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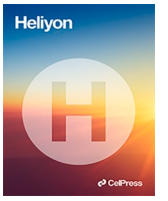

Research article

Pharmacological validation of a novel exopolysaccharide from *Streptomyces* sp. 139 to effectively inhibit cytokine storms

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ABSTRACT

With the rapid development of immunotherapy in recent years, cytokine storm has been recognized as a common adverse effect of immunotherapy. The emergence of COVID-19 has renewed global attention to it. The cytokine storm's inflammatory response results in infiltration of large amounts of monocytes/macrophages in the lungs, heart, spleen, lymph nodes, and kidneys. This infiltration leads to secondary tissue damage, acute respiratory distress syndrome (ARDS), organismal damage, and even death. However, there is currently no designated treatment for cytokine storm and the resulting ARDS. Consequently, there is a pressing need to identify a pharmaceutical agent that can effectively mitigate cytokine storms. Ebosin is a new exopolysaccharide generated by *Streptomyces* sp.139 and pharmacological activity for cytokine storm is investigated in vivo. The results show that Ebosin significantly augments the survival rates of mice, and its effectiveness increases with higher doses. It significantly inhibited the expression of cytokines IL-5, IL-6, IL-9 and chemokine Eotaxin in serum and lung tissues. Ebosin can alleviate the pathological damage in the lungs, liver, and spleen caused by LPS. Additionally, it can inhibit the phosphorylation of IKKα/β, Stat3 and NF-κB p65 upon LPS stimulation in vitro. We hypothesized that Ebosin may decrease cytokine release by inhibiting the phosphorylation of IKKα/β, Stat3, and NF-κB p65, neutrophil infiltration in animals. The article preliminarily elucidated the activity and mechanism of Ebosin against cytokine storm, which provides a reference for the study of anti-cytokine storm activity of microbial natural products.

1. Introduction

Since the end of 2019, there has been a rapid global outbreak of COVID-19 with continuing virus mutations, high transmission rates, and frequent breakthrough infections. The latest statistics from the World Health Organization report as of May 25, 2023, a cumulative total of 7,668, 444, 977 confirmed cases and 6,935,540 deaths worldwide [1]. The COVID-19 infection triggers innate immune responses in the human body, such as mononuclear macrophages and neutrophils, as well as adaptive immune responses that

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are mediated by T cells and B cells. A moderate immune response helps control the virus, while an overly activated antiviral response produces inflammatory cytokines that trigger cytokine storms, leading to accelerated deterioration and organismal damage [2,3]. Cytokine storm, also known as cytokine release syndrome (CRS), is a process in which the uncontrolled and excessive release of inflammatory cytokines and chemokines leads to a disturbance of self-regulation, contributing significantly to severe illness in COVID-19 patients [4]. Of these, inflammatory cytokines including IL-1, IL-2, IL-5, IL-6, IL-9, and MCP-1, have been produced in significant amounts in patients [5]. It was discovered that critically ill patients with SARS-CoV-2 infection exhibited substantial increases in pro-inflammatory cytokines, including IL-6, TNF-a, and IFN-γ, indicative of a cytokine storm. In addition, serum levels of IL-2 and IL-6 were positively associated with disease severity [6].

Cytokine Storm leads to the infiltration of large numbers of monocytes/macrophages into the lungs, heart, spleen, lymph nodes, and kidneys, and can result in physiological deterioration and death through the triggering of acute respiratory distress syndrome (ARDS) or organismal damage [7,8]. However, there is currently no designated treatment for CRS and the resulting ARDS. Glucocorticoids can restrain the expression of inflammatory genes by binding to their receptors, thereby producing an anti-inflammatory effect. Due to hormone therapy can cause adverse reactions and complications, such as lung tissue damage or shock, and may even suppress the immune system and accelerate symptom progression, it is advisable to avoid using glucocorticoids as the primary treatment [9,10]. ACE/Ang II/AT1R pathway inhibitors, such as captopril, enalapril, and losartan, may decrease cytokine storm and alleviate lung injury in COVID-19 patients. Whereas, it is necessary to monitor blood pressure carefully while administering these drugs [11]. Since IL-6 is one of the increased cytokines COVID-19 patients with CRS [12], IL-6 blocking agents have been used for treating people with severe COVID-19. Nonetheless, the effect of sarilumab and the other IL-6 blocking agents on COVID-19 patients with CRS is very uncertain [13]. In addition, the current evidence about efficacy of specific interleukin (IL)-1 inhibitors and GM-CSF blockades are still insufficient [14]. Therefore, the development of multitarget drug that effectively inhibit CRS is now of great significance.

