisms of Action in Mediating the Anti-Inflammatory Effects of Black Cohosh and Related *Cimicifuga* species on Human Primary Blood Macrophages

Cindy L. H. Yang,<sup>†</sup> Stanley C. C. Chik,<sup>†</sup> James C. B. Li,<sup>†,‡</sup> Benny K. W. Cheung,<sup>†,‡</sup> and Allan S. Y. Lau<sup>\*,†,‡</sup>

<sup>†</sup>Molecular Chinese Medicine Laboratory, Li Ka Shing Faculty of Medicine and <sup>‡</sup>Cytokine Biology Group, Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China

Received May 11, 2009

*Cimicifuga* species have been used as traditional medicinal herbs to treat inflammation and symptoms associated with menopause in Asia, Europe, and North America. However, the underlying mechanism of their anti-inflammatory effects remains to be investigated. With bioactivity guided purification involving the use of partitioning extraction and high performance liquid chromatography, we isolated one of the key bioactive constituents from the rhizome extracts of *Cimicifuga racemosa*. By NMR spectroscopy, the molecule was identified to be cimicracemate A (**1**). This compound (140  $\mu$ M) suppressed the lipopolysaccharide-induced TNF- $\alpha$  production in the blood macrophages by  $47 \pm 19\%$  and  $58 \pm 30\%$  at LPS concentrations of 1 ng/mL and 10 ng/mL, respectively. The anti-inflammatory activity of compound **1** may be due to its modulation of a signaling mitogen activated protein kinase and transcription factor nuclear factor-kappaB activities. Compound **1** was found in other *Cimicifuga* species. Our data indicate that compound **1** or its chemical analogues may have the potential to be further developed as a new class of therapeutic agent.

### Introduction

In response to injury and microbial invasion, the human host mounts inflammatory responses to control the pathogen and to initiate the repair process.<sup>1</sup> During inflammation, different immune cells including T-lymphocytes, neutrophils, and macrophages are recruited to the site of infection and produce cytokines to facilitate the immune response. Among these cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is one of the major proinflammatory proteins playing a pivotal role in mediating the immune defense.

In addition to acute phase response, TNF- $\alpha$  has been shown to be involved in the progression of various chronic diseases including tumorigenesis and rheumatoid arthritis. The dysregulation of TNF- $\alpha$  production was demonstrated to be involved in different stages of tumorigenesis including initiation of tumor growth,<sup>2</sup> cell proliferation,<sup>3</sup> and invasion.<sup>4</sup> For tumor cell proliferation, TNF- $\alpha$  upregulates specific growth factors to mediate the malignant growth. The cytokine promotes angiogenesis favoring growth of blood vessels to support the tumor migration, thus playing a key role in tumor metastasis. For example, our previous results showed that glioblastoma migration and induction of metalloproteinases were significantly enhanced in response to TNF- $\alpha$  effects.<sup>5</sup>

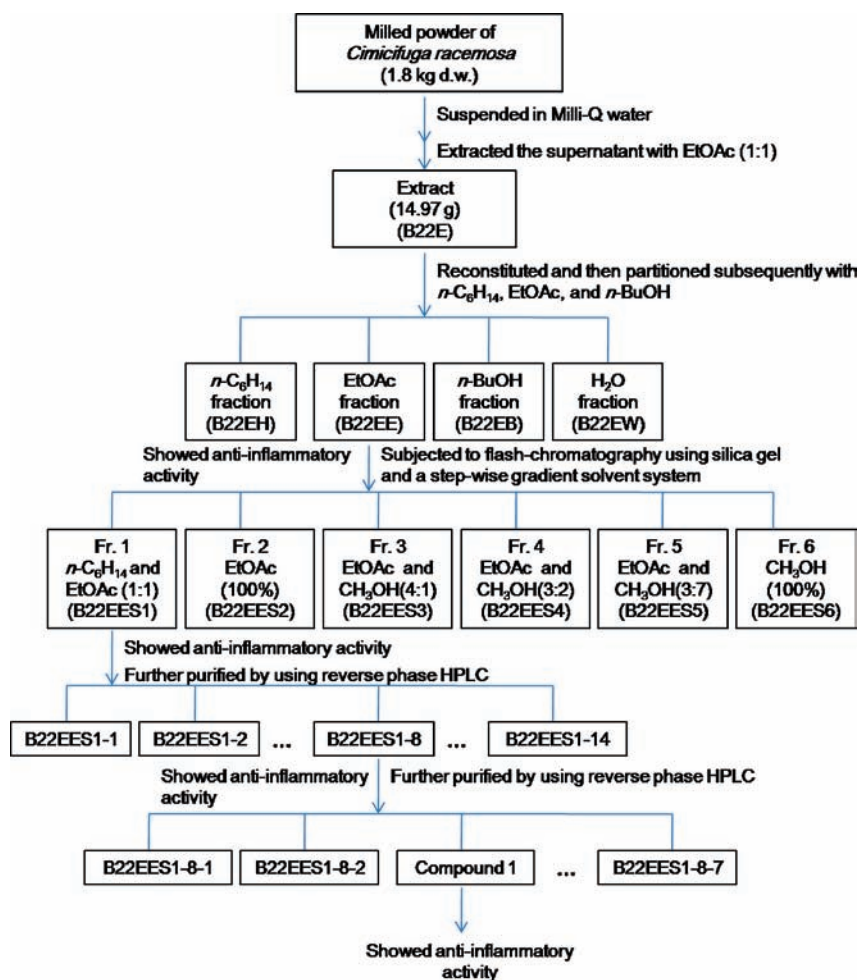
Examples of chronic disease pathogenesis mediated by TNF- $\alpha$  include rheumatoid arthritis and inflammatory bowel diseases.<sup>6,7</sup> In contrast to fulminant response to bacterial invasion in septic individuals, patients with rheumatoid arthritis have a low-grade insidious inflammation in the synovial tissues. It is known that overproduction of TNF- $\alpha$  in the inflamed joints leads to slow destruction of the joint cartilage and surrounding bone.<sup>8</sup>

During the acute phase of sepsis, uncontrolled production of TNF- $\alpha$  is well-known to cause deleterious effects to the host. The clinical outcome of infection leading to sepsis is primarily associated with excessive stimulation of the host immune cells, particularly monocytes or macrophages, by bacterial endotoxins (e.g., lipopolysaccharide [LPS]).<sup>9–11</sup> Macrophages overstimulated by LPS also produce high levels of mediators such as interleukin-1 (IL-1), IL-6, and TNF- $\alpha$ .<sup>12</sup> These mediators are implicated in the pathogenesis of sepsis and found to be contributing factors to the demise of the host. Hence, the development of novel therapies directed toward the inhibition of TNF- $\alpha$  production may help to aid in the treatment of these acute and chronic diseases described above.

Following exposure to pathogens and endotoxins, intracellular signaling pathways including specific kinases and transcription factors are activated to induce the expression of TNF- $\alpha$ . The involvement of mitogen-activated protein kinases (MAPK) and the nuclear factor kappa B (NF- $\kappa$ B) in the pathogen-induced TNF- $\alpha$  expression are well-documented.<sup>13–15</sup> In our previous studies, we demonstrated that mycobacteria, avian influenza viruses, and HIV-1 are the inducers of TNF- $\alpha$  through the MAPK.<sup>16–18</sup> There are three MAPK subtypes including extracellular signal-regulated kinase-1/2 (ERK 1/2),

\*Corresponding author. Allan S. Y. Lau, Molecular Chinese Medicine Laboratory, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China. Tel 852-28554269. Fax 852-28198142. E-mail asylau@hkucc.hku.hk.

