Ebosin Attenuates the Inflammatory Responses Induced by TNF-α through Inhibiting NF-κB and MAPK Pathways in Rat Fibroblast-Like Synoviocytes

Yang Zhang, Lifei Wang, Liping Bai, Rong Jiang, Jianbo Wu, and Yuan Li

1NHC Key Laboratory of Biotechnology of Antibiotics, CAMS Key Laboratory of Synthetic Biology for Drug Innovation, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 1 Tiantan Xili, 100050 Beijing, China
2Beijing Institute of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

Correspondence should be addressed to Yuan Li; yuanwli@hotmail.com

Received 6 July 2021; Revised 4 December 2021; Accepted 22 January 2022; Published 17 March 2022

Academic Editor: Ran Wang

Copyright © 2022 Yang Zhang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Tumor necrosis factor-α (TNF-α) lies at the apex of signal transduction cascades that results in induced destruction of joints in rheumatoid arthritis. It is therefore of great medicinal interest to modulate the cellular responses to TNF-α. Ebosin, a novel exopolysaccharide derived from Streptomyces sp, has been demonstrated to have remarkable therapeutic actions on collagen-induced arthritis in rats, while it also suppressed the production of IL-1β, TNF-α, and IL-6 at both mRNA and protein levels in cultured fibroblast-like synoviocytes. In order to further understand the potential mechanisms involved in the anti-inflammatory effects of ebosin at molecular level, we investigated the impact of it on the activation of MAPK and NF-κB pathways following TNF-α induced in fibroblast-like synoviocytes (FLS). The results showed that the phosphorylation levels of TNF-α-induced p38, JNK1, JNK2, IKKα, IKKβ, and IκB, as well as NF-κB nuclear translocation, were reduced significantly in FLS cells in response to ebosin. Furthermore, we proved that ebosin decreased the level of NF-κB in the nucleus and blocked the DNA-binding ability of NF-κB using electrophoresis mobility gel shift assay. Besides, low levels of matrix metalloproteinases (MMP-1 and MMP-3) and chemokines (interleukin-8 and RANTES) were found in TNF-α-stimulated fibroblast-like synoviocytes treated with ebosin. These results indicate that ebosin can suppress a range of activities in both MAPK and NF-κB pathways induced by TNF-α in rat fibroblast-like synoviocytes, which provides a rationale for examining the use of ebosin as a potential therapeutic candidate for rheumatic arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic destructive disease of the joints and cartilage. Proinflammatory cytokines such as tumor necrosis factor (TNF) produced by fibroblast-like synoviocytes (FLS) and inflammatory cells, which are expressed at high levels in rheumatoid joint tissue, where they contribute significantly to inflammation and articular destruction. Dysfunction of TNF-α is involved in the pathological process of different types of diseases including RA [1, 2], which was the first proinflammatory cytokine fully identified as a therapeutic target for RA [3]. TNF-targeted therapy has convincingly demonstrated significant benefit for the majority of RA patients treated. Using recombinant proteins including infliximab, adalimumab, and golimumab [4] to block TNF has emerged in recent years.

TNF-α elicits its biological activities through binding to two types of cell surface receptors TNF-R1 and TNF-R2. In most of the cells, TNF-R1 was considered to be a key mediator of TNF-α signal [5]. NF-κB is an important downstream target of TNF-α signaling, and it has been identified to be involved in inflammatory responses [6]. TNF-α triggers NF-κB activation by a variety of signaling molecules, including TRAF2 (TNF receptor-associated factor 2), RIP (receptor-interacting protein), and the IKK (IκB kinase) complex. Another signal pathway activated by TNF-α is mitogen-activated protein kinase...
pathways (including p38, JNK, and ERK1/2), which has been strongly associated with many of the processes that mediate the pathological features of RA [7]. The p38 MAPK and JNK (Jun N-terminal kinase) pathway inhibitors attract more attention since they can reduce both the synthesis of proinflammatory cytokine and their intracellular signaling [8]. In addition, TNF-α also induces extracellular matrix (ECM) remodeling by regulating the expression of MMPs involved in joint damage in RA, as well as expression of multiple chemokines including IL-8, MCP-1, CCL3, CCL4, and RANTES, which contribute to chronic inflammatory [9–16]. Currently, anti-inflammatory therapy by inhibiting MAPK and NF-κB pathways has been well recognized [17].

Ebosin, a novel exopolysaccharide (EPS) extracted from Streptomyces sp.139 of soil samples in China, remarkably inhibits the development of CIA (collagen-induced arthritis) in rats [18], which consisted of rhamnose, fucose, arabinose, mannose, xylose, glucose, galactose, and galacturonic acid [19]. This anti-inflammatory effect of ebosin on CIA has been identified to be related to attenuating the production of IL-1β (interleukin-1β), IL-6 (interleukin-6), and TNF-α at the transcriptional and posttranscriptional levels [18].