Ebosin is an exopolysaccharide that was isolated from the supernatant of a fermentation culture of *Streptomyces* sp. 139 by our lab [15]. The previous experiments showed that Ebosin had a significant inhibitory effect on type II collagen rheumatoid arthritis [16]. It has also been demonstrated in vivo that have an inhibitory effect on psoriasis manifestations. After treatment by Ebosin, the expression levels of IL-6, IL-1β, other cytokines and chemokines significantly decreased in imiquimod-mediated psoriasiform skin tissues [17]. Lipopolysaccharide (LPS), a highly potent inducer of cytokine release syndrome (CRS), is also known to cause sepsis and toxic shock syndrome in mice. It is commonly used to stimulate pro-inflammatory cytokine release in both in vivo and in vitro models [18]. Recently, we used RT-PCR to detect the expression of representative cytokines in mouse and human cells at different concentrations of Ebosin after LPS stimulation and observed a significant inhibitory effect of Ebosin on cytokine IL-6 and TNFα [19]. At present study, the anti-cytokine storm activity of Ebosin were further evaluated in vivo for the first time, by means of survival, cytokine expression, and pathology analysis, with the objective of identifying drugs that can effectively inhibit CRS.

2. Materials and methods

2.1. Reagents and chemicals

The Ebosin-producing strain *Streptomyces* sp. 139 was collected from a soil sample in China and maintained in the China General Microbiology Culture Collection Center (No. 0405). This strain was cultured at 28 °C for 96 h with shaking (250 rpm). Ebosin was isolated from the supernatant of *Streptomyces* sp. 139 fermentation culture as previous description [20].

Ebosin (2 mg/mL, lab stock) and Lipopolysaccharide (LPS, 1 μg/mL, Beyotime, China) were dissolved in Phosphate-Buffered Saline (PBS, Corning, Manassas, USA). Filtered through a 0.22 μm filter, they were stored at −80 °C until use. Sodium carboxymethyl cellulose (CMC-Na, Selleckchem, USA) was dissolved in double distilled water at 0.5 mg/mL and used as a solvent for animal experiments. Etoricoxib (5 mM, Macklin, China) were dissolved in DMSO, and were further diluted with CMC-Na (for animal assays). Lianhuaqingwen was purchased from Shijiazhuang, China Ealing Pharmaceutical Co and dissolved in CMC-Na.

2.2. Cell lines and culture

RAW264.7 cell lines (RRID: CVCL_0493) were purchased from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (CAMS, Beijing, China). The RAW264.7 cell line was identified by extracting genomic DNA, amplifying it using 20 site-specific fluorescent primers (Gold Mix (Green), Tsingke, Beijing, China), and comparing the resulting data with the cell line STR database. And the absence of mycoplasma in the cells was determined by Mycoplasma Detector kit (Vazyme, Nanjing, China). The cells were conventionally cultivated in Dulbecco's modified Eagle medium (DMEM) with high glucose (Corning, Manassas, USA). The media were supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and antibiotics (50 I·U./mL penicillin and 50 μg/mL streptomycin) (Corning, Manassas, USA) under standard conditions (37 °C, 5 % CO_2).

2.3. Experimental animals

Female BALB/c mice of bodyweight 18–22 g were purchased from Beijing Huafukang Biotechnology Co., Ltd., allowed to acclimate to a new SPF surrounding (temperature: 22 ± 2 °C, humidity: 40–60 %, light/dark cycle: 12 h) for 1 week, with food and water supplied ad libitum. Animal experimental protocols were performed under NO. IMB-20210929D$_1$02 according to the Chinese National Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Experimental Ethics Committee of Institute of

Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College.

2.4. Lipopolysaccharide challenge model and animal treatment

In the LPS-induced inflammation model, mice were randomly divided into seven groups (n = 8): control group (only CMC-Na as placebo), LPS group (only 4 mg/kg LPS model), Etoricoxib group (LPS with 0.0084 mg/kg Etoricoxib), Lianhuaqingwen group (LPS with 550 mg/kg Lianhuaqingwen), Ebosin-L (LPS with Ebosin low-dose, 100 mg/kg), Ebosin-M (LPS with middle-dose, 200 mg/kg) and Ebosin-H (LPS with high-dose, 400 mg/kg) treatment groups. All groups were applied oral gavage one time every day from day 1 to day 14.1 h after administration to establish the model at day 8, mice were intraperitoneally injected with LPS (4 mg/kg, dissolved in NS) except the placebo group. The changes in the weight of mice were monitored and their survival were observed every day. All mice were euthanized at day 35.