<sup>a</sup> Abbreviations: PBMac, primary blood macrophage; LPS, lipopolysaccharide; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; NF- $\kappa$ B, transcription factor nuclear factor-kappaB.



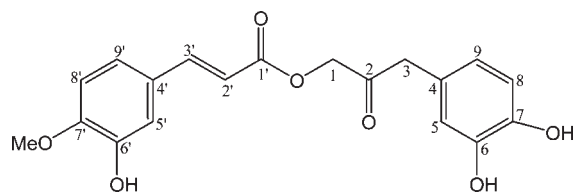
**Figure 1.** Extraction scheme of compound **1** from *C. racemosa*. The herb (1.8 kg) was milled and extracted with milli-Q water for 1 h with continuous sonication. The collected supernatant was then partitioned with ethyl acetate (EtOAc) (1:1). The resulting dried EtOAc extract was reconstituted and then sequentially partitioned with hexane ( $n\text{-C}_6\text{H}_{14}$ ), EtOAc, and butanol ( $n\text{-BuOH}$ ). Using this bioassay guided fractionation scheme, the fractions showing inhibitory effects on LPS-induced TNF- $\alpha$  production were subjected to silica gel 60A (35–75  $\mu\text{m}$ ) chromatography and reversed-phase high-performance liquid chromatography using gradient elution until a single compound with anti-inflammatory activity was obtained.

p38 kinase, and c-Jun N-terminal kinase (JNK)<sup>19–23</sup> known in humans. They transduce a variety of extracellular stimuli through a cascade of protein phosphorylations that lead to the activation of transcription factors such as NF- $\kappa$ B. The activation of NF- $\kappa$ B is crucial in cytokine production including IL-6 and TNF- $\alpha$ .<sup>16–18</sup> The process is achieved by the phosphorylation of I- $\kappa$ B at Ser32 and Ser36 via the I- $\kappa$ B kinase (IKK) signalosome complex, leading to dissociation of I- $\kappa$ B and NF- $\kappa$ B subunits<sup>24</sup> and consequent proteosomal degradation of I- $\kappa$ B.<sup>25</sup> The activated NF- $\kappa$ B is then translocated from the cytoplasm to the nucleus, where it binds to  $\kappa$ B binding sites in the promoter region of responsive genes, leading to the initiation of transcription of pro-inflammatory mediators. Because inappropriate activation of NF- $\kappa$ B is associated with a wide range of human diseases,<sup>26</sup> it has been considered as a plausible target for therapeutic intervention.

Nonsteroid anti-inflammatory drugs (NSAIDs), steroids, and cytotoxic drugs are well-known in treating inflammatory diseases. However, they may not be highly effective, and some of them are associated with severe adverse effects including gastrointestinal irritation and bleeding. In recent years, immunotherapeutics have been developed which aim at the neutralization of TNF- $\alpha$  and suppression of its undesirable

proinflammatory effects. These include soluble TNF- $\alpha$  receptor and anti-TNF- $\alpha$  antibody. Despite their novelty and efficacy in the arrest of disease progression, they are very expensive therapeutic regimens. There is a current trend for discovery of bioactive agents from natural sources, especially from microbes and plants, for the development of novel therapeutics. Plants contain a variety of previously unknown chemicals that may have potent biological effects including anti-inflammatory activities.<sup>27</sup> Identification of lead compounds from plants and delineation of their mechanisms of action are important to assess their potential for clinical uses.

*Cimicifuga rhizome* (black cohosh) has a long and diverse history of medicinal use in Eastern United States and Canada.<sup>28</sup> Historically, native American Indians used it to treat a variety of conditions including malaria, rheumatism, abnormalities in kidney function, sore throat, menstrual irregularities, and menopause.<sup>28–30</sup> In Asian countries including China, Japan, and Korea, *Cimicifuga racemosa* and its counterparts *C. heracleifolia*, *C. fetida*, and *C. dahurica* have been used to treat fever, pain, and inflammation.<sup>31,32</sup> Previous studies demonstrated the inhibitory effects of *C. racemosa* extracts on histamine, bradykinin, and cyclooxygenase-2 (COX-2) mediated inflammatory actions.<sup>33</sup> The extract also has protective



**Figure 2.** Chemical structure of compound 1.

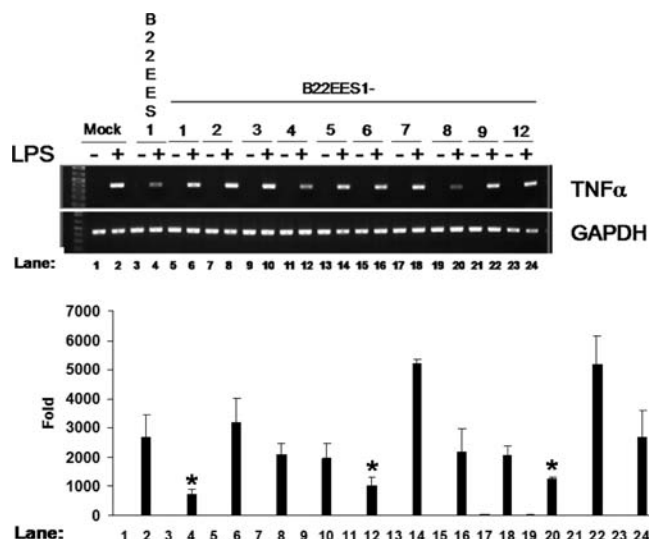
effects against menadione-induced DNA damage through its scavenging effects on reactive oxygen species.<sup>34</sup> In addition, *C. heracleifolia* extracts have been demonstrated to have their antiviral activities against respiratory syncytial virus.<sup>32</sup> In a recent study, *C. fetida* extracts were shown to induce apoptosis and cell cycle arrest of hepatocarcinoma cells, which are critical effects in inhibiting the tumor progression.<sup>35</sup>

In view of the beneficial role of *C. racemosa* and its counterparts as an anti-inflammatory agent, we isolated and identified a single compound from these herbs that possesses potent anti-inflammatory effects. In this project, we used a bioactivity-guided fractionation scheme involving the sequential use of partitioning extraction and high-performance liquid chromatography to purify the specific compound that can inhibit LPS-induced TNF- $\alpha$  production. Following purification, NMR spectroscopy was used and the molecule was identified to be compound 1. We further investigated its mechanism of suppressive action on signaling pathways and effects on NF- $\kappa$ B activity. These findings may have clinical implications for the potential use of the molecule and medicinal plants including *C. racemosa*, *C. heracleifolia*, *C. fetida*, and *C. dahurica* as alternative therapeutics.