Although anti-TNF agents have been shown to improve the outcome of the management of RA, a proportion of patients does not respond well to anti-TNF therapy and has increased the risk of adverse events such as infections [20–24]. Unlike antibody-based agents, ebosin is the first EPS produced by the Streptomyces genus with a novel structure [19]. In a previous study, we found that the inhibitory effect of ebosin on TNF-α secretion was stronger than other cytokines as IL-1β and IL-6 in the CIA rat model and FLS cells, implicating that ebosin may improve rheumatoid arthritis symptoms through the TNF-α signaling pathway [18]. However, ebosin may have a wide application prospect due to its efficacy and high safety which have been proven by a long-term toxicity study (unpublished data). Based on the above, the purpose of this study was to investigate the impact of ebosin on TNF-α-mediated MAPK and NF-κB pathways, as well as on levels of MMPs and chemokines in rat fibroblast-like synoviocytes (FLS), which will help us to better understand the mechanism of action of ebosin.

2. Materials and Methods

2.1. Isolation of FLSs. Male Wistar rats (Certificate No.: SCXK 2005-0013) were obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences, Beijing [18]. All rats were handled humanely and procedures under standard laboratory conditions with the approval of the Institute of Experimental Animals and Use Committee of the Chinese Academy of Medical Sciences. Chicken type II collagen (CII, Sigma)-induced arthritis (CIA) model and isolation of FLS were performed following the protocol described by Zhang et al. [18, 25]. In brief, Synovial tissues were isolated from the knee joint of CIA rats, which were sacrificed on day 30 after immunization, and then digested by 0.4% type II collagenase (Gibco). Isolated FLS were grown in DMEM high-glucose medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and cultured at 37°C, 5% CO2.

2.2. Purification of Ebosin. Ebosin-producing Streptomyces sp.139 was found from a soil sample in China and deposited in the China General Microbiology Culture Collection Center (No. 0405). Ebosin was purified from the supernatant of fermentation culture of Streptomyces sp.139 according to the protocol as described before [19].

2.3. Cytoplasmic and Nuclear Protein Extraction. FLS were cultivated in 6-well plates (at 1 × 10⁴/ml) at 37°C for 24 h. Ebosin (80, 16, or 3.2 μg/ml) was diluted in DMEM and added into FLS for 3 h with TNF-α (10 ng/ml). Cells were collected in 0.25% trypsin-EDTA (HyClone) and harvested by centrifugation at 500 g for 5 min. Cytoplasmic and nuclear were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents following the manufacturer’s protocol [25]. The cytoplasmic and nuclear extracts were stored at -80°C. Determination of protein concentration was performed by the Bradford method [26].

2.4. Western Blot Analysis. The effect of Ebosin on MAPK and NF-κB signaling pathways was determined by western blot assay as described previously [25]. The expression level of protein was detected with antibodies (Cell Signaling Technology) against phosphorylated or nonphosphorylated p38, JNK1, JNK2, ERK, IKKα, IKKβ, 1xβ, and NF-κB p65, respectively. The relative densities for the protein bands were quantitated using ImageQuant 300 (GE Healthcare) with Image J software.

2.5. Enzyme-Linked Immunosorbent Assays (ELISAs). ELISA was used to evaluate the levels of IL-8, RANTES, MMP-1, and MMP-3 in cell culture medium. FLS cells were plated in 24-well plates at 1 × 10⁶/ml and cultivated at 37°C for 24 h and then received proper ebosin treatments (80, 16, and 3.2 μg/ml) for 24 h. Cells were further induced by adding 10 ng/ml TNF-α for 24 h before supernatants were collected. The level of MMP-1, MMP-3, RANTES, and IL-8 in the medium was detected using colorimetric ELISA kits (USCN Life Science Inc. and Boster, Elab).

2.6. Electrophoretic Mobility Shift Analysis (EMSA). EMSA was performed to detect the effect of ebosin on the ability of NF-κB binding to DNA in nuclei using the LightShift Chemiluminescent EMSA Kit (Thermo) following the instructions of the manufacturer [25]. Extraction of nuclear protein was performed as previously described. Oligonucleotides labeling with biotin (5′-AGTTGAGGGACTTCCAGGC-C3′; 3′-TCAA CTCCCCTGAAAAGGTCCG-5′), which contains the κB binding site (κB, 5′-GGACATTC-3′), was synthesized from Beyotime Biotechnology Company. Unlabeled oligonucleotide and a 50-fold excess of cold κB oligonucleotide were used as a control to confirm specific binding.

2.7. Immunofluorescence Analysis. FLS cells were cultivated in 96-well plates (at 1 × 10⁴/ml) at 37°C for 12 h. Ebosin (80 μg/ml) was added in each well followed by cultivation
at 37°C for 12 h and then treated cell with TNF-α (10 ng/ml) for an additional 3 h. The cells were fixed in 5% paraformaldehyde (PFA)/PBS for 10 min at room temperature, permeabilized with PBS/0.1% Triton-X100 for 15 min, and blocked in PBS with 5% bovine serum albumin for 1 h. The cells were incubated with rabbit anti-NF-κB p65 (Cell Signaling Technology) and Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes). The fluorescent signals were detected by fluorescence microscopy (Olympus IX71, Japan).