## Results

**Extraction and Identification of Compound 1.** A light brown powder was obtained by repeated partitioning of the EtOAc fraction prepared from the rhizomes of *C. racemosa* and sequential chromatography on silica gel and reversed-phase HPLC. The detailed procedures were summarized in Figure 1. Using HPLC, a single compound was eluted at approximately 9.4 min with UV absorbance maximized at 290 and 325 nm (Figure S1, Supporting Information), which revealed that it has a conjugated aromatic system. The <sup>13</sup>C NMR spectra of compound 1 showed signals at  $\delta$  68.6 (*t*, C-1), 204.6 (*s*, C-2), 46.4 (*t*, C-3), 126.1 (*s*, C-4), 117.7 (*d*, C-5), 146.7 (*s*, C-6), 145.8 (*s*, C-7), 116.7 (*d*, C-8), 122.1 (*d*, C-9), 168.3 (*s*, C-1'), 115.3 (*d*, C-2'), 147.6 (*d*, C-3'), 128.9 (*s*, C-4'), 114.9 (*d*, C-5'), 148.2 (*s*, C-6'), 151.8 (*s*, C-7'), 112.6 (*d*, C-8'), 123.1 (*d*, C-9'), and 56.5 (*q*, MeO-7') (Figure S2, Supporting Information). Compound 1 showed a [M]<sup>−</sup> ion peak at *m/z* 357.0952 in its HR-ESI-MS (Figure S3, Supporting Information), consistent with the molecular formula C<sub>19</sub>H<sub>17</sub>O<sub>7</sub> (calc. 357.0974). In comparison of the spectroscopic data with the data reported in the literature, compound 1 was identified as shown in Figure 2.<sup>36</sup>

**Isolation of Compound 1 Using Bioassay Guided Approach.** Isolation of compound 1 from the extract of *C. racemosa* was done following our bioassay guided fractionation scheme. Individual extracts or fractions obtained from *C. racemosa* were examined for their suppressive effects on LPS-induced TNF- $\alpha$  induction in PBMac. The EtOAc extract of *C. racemosa* with anti-inflammatory activity was further separated into six fractions (Figure 1) and their bioactivities

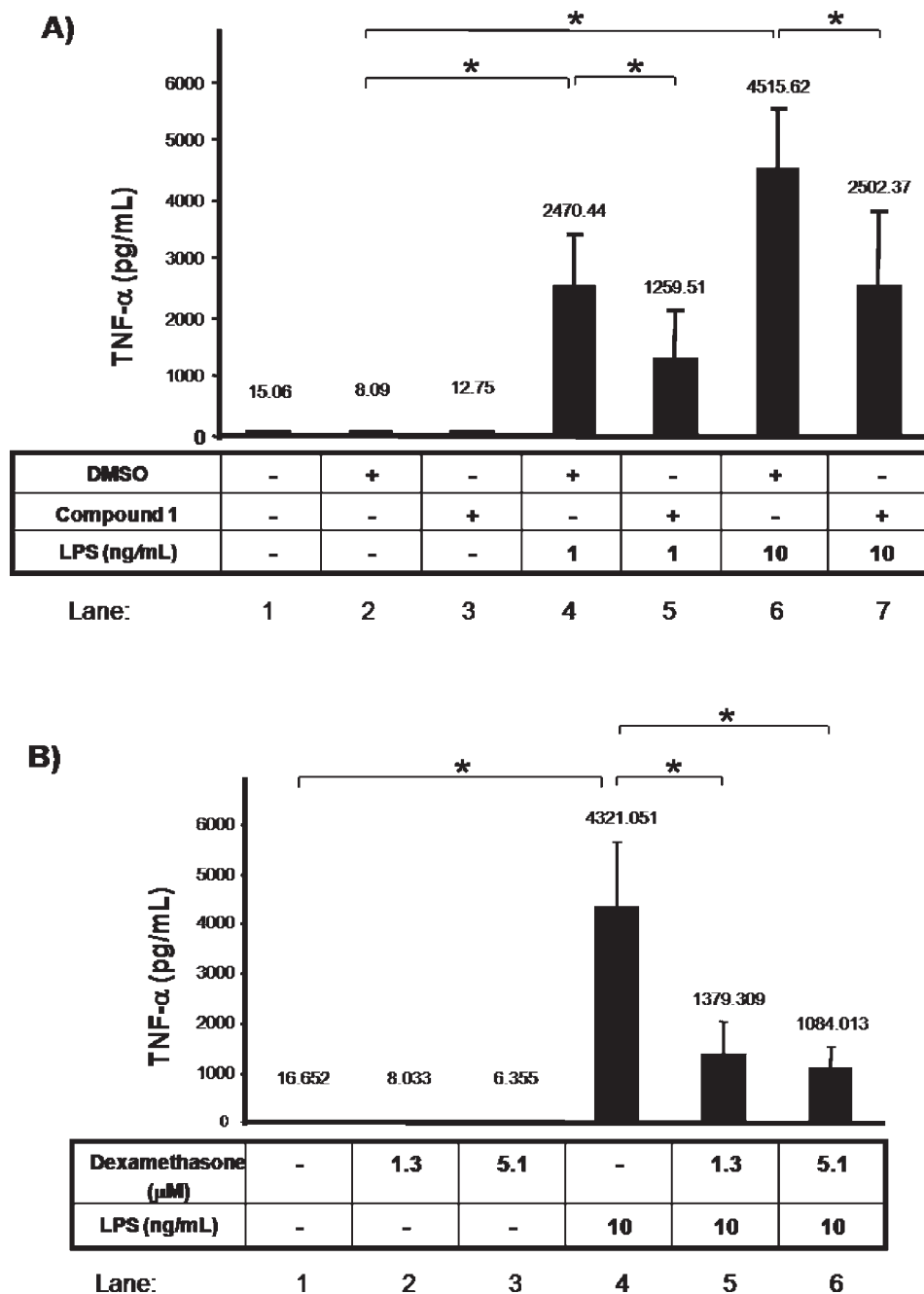


**Figure 3.** Bioassay guided fractionation of *C. racemosa*. PBMac were treated with different *C. racemosa* fractions at 100  $\mu$ g/mL for 24 h prior to the addition of 20 ng/mL LPS for 3 h. RT-PCR (upper panel) and quantitative RT-PCR (lower panel) assays of TNF- $\alpha$  and GAPDH were performed afterward. The results shown are representative of at least three independent experiments, with cells obtained from different donors. \*  $P < 0.05$ , compared with the corresponding control.

were shown in Figure S4 (Supporting Information). Using bioactivity assay, the fraction B22EES1 was shown to have inhibitory effects on the LPS-induced TNF- $\alpha$  mRNA expression, when compared to the mock (Figure 3, lanes 2 vs 4). Among the subfractions of B22EES1, only B22EES1-4 and B22EES1-8 retained the suppressive activities for TNF- $\alpha$  induction (Figure 3, lanes 12 and 20). With additional purification steps, B22EES1-8 was further subfractionated and purified to obtain compound 1.

**Effects of Compound 1 on LPS-Induced Cytokine Production.** To confirm the activities of compound 1 in suppressing TNF- $\alpha$  production, compound 1 was incubated with PBMac for 24 h prior to the addition of LPS at concentrations of 1 ng/mL and 10 ng/mL. The culture supernatants were collected and measured for the levels of secreted TNF- $\alpha$  by ELISA. The results showed that compound 1 (140  $\mu$ M) inhibited the LPS-induced TNF- $\alpha$  protein production by  $47 \pm 19\%$  and  $58 \pm 30\%$  at LPS concentrations of 1 ng/mL and 10 ng/mL, respectively (Figure 4A, lane 4 vs 5 and lane 6 vs 7). The range of variation is due to the fact that primary blood macrophages are used to perform the experiments. Dexamethasone, a potent immunosuppressive corticosteroid, was used as a prototype drug to cause a significant inhibition of LPS-induced TNF- $\alpha$  production by  $32 \pm 7.5\%$  and  $25 \pm 6.3\%$  at concentrations of 1.3 and 5.1  $\mu$ M (Figure 4B), respectively.

**Mechanisms of Cytokine Downregulation by Compound 1.** Next, we further elucidated the molecular pathways involved in the inhibition of LPS-induced TNF- $\alpha$  production by compound 1. It is well-documented that the activation of cytokine production in LPS-treated cells is initiated by the binding of LPS to its receptor.<sup>37</sup> After binding to the receptor, a cascade of signaling kinases is activated. Among the activated kinases, MAPK play a crucial role in LPS-induced cytokine production. Previous studies including ours illustrated that the induction of TNF- $\alpha$  by LPS and other pathogens required the phosphorylation and activation



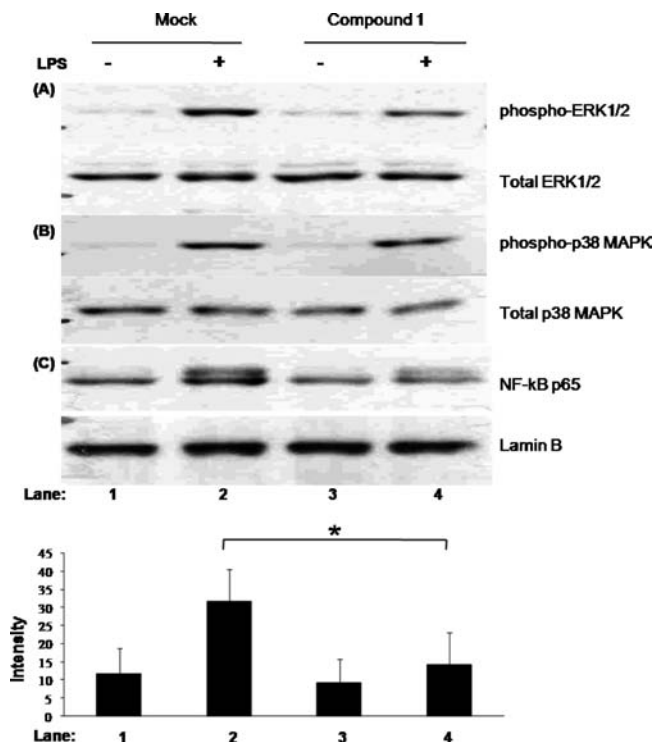
**Figure 4.** Inhibition of LPS-induced TNF- $\alpha$  production by compound **1** and dexamethasone. PBMac were incubated with (A) 140  $\mu$ M compound **1** or (B) 1.3 or 5.1  $\mu$ M of dexamethasone for 24 h prior to the addition of 1 ng/mL and 10 ng/mL LPS for another 24 h. The culture supernatants were collected and assayed for TNF- $\alpha$  by ELISA. The results shown are the mean values  $\pm$  standard derivation (S.D.) of six independent experiments, with cells obtained from different donors. \*  $P < 0.05$ , compared with the corresponding control.

of ERK1/2 and p38 MAPK.<sup>16,18,38</sup> In order to investigate the effect of compound **1** on LPS stimulation of MAPK, PBMac was treated with compound **1** for 24 h and followed by the addition of LPS for 15 min. The Western blot results showed that the ERK1/2 and p38 MAPK phosphorylations were induced after LPS treatment as expected (Figure 5, lane 2). Pretreatment with compound **1** suppressed the LPS-induced phosphorylation of ERK1/2 (Figure 5A, lane 2 vs 4) but not p38 MAPK (Figure 5B, lane 2 vs 4). These results demonstrated that the anti-inflammatory activity of compound **1** may be due to its inhibition of ERK1/2 phosphorylation.

Along the signaling pathways regulated by MAPK in response to LPS treatment, activation of the transcription

factor NF- $\kappa$ B plays a critical role in the induction of TNF- $\alpha$ .<sup>39</sup> The activation of NF- $\kappa$ B involves degradation of its specific inhibitor I $\kappa$ B and translocation of NF- $\kappa$ B subunits from the cytoplasm to the nucleus. In our experiments, the results showed that the addition of compound **1** to PBMac for 24 h prior to the addition of LPS reduced the amount of p65NF- $\kappa$ B in the nuclear fraction (Figure 5C, lane 2 vs 4), indicating that the translocation of p65NF- $\kappa$ B to the nucleus was inhibited by compound **1**. In general, our results revealed that compound **1** could inhibit LPS-induced kinase activities and their consequent activation of the nuclear transcription factor for TNF- $\alpha$  transcription.





**Figure 5.** The effects of compound **1** on LPS-induced phosphorylation (phospho-) of ERK1/2, p38 MAPK, and nuclear translocation of NF- $\kappa$ B p65. PBMac were incubated with compound **1** (140  $\mu$ M) for 24 h prior to the addition of 10 ng/mL LPS for an additional 15 min. Cytoplasmic (Figure 5A,B) and nuclear proteins (Figure 5C) were harvested for Western Blotting: (A) Cytoplasmic proteins: phospho-ERK1/2 and total ERK1/2. (B) Cytoplasmic proteins: phospho-p38 and total p38 kinase. (C) Nuclear proteins: upper panel, NF- $\kappa$ B p65 and lamin B; lower panel, the intensity of corresponding lanes in the gel photograph of NF- $\kappa$ B p65 was shown. The results shown are representative of at least three independent experiments, with cells obtained from different donors. \*  $P < 0.05$ , compared with the corresponding control.

**Determination of Compound 1 in Different Species of *Cimicifuga*.** Under the same UPLC and ESI-MS conditions, the retention time and the mass-to-charge ratio of compound **1** were compared to the chromatograms and spectra of CF22EES1–8 and CH22EES1–8. In Figure 6B and C, both samples had a peak with retention time at approximate 6 min with an ion peak at  $m/z$  357 that was the same as that of compound **1** (Figure 6A). The results revealed that herbs including *C. fetida* and *C. heracleifolia* contained compound **1**. These herbs containing compound **1** may exert similar biological effects.

## Discussion and Conclusions

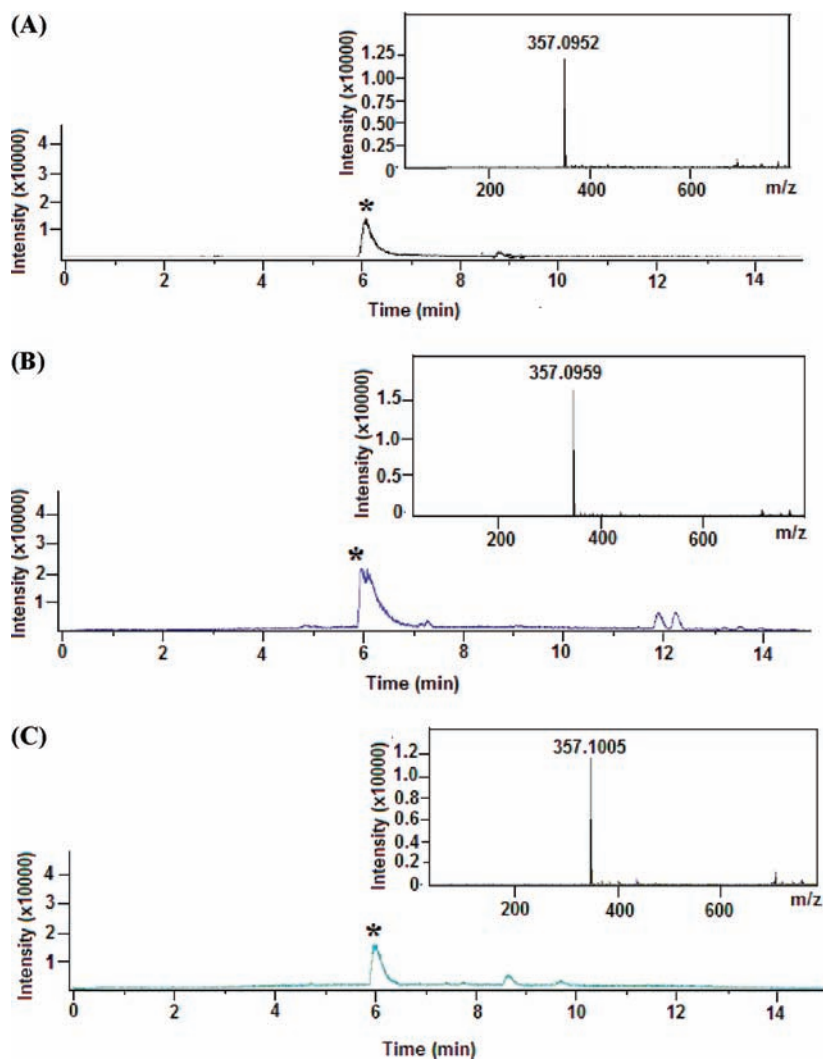
In the current treatment for advanced stages of rheumatoid arthritis, psoriasis, psoriatic arthritis, and ankylosing spondylitis, monoclonal TNF- $\alpha$  antibody plays an important role in the control of disease progression. Similarly, several randomized, double-blind, placebo-controlled clinical trials had been performed in patients with Crohn's disease. The results of these clinical trials showed that the anti-TNF- $\alpha$  antibody has beneficial effects for the patients.<sup>40</sup> Additionally, recent studies showed that inflammatory responses including TNF- $\alpha$  production may play an important role in the pathogenesis of cardiovascular diseases. It was suggested that TNF- $\alpha$  may destabilize the atherogenesis and atherosclerotic plaques