2.8. Statistical Analysis. Data are presented as mean ± SD values. Evaluation of significance of differences between sample groups was performed by GraphPad Prism software (Version 6.0) using Student’s t-test. All P value < 0.05 was considered significant (*).

3. Results

3.1. Ebosin Downregulates TNF-α-Induced p38 MAPK Activation. Phosphorylation of p38 MAPK is involved in the activation of proinflammatory cytokines including TNF-α [27]. In order to investigate the effect of ebosin on TNF-α-induced p38 MAPK activation, FLS cells were treated with ebosin at a concentration of 0.128, 0.64, 3.2, 16, 80, and 400 μg/ml for 12 h. Phosphorylated active forms of p38 MAPK were detected by western blot in FLS cells following TNF-α stimulation. As shown in Figure 1(a), ebosin markedly reduced the level of phosphorylation of p38 MAPK by 63.67% (P < 0.01), 38.94% (P < 0.001), 25.21% (P < 0.05), 16.51% (P < 0.01), 10.16% (P < 0.05), and 3.62%, respectively, in a dose-dependent manner, while the levels of nonphosphorylated p38 in FLS did not change (Figure 1(a)), indicating that ebosin can downregulate p38 MAPK activation induced by TNF-α.

3.2. Ebosin Reduces the Production of Phosphorylated JNK1 and JNK2 MAPK. JNK is one major group of MAPK cascades, which are activated by TNFR superfamily members [27]. To understand the effect of ebosin on TNF-α-induced expression of JNKs in FLS, the supernatant of lysed cells was assessed by western blot after treating cells with TNF-α in the presence of ebosin. The results showed that ebosin in the range of 0.128–400 μg/ml significantly reduced the expression levels of phosphorylated JNK1 by 50.32% (P < 0.01), 34.84% (P < 0.001), 27.60% (P < 0.01), 13.51% (P < 0.01), 8.95%, and 1.19%, respectively (Figure 1(b)), while the phosphorylated JNK2 levels decreased by 59.09% (P < 0.01), 50.07% (P < 0.01), 43.00% (P < 0.01), 21.96% (P < 0.05), 13.29%, and 8.30%, respectively (Figure 1(b)), but does not affect nonphosphorylated forms of JNK1 and JNK2 (Figure 1(b)) at the same dosages.

3.3. The Expression Levels of p42/44 MAPK (ERK1/2) Are Not Influenced by Ebosin. The activation of p42/44 MAPK is associated with inflammatory response, synovial proliferation, and angiogenesis in RA [27]. To evaluate effect of ebosin on TNF-α-induced expression of p42/44 MAPK in FLS, we treated FLS cells with TNF-α in the presence of ebosin, and then, the phosphorylated (or nonphosphorylated) p42/44 protein level in the supernatant of lysed cells was determined using western blot. The results found that ebosin did not exert any action on both phosphorylated and nonphosphorylated forms of p42/44 MAPK (Figure 1(c)).

3.4. Effect of Ebosin on Expression of IKKα and IKKβ. The IKK complex has consisted of two catalytic subunits, IKKα and IKKβ, and a noncatalytic subunit IKKγ. Activation of IKK complex by TNF-α stimulation requires the phosphorylation of IKKαβ in its activation loop and polyubiquitination of IKKγ [28]. To expand these studies, we have analyzed the impact of ebosin on the phosphorylation level of IKKα and IKKβ in response to TNF-α. Western blot results showed that 80, 16, and 3.2 μg/ml ebosin decreased the protein levels of phosphorylated IKKα by 53.85% (P < 0.01), 19.90% (P < 0.05), and 0.85% separately and IKKβ by 71.56% (P < 0.01), 58.11% (P < 0.05), and 40.28% (P < 0.05), respectively (Figure 2(a)), but not nonphosphorylated IKKα and IKKβ (Figure 2(a)).

3.5. Ebosin Suppresses the Production of IκBs. IκBs is an inhibitory factor of NF-κB, and ubiquitination of IκBs leads to release NF-κB entering the nucleus and activating transcription of appropriate gene targets [28]. Using western blot, we measured the expression level of TNF-α-induced IκBα with ebosin in FLS cells. As shown in Figure 2(b), ebosin reduced the expression levels of phosphorylated IκBα by 35.27% (P < 0.05), 14.99% (P < 0.05), and 6.93%, respectively, at dosages 80, 16, and 3.2 μg/ml, but the levels of nonphosphorylated IκBα were enhanced by 55.12% (P < 0.05), 45.08% (P < 0.05), and 32.78% (P < 0.05), respectively, at the same dosages (Figure 2(b)).

3.6. Ebosin Attenuates NF-κB DNA-Binding Activity. Once released from its inactive form complexed with IκBs, NF-κB is presumably translocated to the nucleus and interacted with specific DNA-binding sequences to regulate gene transcription [29]. To confirm if ebosin can affect the NF-κB’s activity through interfering with its specific DNA, an electrophoretic mobility shift analysis (EMSA) was performed in FLS cells stimulated by TNF-α and then incubation with varying concentrations of ebosin. Results in Figure 3 showed that ebosin significantly blocked NF-κB-DNA binding in a dose-dependent manner.