leading to their rupture, resulting in myocardial infarction or stroke in the susceptible patients.

During microbial infection, macrophages are activated to produce cytokines to mediate immune response. Depending on the invading microbe and its biological properties, the host immune system utilizes different sets of cytokines to combat the invading pathogen locally and systemically. A good example is mycobacterial infection, in which the proinflammatory cytokines TNF- $\alpha$ , induced via PKR and MAPK activity,<sup>16,41,42</sup> plays a critical role in host survival by propagating inflammation to contain the microbes by the formation of granuloma.<sup>43</sup> The protective role of TNF- $\alpha$  in controlling mycobacterial growth is exemplified by the resurgence of tuberculosis in patients who are receiving anti-TNF- $\alpha$  therapy including specific antibody or its soluble TNF- $\alpha$  receptor.<sup>44</sup> Although the effects of proinflammatory cytokines are protective, their overproduction may have deleterious effects to the host. In fact, uncontrolled induction of proinflammatory cytokines can lead to complications such as hypotension, organ failure, and even death in patients.<sup>45,46</sup> Indeed, the overproduction of TNF- $\alpha$  in endotoxemia patients leads to serious consequences including shock and kidney failure. In chronic diseases such as rheumatoid arthritis, TNF- $\alpha$  overexpression is known to be a major damaging factor and is associated with progressive joint destruction.<sup>47</sup>

In view of the efficacy of anti-TNF- $\alpha$  treatment for several autoimmune diseases, we used a bioassay guided approach to identify novel anti-inflammatory molecules in medicinal herbs. This approach can be used to investigate the biological activities of different herbal extracts and may be useful for the purification and identification of bioactive constituents. After five rounds of extraction, we were able to identify a single compound known as compound **1** that possesses our targeted biological activities, i.e., inhibition of TNF- $\alpha$  induction. With its previously unknown immunomodulatory property, this compound was described as having protective properties against Menadione-induced DNA damage.<sup>34</sup> After the identification of compound **1**, we specifically compared its biological activities with dexamethasone, a commonly used drug for immunosuppression. We observed that incubation with compound **1** ameliorated the LPS-upregulated TNF- $\alpha$  production by 47% and 58% with concentrations of LPS as inducers at 1 ng/mL and 10 ng/mL, respectively (Figure 4A). As the active concentration of compound **1** is 100 times higher than the tested dexamethasone, it seems less effective than dexamethasone (Figure 4B). Dexamethasone is an effective drug used in the treatment of many autoimmune diseases. However, it is well-known to have side effects to the patients. Since compound **1** was isolated from the herbs of *Cimicifuga* species, there is a long historical record of the herb's usage for inflammatory conditions. With the use of current chemical engineering technology, compound **1** can be further modified to increase the efficacy and lower the toxicity. Thus, compound **1** may be used as a lead compound for further development into a drug, and hopefully with fewer adverse effects.

The MAPK signal transduction pathway is well-known to play a crucial role in many aspects of immune mediated inflammatory responses.<sup>48,49</sup> Upon activation of MAPK, the transcription factors are phosphorylated and activated to result in a biological response. By blocking the kinases cascade, the pathogen-activated signaling cascade and consequent induction of inflammatory cytokines will be abrogated. Therefore, these kinases have become attractive targets for anti-inflammatory therapy.<sup>50</sup> We found that compound **1** strongly inhibited ERK1/2 induced by LPS in PBMac, resulting



**Figure 6.** UPLC chromatograms and HRESI-MS spectra of (A) compound **1**, (B) CF22EES1–8, and (C) CH22EES1–8. Herbs *C. fetida* and *C. heracleifolia* were extracted following the extraction procedure of *C. racemosa*. Their fractions (CF22EES1–8 and CH22EES1–8) were injected into an UPLC-coupled high-resolution ESI-TOF-MS using the same condition as that of compound **1**. The chromatograms showed the presence of a compound (with \*) with retention time at approximately 6 min and with an ion peak at 357 *m/z*.

in the attenuation of TNF- $\alpha$  production. It is possible that compound **1** may function similarly to an existing MEK inhibitor, 2-(2-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluoro-benzamide, by inhibiting ERK activity.<sup>51</sup>

NF- $\kappa$ B is the key transcription factor responsible for transcriptional activation of TNF- $\alpha$ . During macrophage activation, I- $\kappa$ B was dissociated from the NF- $\kappa$ B/I- $\kappa$ B cytosolic complex, leading to the translocation of p65NF- $\kappa$ B to the nucleus resulting in transcription of specific proinflammatory genes. Here, we found that compound **1** inhibited the translocation of p65NF- $\kappa$ B to the nucleus. These results indicated that compound **1** may inhibit LPS induction of TNF- $\alpha$  production by perturbing the NF- $\kappa$ B signaling pathway.

Triterpene glycosides and aromatic acids are the main classes of compounds from *C. racemosa*.<sup>52</sup> Among them, a previous report showed that phenolic acids such as ferulic and isoferulic acids have an anti-inflammatory effect by inhibiting the production of macrophage inflammatory protein-2 (MIP-2) in a murine macrophage cell line (RAW264.7) in response to respiratory syncytial virus infection.<sup>53</sup> In our report, compound **1** was isolated as a phenylpropanoid ester formed between isoferulic acid and 3-(30,40-dihydroxyphenyl)-2-keto-propanol<sup>54</sup>

with a hydroxyl and a methoxy substituent on the aromatic ring. As compound **1** has a structure similar to that of isoferulic and ferulic acids, this may explain why they have similar anti-inflammatory activity.

In summary, we have isolated and identified compound **1** from *C. racemosa* and its Chinese herbal counterparts using our custom-designed isolation and bioassay-guided procedures. Our results showed the effects of compound **1** on the regulation of cytokines via its activity in the modulation of signaling kinase and transcription factor activities. Compound **1** and its analogues may have the potential to be developed further into a new class of drugs. Additionally, our study demonstrated that compound **1** suppresses mitogen-induced inflammatory responses, which may suggest additional uses of this molecule for other clinical conditions. Since overproduction of TNF- $\alpha$  is deleterious to the host and can result in severe complications, limiting the overwhelming inflammatory response may be beneficial to patients in clinical management. Here, we have identified a novel function for compound **1** with its active anti-inflammatory property in *C. racemosa* and its Chinese counterparts, and that may open up a new area in the potential therapeutic uses of the herbs.

Additional experiments will be done to prove the anti-inflammatory activity of black cohosh and related herbs *in vivo*.

## Materials and Methods

**Plant Material.** *Cimicifuga racemosa* was purchased from the Glenbrook Farms Herbs and Such, Campbellsville, Kentucky, lot number 9459).

**Extraction and Isolation of the Bioactive Molecules.** The procedures for plant extraction were shown in Figure 1. Briefly, *C. racemosa* (1.8 kg) was milled, homogenized, and then suspended in (1:5) milli-Q water for 1 h with continuous sonication. The supernatant was filtered through an analytical filter paper and then partitioned three times with ethyl acetate (EtOAc) (1:1). The resulting EtOAc extract was concentrated to dryness *in vacuo* (35 °C) to yield 14.97 g of a dark brown residue. The residue was reconstituted in methanol (MeOH) and then fractionated by partitioning with hexane (*n*-C<sub>6</sub>H<sub>14</sub>). The MeOH fraction was concentrated and reconstituted in H<sub>2</sub>O and then partitioned sequentially with EtOAc and butanol (*n*-BuOH). Four fractions, namely, *n*-C<sub>6</sub>H<sub>14</sub>, EtOAc, *n*-BuOH, and H<sub>2</sub>O fractions were obtained. Using our bioactivity guided fractionation scheme, the fraction which showed inhibitory effects on LPS-induced TNF- $\alpha$  production was subjected to additional silica gel 60A (35–75  $\mu$ m) chromatography using *n*-C<sub>6</sub>H<sub>14</sub>, EtOAc, and MeOH to yield six fractions. The active fractions were further purified by reversed-phase high-performance liquid chromatography (HPLC) (Lichrospher 100 RP C<sub>18</sub> EC 5  $\mu$ m, 250  $\times$  4.6 mm ID) using a gradient elution from 25% acetonitrile (CH<sub>3</sub>CN) to 90% CH<sub>3</sub>CN at a flow rate of 1 mL/min. Peak detection was achieved using an Agilent 1200 series of fast scanning photodiode array detector set at 210, 254, and 280 nm. Eluting peaks were scanned between 200 and 300 nm with 1 nm intervals to determine absorbance maxima and minima. By repeating the purification process using HPLC, a pure compound (29 mg) with purity greater than 95% was obtained. (Figure S1, Supporting Information).

**Elucidation of the Molecular Structure.** The structure of the resulting pure compound **1** was elucidated by using a Bruker 500 MHz DRX NMR spectrometer, operating at 500 MHz for <sup>1</sup>H and at 125.765 MHz for <sup>13</sup>C NMR, using methanol-*d* as the solvent. Distortionless enhancement by polarization transfer (DEPT) experiments was performed using a transfer pulse of 135° to obtain positive signals for CH and CH<sub>3</sub> and negative signals for CH<sub>2</sub>. HR-ESI-MS was performed on a microTOF II ESI-TOF mass spectrometer (Bruker Daltonics). Data sets were acquired in negative electrospray (ESI) mode in a scan ranging from 100 to 1600 *m/z* at a sampling rate of 2 Hz. ESI parameters were as follows: capillary, 3.2 kV; nebulizer pressure, 4 bar; dry gas flow, 8 L/min; and dry gas temperature, 200 °C.

**Cell Culture and Primary Blood Macrophage Isolation.** Human peripheral blood monocytic cells were isolated from the buffy coat of healthy donor blood supplied by Hong Kong Red Cross by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation as described in our previous reports.<sup>16–18</sup> Briefly, the buffy coat was spun at 3000 rotations per min (rpm) for 15 min to separate the blood cells and the plasma. The heat inactivated serum was filtered for future use. The cell layer was diluted with phosphate buffered saline (PBS) in a ratio of 1:1. The diluted cells were overlaid on Ficoll-Paque slowly and centrifuged at 2300 rpm for 20 min for separation of mononuclear cells from erythrocytes. The mononuclear cell layer was removed and washed with RPMI 1640 medium until the supernatant was clear. The cells were finally resuspended in RPMI 1640 medium supplemented with 5% autologous serum and cultured for 1 h. The nonadherent cells were removed afterward, and the remaining adherent cells were further incubated for another 24 h at 37 °C in 5% carbon dioxide (CO<sub>2</sub>). The adherent monocytic cells were detached and seeded onto tissue culture plates and incubated for another 7–14 days

in order to differentiate the freshly isolated monocytic cells to primary blood macrophages (PBMac).

**Isolation of RNA and Reverse Transcription.** Total RNA from PBMac with or without treatment of *C. racemosa* extracts/fractions was extracted by TRIzol (Invitrogen). Reverse transcription (RT) of mRNA (mRNA) to cDNA (cDNA) was done by using the SuperScript II system (Invitrogen) as per the manufacturer's instruction.

**Polymerase Chain Reaction (PCR) and Quantitative RT-PCR.** Semiquantitative PCR assays of targeted genes were performed in a 25  $\mu$ L reaction mixture containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleoside triphosphate, 0.25  $\mu$ M of each primer, 2 U of *Taq* polymerase (Amersham Pharmacia Biotech, Piscataway, NJ), and 1  $\mu$ L of cDNA. PCR primer sets for TNF- $\alpha$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows. TNF- $\alpha$  (upstream, 5'-GGCTCCAGGCGGTGCTTGTC-3'; downstream, 5'-AG-ACGGCGATGCGGCTGATG-3'), and GAPDH (upstream, 5'-ACCACAGTCCATGCCATCAC-3'; downstream, 5'-TCC-ACCACCCTGTTGCTGTA-3'). The thermal cycling condition for PCR was 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The cycling reactions were repeated for 24 more cycles.

For Quantitative RT-PCR, it was performed according to the manufacturer's instructions by using Applied Biosystems TaqMan Universal Master Mix. The TNF- $\alpha$  TaqMan probes were purchased from Applied Biosystems (Foster City, CA), and GAPDH RNA was used as an internal control. Samples were allowed to run in triplicate in each Quantitative RT-PCR assay.

**Enzyme-Linked ImmunoSorbent Assay (ELISA).** Culture supernatants of the LPS-treated PBMac with or without compound **1** pretreatment were collected at different time intervals and stored at –70 °C. The levels of the secreted TNF- $\alpha$  were measured by ELISA kits specific for the cytokine (R&D system, Minneapolis, MN).

**Preparation of Cellular Extracts.** For collection of whole cell lysates, PBMac were washed with cold PBS and incubated in cold lysis buffer (50 mM tris(hydroxymethyl)aminomethane-chloride (Tris-Cl) [pH 7.4]; 150 mM sodium chloride (NaCl); 50 mM sodium fluoride (NaF); 10 mM  $\beta$ -glycerophosphate; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 10% glycerol; 1% Triton X-100; 1 mM phenylmethanesulfonylfluoride (PMSF); 1 mM sodium orthovanadate; 2  $\mu$ g/mL pepstatin A; 2  $\mu$ g/mL aprotinin; and 2  $\mu$ g/mL leupeptin) for 20 min. The lysate was then centrifuged at 4 °C for 20 min. The supernatant was collected and stored at –70 °C until use.