3.7. Ebosin Inhibit NF-κB Nuclear Translocation. For understanding the effect of ebosin on the activity of NF-κB, we detected the NF-κB protein level in the cytoplasm and nucleus using the NF-κB p65 antibody. Western blot analysis showed (Figure 2(c)) that ebosin markedly enhanced the protein level of p65 in the cytoplasm induced with TNF-α by 51.72% (P < 0.01), 46.84% (P < 0.05), 40.33% (P < 0.05), 30.34% (P < 0.05), 23.12% (P < 0.05), and 14.34%, respectively, at dosages 400, 80, 16, 3.2, 0.64, and 0.128 μg/ml. However, it decreased the expression of NF-κB in the nucleus of cells by 80.11% (P < 0.001), 77.02% (P < 0.001), 53.77% (P < 0.001), 19.25% (P < 0.05), 10.07% (P < 0.05), and 5.25%, respectively, at same dosages (Figure 2(c)). For further understanding the nuclear events that govern NF-κB function by ebosin, immunofluorescence was performed to analyze the translocation of activated NF-κB in the
nucleus (Figure 4). The cells were treated with ebosin at 80 μg/ml after TNF-α inducing and then incubated with rabbit NF-κB p65 antibody. From these results, we observed that the process of NF-κB from the cytoplasm into the nucleus was significantly suppressed in FLS by ebosin (Figure 4).

3.8. Ebosin Reduced the Secretion of MMP1 and MMP3. The increased level of MMP expression has been associated with destroy collagenous components of cartilage in RA [30]. In order to study the therapeutic effects of ebosin, we measured the influence of ebosin on MMP-1 and MMP-3 secretion in TNF-α-induced FLS cells by ELISA. As shown in Figure 5 (a), the concentration of MMP-1 in cell-cultured supernatant decreased by 44.16% \((P < 0.01)\), 33.63% \((P < 0.05)\), and 26.94% \((P < 0.05)\), respectively, at dosages of 80, 16, and 3.2 μg/ml of ebosin, and meanwhile, the productions of MMP-3 in FLS were suppressed by 83.01% \((P < 0.01)\), 70.81% \((P < 0.01)\), and 32.63% \((P < 0.05)\), respectively (Figure 5(b)).

3.9. Effect of Ebosin on Secretion of Chemokines. Clinical studies have demonstrated chemokines, produced by FLS cells, promoting inflammation and cartilage destruction in response to TNF-α [31]. In this study, we detect the concentration of TNF-α-induced release of chemokines comprising RANTES and IL-8 in FLS treated with ebosin by ELISA. The results showed that ebosin at dosages 80, 16, and 3.2 μg/ml decreased the production of IL-8 by 68.08% \((P < 0.001)\), 57.52% \((P < 0.001)\), and 57.28% \((P < 0.001)\), respectively (Figure 5(c)), and at the meantime, diminished the expression levels of RANTES by 53.08% \((P < 0.001)\), 29.94% \((P < 0.01)\), and 19.93% \((P < 0.01)\) separately (Figure 5(d)).

4. Discussion

In the current study, we demonstrated that ebosin affects the TNF-α-induced inflammatory responses in FLS largely due to its intervention in TNF-α-induced MAPKs and NF-κB pathways (Figure 6).
Figure 2: The influences of ebosin on the NF-κB signaling pathway mediated by TNF-α in FLS. (a) Effects of ebosin on the expression of phosphorylated and nonphosphorylated IKKα and IKKβ. FLS cells (1 × 10^5/ml) were incubated in the presence or absence of ebosin for 12 h then with TNF-α (10 ng/ml) 3 h. The production of phosphorylated and nonphosphorylated IKKα and IKKβ was analyzed with western blot. (b) Effects of ebosin on production of phosphorylated IκBα and nonphosphorylated IκBα. The expression levels of phosphorylated and nonphosphorylated IκBα were identified with the same protocols as before. (c) Effect of ebosin on nuclear and cytoplasm NF-κB of FLS. The production of nuclear and cytoplasm NF-κB was determined by western blot also. All of the data are expressed as the means ± SD from at least 3 independent experiments. *P < 0.001*, **P < 0.01**, and ***P < 0.05*** compared to the control 2 (FLS incubated with TNF-α) individually for NF-κB, IKKα, IKKβ, and IκBα.

Figure 3: Ebosin inhibits NF-κB DNA binding activity in FLS mediated by TNF-α. The DNA binding activity in the nuclear extracts of FLS mediated by TNF-α was assessed in an electrophoretic mobility shift assay (EMSA) by a specific probe, an oligonucleotide labeled with biotin. A LightShift Chemiluminescent EMSA Kit was used following the instructions of the manufacturer. Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