To collect nuclear protein extracts, the treated cells were washed with PBS and resuspended in buffer A (10 mM N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) [pH 7.9], 10 mM potassium chloride (KCl), 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), 2  $\mu$ g aprotinin, 1 mM sodium orthovanadate, 2  $\mu$ g/mL pepstatin A, 2  $\mu$ g/mL leupeptin, and 50 mM NaF) for 15 min. After that, NP-40 at a final concentration of 0.625% was added and mixed vigorously for cell lysis. The cell lysate was centrifuged, and the supernatant containing cytoplasmic proteins was collected for storage at –70 °C. The nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) for 15 min on ice to complete the lysis of nuclear membrane. The nuclear lysate was then centrifuged, and the supernatant containing the nuclear proteins was collected and stored at –70 °C.<sup>16,55</sup>

**Western Blot Analysis.** Whole cell lysate (20  $\mu$ g) or nuclear protein (2  $\mu$ g) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for probing overnight with the respective antibodies specific for the phosphorylated or total form of ERK1/2 and p38 MAPK (Cell Signaling Technology, Beverly, MA), NF- $\kappa$ B p65 protein, and lamin B (Santa Cruz



Biotechnology, Santa Cruz, CA). The membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (BD Transduction Lab, San Diego, CA). The signal was visualized by using enhanced chemiluminescence kit (Amersham Pharmacia Biotech). In order to quantify the results from the Western blots, the gels were scanned and the intensity of the bands were analyzed by a computer program Quantity One from BioRad.

**Determination the Presence of Compound 1 in *C. fetida* and *C. heracleifolia*.** *C. fetida* and *C. heracleifolia* were provided by Purafarm International (H.K.) Ltd. They were extracted following the extraction procedure of *C. racemosa* as described above. Compound 1 and the other fractions of *C. fetida* and *C. heracleifolia* (CF22EES1–8 and CH22EES1–8) were injected separately into an Acquity UPLC system (Waters, USA) equipped with an Xterra MS C18 column (150 × 2.1 mm ID, 3.5 μm). Chromatographic separations were performed using a gradient elution from 25% acetonitrile (CH<sub>3</sub>CN) to 90% CH<sub>3</sub>CN at a flow rate of 200 μL/min. Eluted compounds were detected using a micrOTOF II ESI-TOF mass spectrometer (Bruker Daltonics). Data sets were acquired in negative electrospray (ESI) mode in a scan ranging from 100 to 1600 *m/z* at a sampling rate of 2 Hz. ESI parameters were as follows: capillary, 3.2 kV; nebulizer pressure, 4 bar; dry gas flow, 8 L/min; and dry gas temperature, 200 °C.

**Statistical Analysis.** Differences between the means of the experimental groups were analyzed with the two-tailed Student's *t* test. A value of *p* less than 0.05 was considered significant.

**Acknowledgment.** This project was supported in part by grants from Prof Francis SK Lau and Mr. William Au Research Fund as well as Purapharm International awarded to Dr. A. Lau. We thank Genome Research Centre of The University of Hong Kong for facility support. We also thank Prof. PY Qian from The University of Science and Technology for providing UPLC-TOF-ESI-MS facilities for spectroscopic analysis and Miss S. Dash for her time and assistance in this regard. Both SCCC and JCBL contributed equally to this manuscript.

**Supporting Information Available:** List of chromatographic and spectroscopic data of the active compound as well as the bioassay result. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- Tracey, K. J. The inflammatory reflex. *Nature* **2002**, *420*, 853–859.
- Aggarwal, B. B.; Shishodia, S.; Sandur, S. K.; Pandey, M. K.; Sethi, G. Inflammation and cancer: how hot is the link? *Biochem. Pharmacol.* **2006**, *72*, 1605–1621.
- Woodworth, C. D.; McMullin, E.; Iglesias, M.; Plowman, G. D. Interleukin 1 alpha and tumor necrosis factor alpha stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2840–2844.
- Montesano, R.; Soulie, P.; Eble, J. A.; Carrozzino, F. Tumour necrosis factor alpha confers an invasive, transformed phenotype on mammary epithelial cells. *J. Cell. Sci.* **2005**, *118*, 3487–3500.
- Cheng, S. M.; Xing, B.; Li, J. C.; Cheung, B. K.; Lau, A. S. Interferon-gamma regulation of TNFalpha-induced matrix metalloproteinase 3 expression and migration of human glioma T98G cells. *Int. J. Cancer* **2007**, *121*, 1190–1196.
- Brennan, F. M.; Gibbons, D. L.; Mitchell, T.; Cope, A. P.; Maini, R. N.; Feldmann, M. Enhanced expression of tumor necrosis factor receptor mRNA and protein in mononuclear cells isolated from rheumatoid arthritis synovial joints. *Eur. J. Immunol.* **1992**, *22*, 1907–1912.
- Komatsu, M.; Kobayashi, D.; Saito, K.; Furuya, D.; Yagihashi, A.; Araake, H.; Tsuji, N.; Sakamaki, S.; Niitsu, Y.; Watanabe, N. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clin. Chem.* **2001**, *47*, 1297–1301.
- Goldring, S. R. Bone and joint destruction in rheumatoid arthritis: what is really happening? *J. Rheumatol. Suppl.* **2002**, *65*, 44–48.
- Raetz, C. R. Biochemistry of endotoxins. *Annu. Rev. Biochem.* **1990**, *59*, 129–170.
- Bone, R. C. Gram-negative sepsis. Background, clinical features, and intervention. *Chest* **1991**, *100*, 802–808.
- Raetz, C. R.; Ulevitch, R. J.; Wright, S. D.; Sibley, C. H.; Ding, A.; Nathan, C. F. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* **1991**, *5*, 2652–2660.
- Tracey, K. J.; Cerami, A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* **1994**, *45*, 491–503.
- Shakhov, A. N.; Collart, M. A.; Vassalli, P.; Nedospasov, S. A.; Jongeneel, C. V. Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J. Exp. Med.* **1990**, *171*, 35–47.
- Ziegler-Heitbrock, H. W.; Sternsdorf, T.; Liese, J.; Belohradsky, B.; Weber, C.; Wedel, A.; Schreck, R.; Bauerle, P.; Strobel, M. Pyrrolidine dithiocarbamate inhibits NF-kappa B mobilization and TNF production in human monocytes. *J. Immunol.* **1993**, *151*, 6986–6993.
- Trede, N. S.; Tsytsykova, A. V.; Chatila, T.; Goldfeld, A. E.; Geha, R. S. Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. *J. Immunol.* **1995**, *155*, 902–908.
- Cheung, B. K.; Lee, D. C.; Li, J. C.; Lau, Y. L.; Lau, A. S. A role for double-stranded RNA-activated protein kinase PKR in Mycobacterium-induced cytokine expression. *J. Immunol.* **2005**, *175*, 7218–7225.
- Lee, D. C.; Cheung, C. Y.; Law, A. H.; Mok, C. K.; Peiris, M.; Lau, A. S. p38 mitogen-activated protein kinase-dependent hyperinduction of tumor necrosis factor alpha expression in response to avian influenza virus H5N1. *J. Virol.* **2005**, *79*, 10147–10154.
- Li, J. C.; Lee, D. C.; Cheung, B. K.; Lau, A. S. Mechanisms for HIV Tat upregulation of IL-10 and other cytokine expression: kinase signaling and PKR-mediated immune response. *FEBS Lett.* **2005**, *579*, 3055–3062.
- Davis, R. J. The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **1993**, *268*, 14553–14556.
- Su, B.; Karin, M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **1996**, *8*, 402–411.
- Chan-Hui, P. Y.; Weaver, R. Human mitogen-activated protein kinase kinase kinase mediates the stress-induced activation of mitogen-activated protein kinase cascades. *Biochem. J.* **1998**, *336*, 599–609.
- Herlaar, E.; Brown, Z. p38 MAPK signalling cascades in inflammatory disease. *Mol. Med. Today* **1999**, *5*, 439–447.
- Ichijo, H. From receptors to stress-activated MAP kinases. *Oncogene* **1999**, *18*, 6087–6093.
- Abate, A.; Oberle, S.; Schroder, H. Lipopolysaccharide-induced expression of cyclooxygenase-2 in mouse macrophages is inhibited by chloromethylketones and a direct inhibitor of NF-kappa B translocation. *Prostaglandins Other Lipid Mediat.* **1998**, *56*, 277–290.
- Pando, M. P.; Verma, I. M. Signal-dependent and -independent degradation of free and NF-kappa B-bound IkappaBalpha. *J. Biol. Chem.* **2000**, *275*, 21278–21286.
- Chen, F.; Castranova, V.; Shi, X.; Demers, L. M. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **1999**, *45*, 7–17.
- Habtemariam, S. Natural inhibitors of tumour necrosis factor-alpha production, secretion and function. *Planta Med.* **2000**, *66*, 303–313.
- Blumenthal, M.; Goldberg, A.; Brinkman, J. Black cohosh root. In *Herbal Medicine: The Expanded Commission E Monographs*, Blumenthal, M., Ed.; American Botanical Council: Newton, MA, 2000; pp 22–27.
- Boon, H.; Smith, M. *The Botanical Pharmacy: The Pharmacology of 47 Common Herbs*; Quarry Health Books: Kingston, Ontario, Canada, 1999; pp 41–45.
- Kronenberg, F.; Fugh-Berman, A. Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials. *Ann. Intern. Med.* **2002**, *137*, 805–813.
- Sakurai, N.; Nagai, M. [Chemical constituents of original plants of Cimicifugae rhizoma in Chinese medicine]. *Yakugaku Zasshi* **1996**, *116*, 850–865.
- Sakai, S.; Kawamata, H.; Kogure, T.; Mantani, N.; Terasawa, K.; Umatake, M.; Ochiai, H. Inhibitory effect of ferulic acid and isoferulic acid on the production of macrophage inflammatory