Figure 4: The effect of ebosin on nuclear translocation of NF-κB mediated by TNF-α in FLS. Indirect immunofluorescence with the specific anti-NF-κB p65 antibody was performed. Using a fluorescence microscope, the cells were counterstained by Hoechst 33528 for nuclear staining.
Inflammation, a representative innate immune response and the cause of numerous diseases including cancer and arthritis [32], is characterized by the involvement of a common set of genes and endogenous mediators including growth factors, inflammatory cytokines, chemokines, MMPs, and toxic molecules (nitric oxide or free radicals) [33]. Efforts have been made to elucidate the mechanisms underlying the inflammatory responses to identify novel anti-inflammatory drug targets including the inflammation mediators such as TNF and IL-1 [34]. Several anti-TNF antibodies, including infliximab and adalimumab, have been approved by the FDA for the treatment of RA [20, 23]. However, long-term use of anti-TNF-α agents has also been reported to be closely associated with increasing risk of adverse events like serious infections, malignancies, skin, tuberculosis, and cancer [24]. Different from antibody-based agents, ebosin is a natural product that originates as secondary metabolites from *Streptomyces* sp.139 with efficacy and high safety properties, which may provide a more effective treatment option for RA.

Exopolysaccharides play a crucial role in several biological activities and have also remarkable industrial applications such as biothickeners in foods [35]. Numerous reports suggested that they can confer health benefits including anti-inflammation [36], cholesterol-lowering properties [37], antitumor activity [38], and antidiabetic activity [39]. EPSs isolated from *Trichoderma erinaceum* DG-312 exhibited a strong anti-inflammatory activity in inflamed mice [40]. Nowak et al. reported that EPS derived from *Lactobacillus rhamnosus* can significantly inhibit the production of arthritogenic antibodies, hence suppressing active CIA [41]. Recently, there has been growing interests in microbial EPS due to its broad medical applications in the field of immune regulation and antiviral activity, even against the coronavirus disease 2019 (COVID-19) [36, 42–45].

ERK1/2, JNK, and p38 MAPKs are the three major members of the MAPK family that respond to distinct signaling cascades [46]. The employment of p38 and JNK inhibitors has emerged as an attractive strategy to reduce both proinflammatory cytokine synthesis and its intracellular signaling [47]. Our results in this study showed that ebosin has a remarkably dose-dependent effect on reducing the phosphorylated p38, JNK1, and JNK2 MAPK protein levels in FLS induced by TNF-α. More than 50% phosphorylated protein was inhibited by ebosin at high dosage (400 μg/ml) that has no significant influence on the cell viability by cytotoxicity assay as described in our previous study [18]. Meanwhile, our research indicated that ebosin did not affect the expression of p42/44 MAPK (ERK1/2) which plays a key role in cell proliferation, differentiation, and migration [48].

The transcription factor NF-κB, which initially exists in the cytoplasm in an inactive complex with IκB, is a
pivotal regulator in the regulation of inflammation and immune responses by eliciting the transcriptional responses following different stimuli such as TNF-α or IL-1β [28]. It has been reported that the activation of NF-κB appears to precede disease onset, which suggested that inhibition of NF-κB by different means may contribute to reduce the severity of disease [49]. In this study, we focused on the capacity of ebosin to counteract the level of NF-κB in FLS induced by TNF-α. Our results showed decreased levels of phosphorylated IKKα and IKKβ after treating with ebosin. Besides, ebosin was capable of downregulating the levels of phosphorylated IκBα. EMSA indicated that ebosin decreased the DNA-binding activity of NF-κB in the nucleus, probably through affecting the phosphorylation of NF-κB itself. Furthermore, we also demonstrated that ebosin can significantly inhibit the NF-κB nuclear translocation process using western blot analysis and fluorescence microscopy. All of the results
mentioned above suggest that ebosin is an inhibitor of the NF-κB-driven signaling pathway, which may be responsible for its anti-inflammatory effects in vivo [18].

MMP-1 and MMP-3, as targets of NF-κB and major collagenolytic enzymes involved in tissue destruction, have been reported to be significantly elevated in the synovial fluid of RA patients [50]. In addition to MMPs, FLS produces chemokines into synovial tissue upon stimulation by proinflammatory cytokines that further enhance inflammation, hyperplasia, and cartilage destruction [51]. As a member of the CC subfamily of chemokines, RANTES is involved in the pathogenesis of RA by promoting leukocyte infiltration [52]. Another important chemokine, IL-8 has been reported to be strongly associated with leukocyte accumulation and inflammation in RA. In the current study, ELISA analysis demonstrated that MMP-1, MMP-3, RANTES, and IL-8 levels were suppressed by ebosin in FLS induced with TNF-α. These results suggested that inhibition of ebosin on NF-κB activation contributes to reduce the secretion of MMPs and chemokines, thereby protecting RA patients from joint destruction.

In conclusion, this study has demonstrated that ebosin is capable of inhibiting the inflammatory responses induced by TNF-α in isolated FLS, acting as an effective in vitro inhibitor of the MAPKs and NF-κB signaling pathways (Figure 6). It is assumed that the anti-inflammatory activity of ebosin is at least partially due to inhibition of these pathways. Therefore, ebosin may be developed as a potential candidate for the treatment of RA. Additional investigations to identify its clinical usefulness should be explored.

Abbreviations

CIA: Collagen-induced arthritis
FLS: Fibroblast-like synoviocytes
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IL-1β: Interleukin-1β
TNF-α: Tumor necrosis factor-α
MAPK: Mitogen-activated protein kinase
IKK: IκB kinase
NF-κB: Nuclear factor-kappa B
p38: p38 mitogen-activated protein kinase
JNK: Jun N-terminal kinase
ELISA: Enzyme-linked immunosorbent assay
EMSA: Electrophoretic mobility shift assay
MMP: Matrix metalloproteinase
RANTES: Reduced upon activation normal T expression and secreted
IL-8: Interleukin-8
TRAF2: TNF receptor-associated factor
RIP: Receptor interacting protein
DMEM: Dulbecco’s modified Eagle’s medium
DMSO: Dimethyl sulfoxide
RIPA: Radio immunoprecipitation assay
HRP: Horseradish peroxidase
PVDF: Polyvinylidene fluoride
EDTA: Ethylene diamino tetraacetic acid
PBS: Phosphate-buffered saline
BCA: Bicinchoninic acid.

Data Availability

The original data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare no conflict of interest.

Authors’ Contributions

Yang Zhang and Lifei Wang contributed equally to this work.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (NSFC 30530830 and 82070627), the National Key Project of New Drug Study of China (2012ZX09301002-001-023-02), the Beijing Municipal Institute of Public Medical Research Development and Reform Pilot Project (2021-10), the Research Fund of Beijing Institute of Hepatology (Y-2021-6), and the Open Project of NHC Key Laboratory of Biotechnology of Antibiotics (NHC-KLBA201903).

References

[1] D. I. Jang, A. H. Lee, H. Y. Shin et al., “The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics,” International Journal of Molecular Sciences, vol. 22, no. 5, p. 2719, 2021.
[2] J. Holbrook, S. Lara-Reyna, H. Jarosz-Gri, and M. McDermott, “Tumour necrosis factor signalling in health and disease,” F1000Res, vol. 8, 2019.
[3] P. C. Taylor and M. Feldmann, “Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis,” Nature Reviews Rheumatology, vol. 5, no. 10, pp. 578–582, 2009.
[4] J. Alam, I. Jantan, and S. N. A. Bukhari, “Rheumatoid arthritis: recent advances on its etiology, role of cytokines and pharmacotherapy,” Biomedicine & Pharmacotherapy, vol. 92, pp. 615–633, 2017.
[5] M. Noack and P. Miossec, “Selected cytokine pathways in rheumatoid arthritis,” Seminars in Immunopathology, vol. 39, no. 4, pp. 365–383, 2017.
[6] E. Jimi, N. Takakura, F. Hiura, I. Nakamura, and S. Hirata-Tsuchiya, “The role of NF-κB in physiological bone development and inflammatory bone diseases: is NF-κB inhibition “killing two birds with one stone”?,” Cell, vol. 8, no. 12, p. 1636, 2019.
[7] Y. Shen, L. Teng, Y. Qu et al., “Anti-proliferation and anti-inflammation effects of corilagin in rheumatoid arthritis by downregulating NF-κB and MAPK signaling pathways,” Journal of Ethnopharmacology, vol. 284, article 114791, 2022.
[8] Q. Fang, C. Zhou, and K. S. Nandakumar, “Molecular and cellular pathways contributing to joint damage in rheumatoid arthritis,” Mediators of Inflammation, vol. 2020, Article ID 3830212, 20 pages, 2020.
[9] G. Zhang, B. Liu, Z. Zeng, Q. Chen, Y. Feng, and X. Ning, “Oxymatrine hydrizone (OMTH) synthesis and its protective effect for rheumatoid arthritis through downregulation of
MEK/NF-κB pathway,” *Environmental Toxicology*, vol. 36, no. 12, pp. 2448–2453, 2021.

[10] N. Akhter, A. Wilson, R. Thomas et al., “ROS/TNF-α crosstalk triggers the expression of IL-8 and MCP-1 in human monocyctic THP-1 cells via the NF-κB and ERK1/2 mediated signaling,” *International Journal of Molecular Sciences*, vol. 22, no. 19, p. 10519, 2021.

[11] Z. Yu, Y. Wang, Y. Li et al., “Effect of miobushing on the serum levels of MMP-1, MMP-3, and VEGF in patients with rheumatoid arthritis,” *Evidence-based Complementary and Alternative Medicine*, vol. 2020, Article ID 7150605, 2020.

[12] X. Tong, H. Zeng, P. Gu, K. Wang, H. Zhang, and X. Lin, “Monocyte chemoattractant protein-1 promotes the proliferation, migration and differentiation potential of fibroblast-like synoviocytes via the PI3K/P38 cellular signaling pathway,” *Molecular Medicine Reports*, vol. 21, no. 3, pp. 1623–1632, 2020.

[13] S. Sindhu, S. Kochumon, S. Shenouda, A. Wilson, F. Al-Mulla, and R. Ahmad, “The cooperative induction of CCL4 in human monocyctic cells by TNF-α and palmitate requires MyD88 and involves MAPK/NF-κB signaling pathways,” *International Journal of Molecular Sciences*, vol. 20, no. 18, p. 4658, 2019.