- protein-2 in response to respiratory syncytial virus infection in RAW264.7 cells. *Mediators Inflamm.* **1999**, *8*, 173–175.
- (33) Kim, S. J.; Kim, M. S. Inhibitory effects of cimicifugae rhizoma extracts on histamine, bradykinin and COX-2 mediated inflammatory actions. *Phytother. Res.* **2000**, *14*, 596–600.
- (34) Burdette, J. E.; Chen, S. N.; Lu, Z. Z.; Xu, H.; White, B. E.; Fabricant, D. S.; Liu, J.; Fong, H. H.; Farnsworth, N. R.; Constantinou, A. I.; Breemen, Van; Pezzuto, R. B.; Bolton, J. M.; Black, J. L. cohosh (*Cimicifuga racemosa* L.) protects against menadione-induced DNA damage through scavenging of reactive oxygen species: bioassay-directed isolation and characterization of active principles. *J. Agric. Food Chem.* **2002**, *50*, 7022–7028.
- (35) Tian, Z.; Pan, R.; Chang, Q.; Si, J.; Xiao, P.; Wu, E. Cimicifuga foetida extract inhibits proliferation of hepatocellular cells via induction of cell cycle arrest and apoptosis. *J. Ethnopharmacol.* **2007**, *114*, 227–233.
- (36) Chen, S. N.; Fabricant, D. S.; Lu, Z. Z.; Zhang, H.; Fong, H. H.; Farnsworth, N. R. Cimicifugoside A-D, propanoid esters from the rhizomes of *Cimicifuga racemosa*. *Phytochemistry* **2002**, *61*, 409–413.
- (37) Lu, Y. C.; Yeh, W. C.; Ohashi, P. S. LPS/TLR4 signal transduction pathway. *Cytokine* **2008**, *42*, 145–151.
- (38) Kim, S. H.; Kim, J.; Sharma, R. P. Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. *Pharmacol. Res.* **2004**, *49*, 433–439.
- (39) Blackwell, T. S.; Christman, J. W. The role of nuclear factor-kappa B in cytokine gene regulation. *Am. J. Respir. Cell Mol. Biol.* **1997**, *17*, 3–9.
- (40) Panes, J.; Gomollon, F.; Taxonera, C.; Hinojosa, J.; Clofent, J.; Nos, P. Crohn's disease: a review of current treatment with a focus on biologics. *Drugs* **2007**, *67*, 2511–2537.
- (41) Yeung, M. C.; Liu, J.; Lau, A. S. An essential role for the interferon-inducible, double-stranded RNA-activated protein kinase PKR in the tumor necrosis factor-induced apoptosis in U937 cells. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12451–12455.
- (42) Chan, M. M.; Cheung, B. K.; Li, J. C.; Chan, L. L.; Lau, A. S. A role for glycogen synthase kinase-3 in antagonizing mycobacterial immune evasion by negatively regulating IL-10 induction. *J. Leukoc. Biol.* **2009**, *86*, 283–291.
- (43) Saunders, B. M.; Britton, W. J. Life and death in the granuloma: immunopathology of tuberculosis. *Immunol. Cell Biol.* **2007**, *85*, 103–111.
- (44) Clay, H.; Volkman, H. E.; Ramakrishnan, L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity* **2008**, *29*, 283–294.
- (45) Tracey, K. J.; Fong, Y.; Hesse, D. G.; Manogue, K. R.; Lee, A. T.; Kuo, G. C.; Lowry, S. F.; Cerami, A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* **1987**, *330*, 662–664.
- (46) Ohlsson, K.; Bjork, P.; Bergenfeldt, M.; Hageman, R.; Thompson, R. C. Interleukin-1 receptor antagonist reduces mortality from endotoxin-shock. *Nature* **1990**, *348*, 550–552.
- (47) Schett, G. Review: Immune cells and mediators of inflammatory arthritis. *Autoimmunity* **2008**, *41*, 224–229.
- (48) Kaminska, B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. *Biochim. Biophys. Acta* **2005**, *1754*, 253–262.
- (49) Ghosh, S.; May, M. J.; Kopp, E. B. NF-kappaB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **1998**, *16*, 225–260.
- (50) Hommes, D. W.; Peppelenbosch, M. P.; van Deventer, S. J. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* **2003**, *52*, 144–151.
- (51) Sebolt-Leopold, J. S.; Dudley, D. T.; Herrera, R.; Van Becelaere, K.; Wiland, A.; Gowan, R. C.; Tecle, H.; Barrett, S. D.; Bridges, A.; Przybranowski, S.; Leopold, W. R.; Saltiel, A. R. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat. Med.* **1999**, *5*, 810–816.
- (52) Chen, S. N.; Fabricant, D. S.; Lu, Z. Z.; Fong, H. H.; Farnsworth, N. R. Cimicifugosides I-P, new 9,19-cyclolanostane triterpene glycosides from *Cimicifuga racemosa*. *J. Nat. Prod.* **2002**, *65*, 1391–1397.
- (53) Sakai, S.; Ochiai, H.; Nakajima, K.; Terasawa, K. Inhibitory effect of ferulic acid on macrophage inflammatory protein-2 production in a murine macrophage cell line, RAW264.7. *Cytokine* **1997**, *9*, 242–248.
- (54) Chen, S. N.; Fabricant, D. S.; Pauli, G. F.; Fong, H. H.; Farnsworth, N. R. Synthesis of cimicifugoside B, a phenylpropanoid found in *Cimicifuga racemosa*. *Nat. Prod. Res.* **2005**, *19*, 287–290.
- (55) Schreiber, E.; Matthias, P.; Muller, M. M.; Schaffner, W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **1989**, *17*, 6419.