[14] P. Cai, T. Jiang, B. Li et al., “Comparison of rheumatoid arthritis (RA) and osteoarthritis (OA) based on microarray profiles of human joint fibroblast-like synoviocytes,” *Cell Biochemistry and Function*, vol. 37, no. 1, pp. 31–41, 2019.

[15] W. Sun, N. Meednu, A. Rosenberg et al., “B cells inhibit bone formation in rheumatoid arthritis by suppressing osteoblast differentiation,” *Nature Communications*, vol. 9, no. 1, p. 5127, 2018.

[16] P. S. Burrage, K. S. Mix, and C. E. Brinckerho, “Matrix metalloproteinases: role in arthritis,” *Frontiers in Bioscience*, vol. 11, no. 1, pp. 529–543, 2006.

[17] H. Okamoto, T. P. Cujec, H. Yamanaka, and N. Katamata, “Molecular aspects of rheumatoid arthritis: role of transcription factors,” *The FEBS Journal*, vol. 275, no. 18, pp. 4463–4470, 2008.

[18] Y. Zhang, L. F. Wang, J. Y. Bai et al., “Anti-inflammatory effect of ebosin on rat collagen-induced arthritis through suppressing production of interleukin-1β, interleukin-6 and tumor necrosis factor-a,” *Journal of Inflammation*, vol. 11, no. 3, pp. 697–708, 2013.

[19] C. Jing, W. Jianbo, L. Yuan, J. Rong, and L. Baoyi, “A new IL-1 receptor inhibitor 139A: fermentation, isolation, physico-chemical properties and structure,” *Journal of Antibiotics (Tokyo)*, vol. 56, no. 2, pp. 87–90, 2003.

[20] M. Pap, I. Sapina, N. Laktasic Zerjavic et al., “Anti-TNF therapy and the risk of malignancies and infections in inflammatory rheumatic diseases- our experience,” *Psychiatria Danubina*, vol. 33, Suppl 4, pp. 625–631, 2021.

[21] S. Zhao, E. Mysler, and R. J. Moots, “Etanercept for the treatment of rheumatoid arthritis,” *Immunotherapy*, vol. 10, no. 6, pp. 433–445, 2018.

[22] S. Bek, A. B. Bojesen, J. V. Nielsen et al., “Systematic review and meta-analysis: pharmacogenetics of anti-TNF treatment response in rheumatoid arthritis,” *The Pharmacogenomics Journal*, vol. 17, no. 5, pp. 403–411, 2017.

[23] P. A. van Schouwenburg, T. Rispens, and G. J. Wolbink, “Immunogenicity of anti-TNF biologic therapies for rheumatoid arthritis,” *Nature Reviews Rheumatology*, vol. 9, no. 3, pp. 164–172, 2013.

[24] J. Li, Z. Zhang, X. Wu, J. Zhou, D. Meng, and P. Zhu, “Risk of adverse events after anti-TNF treatment for inflammatory rheumatological disease. A meta-analysis,” *Frontiers in Pharmacology*, vol. 12, article 746396, 2021.

[25] Y. Zhang, L. Wang, L. Bai et al., “Effect of ebosin on modulating interleukin-1β-induced inflammatory responses in rat fibroblast-like synoviocytes,” *Cellular & Molecular Immunology*, vol. 13, no. 5, pp. 584–592, 2016.

[26] M. M. Bradford, “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding,” *Analytical Biochemistry*, vol. 72, no. 1–2, pp. 248–254, 1976.

[27] G. Sabio and R. J. Davis, “TNF and MAP kinase signalling pathways,” *Seminars in Immunology*, vol. 26, no. 3, pp. 237–245, 2014.

[28] S. Mitchell, J. Vargas, and A. Hoffmann, “Signaling via the NFκB system,” *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, vol. 8, no. 3, pp. 227–241, 2016.

[29] M. S. Hayden and S. Ghosh, “Regulation of NF-κB by TNF family cytokines,” *Seminars in Immunology*, vol. 26, no. 3, pp. 253–266, 2014.

[30] F. Javed, H. B. Ahmed, T. Mikami, K. Almas, G. E. Romanos, and K. Al-Hezaimi, “Cytokine profile in the gingival crevicular fluid of rheumatoid arthritis patients with chronic periodontitis,” *Journal of Investigative and Clinical Dentistry*, vol. 5, no. 1, pp. 1–8, 2014.

[31] S. E. Sweeney and G. S. Firestein, “Rheumatoid arthritis: regulation of synovial inflammation,” *The International Journal of Biochemistry & Cell Biology*, vol. 36, no. 3, pp. 372–378, 2004.

[32] I. M. Rea, D. S. Gibson, V. McGilligan, S. E. Mc Nerlan, H. D. Alexander, and O. A. Ross, “Age and age-related diseases: role of inflammation triggers and cytokines,” *Frontiers in Immunology*, vol. 9, p. 586, 2018.

[33] K. D. Deane, M. K. Demoruelle, L. B. Kelmenson, K. A. Kuhn, J. M. Norris, and V. M. Holers, “Genetic and environmental risk factors for rheumatoid arthritis,” *Best Practice & Research. Clinical Rheumatology*, vol. 31, no. 1, pp. 3–18, 2017.

[34] Z. Chen, A. Bozec, A. Ramming, and G. Schett, “Anti-inflammatory and immune-regulatory cytokines in rheumatoid arthritis,” *Nature Reviews Rheumatology*, vol. 15, no. 1, pp. 9–17, 2019.

[35] I. C. Boels, M. Kleerebezem, and W. M. de Vos, “Engineering of carbon distribution between glycosylation and sugar nucleotide biosynthesis in Lactococcus lactis,” *Applied and Environmental Microbiology*, vol. 69, no. 2, pp. 1129–1135, 2003.

[36] L. Q. Li, A. X. Song, J. Y. Yin, K. C. Siu, W. T. Wong, and J. Y. Lau, “Anti-inflammatory activity of exopolysaccharides produced by a medicinal fungus Cordyceps sinensis Cs-HK1 in cell and animal models,” *International Journal of Biological Macromolecules*, vol. 149, pp. 1042–1050, 2020.

[37] E. Korcz, Z. Kerenyi, and L. Varga, “Dietary fibers, prebiotics, and exopolysaccharides produced by lactic acid bacteria: potential health benefits with special regard to cholesterol-lowering effects,” *Food & Function*, vol. 9, no. 6, pp. 3057–3068, 2018.

[38] J. Angelin and M. Kavitha, “Exopolysaccharides from probiotic bacteria and their health potential,” *International Journal of Biological Macromolecules*, vol. 162, pp. 853–865, 2020.

[39] M. Jin, Z. Lu, M. Huang, Y. Wang, and Y. Wang, “Effects of Se-enriched polysaccharides produced by _Enterobacter cloacae_ Z0206 on alloxan-induced diabetic mice,” *International Journal of Molecular Sciences*, vol. 19, no. 8, pp. 2120–2131, 2018.
[40] J. H. Joo and J. W. Yun, “Structure and molecular characterization of extracellular polysaccharides produced by Trichoderma erinaceum DG-312,” *Journal of Microbiology and Biotechnology*, vol. 15, pp. 1250–1257, 2005.

[41] B. Nowak, M. Ciszek-Lenda, M. Srottek et al., “Lactobacillus rhamnosus exopolysaccharide ameliorates arthritis induced by the systemic injection of collagen and lipopolysaccharide in DBA/1 mice,” *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, vol. 60, no. 3, pp. 211–220, 2012.

[42] M. Andrew and G. Jayaraman, “Marine sulfated polysaccharides as potential antiviral drug candidates to treat coronavirus disease (COVID-19),” *Carbohydrate Research*, vol. 505, article 108326, 2021.

[43] L. Xiao, X. Ge, L. Yang et al., “Anticancer potential of an exopolysaccharide from Lactobacillus helveticus MB2-1 on human colon cancer HT-29 cells via apoptosis induction,” *Food & Function*, vol. 11, no. 11, pp. 10170–10181, 2020.

[44] O. Y. A. Costa, J. M. Raaijmakers, and E. E. Kuramae, “Microbial extracellular polymeric substances: ecological function and impact on soil aggregation,” *Frontiers in Microbiology*, vol. 9, p. 1636, 2018.

[45] M. Moscovici, “Present and future medical applications of microbial exopolysaccharides,” *Frontiers in Microbiology*, vol. 6, p. 1012, 2015.

[46] K. Flores, S. S. Yadav, A. A. Katz, and R. Seger, “The nuclear translocation of mitogen-activated protein kinases: molecular mechanisms and use as novel therapeutic target,” *Neuroendocrinology*, vol. 108, no. 2, pp. 121–1231, 2019.

[47] E. K. Kim and E. J. Choi, “Pathological roles of MAPK signaling pathways in human diseases,” *Biochimica et Biophysica Acta*, vol. 1802, no. 4, pp. 396–405, 2010.

[48] M. Beloueche-Babari, L. E. Jackson, N. M. Al-Saffar, P. Workman, M. O. Leach, and S. M. Ronen, “Magnetic resonance spectroscopy monitoring of mitogen-activated protein kinase signaling inhibition,” *Cancer Research*, vol. 65, no. 8, pp. 3356–3363, 2005.

[49] J. C. Keith Jr., L. M. Albert, Y. Leathurby et al., “The utility of pathway selective estrogen receptor ligands that inhibit nuclear factor-kappa B transcriptional activity in models of rheumatoid arthritis,” *Arthritis Research & Therapy*, vol. 7, no. 3, pp. R427–R438, 2005.

[50] C. J. Malemud, “Matrix metalloproteinases and synovial joint pathology,” *Progress in Molecular Biology and Translational Science*, vol. 148, pp. 305–325, 2017.

[51] B. Bartok and G. S. Firestein, “Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis,” *Immunological Reviews*, vol. 233, no. 1, pp. 233–255, 2010.

[52] D. Wen, X. Du, Y. Qiao, J. Z. Dong, and C. S. Ma, “RANTES gene polymorphisms are not associated with rheumatoid arthritis and atopic dermatitis: a meta-analysis,” *International Reviews of Immunology*, vol. 34, no. 6, pp. 500–508, 2015.