2.5. Measurement of serum cytokines and chemokines

All the mice were anesthetized with 1 % pentobarbital sodium (0.55 mL/100 g body weight, i.p.) at 24 h after LPS injection. Blood of each mouse was drawn from orbit under anesthesia, and was allowed to stand for 1 h after removal and centrifuged at 2000 rpm for 15 min. Mouse serum was isolated and stored at −80 °C until analysis. The mouse right lungs were collected immediately and homogenized in RIPA for cytokine and chemokine analysis, whereas the left lungs were collected for Histological analysis and Immunohistochemistry (IHC) staining. Serum and lung cytokines and chemokines were quantitated with the Bio-Plex Mouse Cytokine 23-plex panel (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17 A, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES, TNF-α; Bio-rad, USA) using a Luminex X-200 with Milliplex Analysis Version 5.1.

2.6. Histological analysis and immunohistochemistry (IHC) staining

The lungs, livers and spleens of the mice were fixed in 4 % neutral paraformaldehyde for 24 h and were then embedded in paraffin. The paraffin block was cut into 4 μm slices. For H&E staining, the slices were stained with 3′-diaminobenzidene (DAB, Sigma-Aldrich) and were counterstained by hematoxylin and eosin (H&E). Scoring was based on histopathological changes, which in the case of the lung included bronchial mucosal cell necrosis, alveolar pneumonia, interstitial pneumonia and neutrophilic infiltration; in the case of the liver included hepatocellular necrosis and neutrophilic infiltration; and in the case of the spleen, included lymphocyte hyperplasia and macrophage hyperplasia. Each indicator was scored on a 5-point scale: 0 = minimal damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage, and 4 = maximum damage. The severity of tissue damage was based on the sum of the three criteria [21, 22]. For immunohistochemistry staining, slices were incubated with primary monoclonal antiCD11b (Abcam, ab133357) antibody at a dilution of 1: 2000 at 4 °C overnight, then with secondary antibody HPR-anti-Rabbit IgG (CST) [23]. Slices were imaged at a magnification of 200 × , and the integrated optical density (IOD) of CD11b was measured using LAS X software (Leica).

2.7. Western blot analysis

The Western blotting protocol was previously reported by Farahzadi et al. [24] In brief, total protein samples of cells were prepared by RIPA buffer (Boster, China) with phosphatase inhibitor (1:1000, Applygen, Beijing, China) on ice-bath for 30 min. The supernatant was collected by centrifugation at 14,000 rpm for 20 min at 4 °C, and the protein concentration was measured by a BCA protein assay kit (Applygen, Beijing, China). The protein samples were loaded to a 10 % SDS-PAGE gel and was transferred to a PVDF membrane (Millipore, MA, USA). The PVDF membranes were blocked with 5 % (w/v) skimmed milk powder in TBST for 2 h, and were subsequently incubated with primary anti–NF–κB p65 (#8242, Cell Signaling Technology, Boston, USA), anti-phospho–NF–κB p65 (#3033, Ser536, Cell Signaling Technology, Boston, USA), anti Stat3 (#4904, Calbiochem, New Jersey, USA), anti phospho-Stat3 (#9145 S, Tyr705, Cell Signaling Technology, Boston, USA), anti-IKKβ (#8943, Cell Signaling Technology, Boston, USA), anti-phospho-IKKα/β (#2697, Ser176/180, Cell Signaling Technology, Boston, USA), anti IκBα (#4812, Cell Signaling Technology, MA, USA), anti--phospho-IκBα (#2859, Ser32, Cell Signaling Technology, MA, USA) and anti GAPDH (AMM04703G, Santa Cruz Biotechnology, Beijing, China) at 4 °C overnight. Then the membranes were washed with TBST and were incubated with secondary HRP-conjugated goat anti-rabbit or anti-mouse IgG antibody (1:10,000) for 2 h at room temperature. The blots were detected using Immobilon™ Western Chemiluminescent HRP substrate (Millipore, MA, USA) on a Bio-Rad Gel imaging system (733BR-2008, Bio-Rad, CA, USA) and were analyzed by Image J software.

2.8. Statistical analysis

All data were analyzed using Graph Pad Prism 8.4.2 (Graph Pad Software, Inc., San Diego, CA) and presented as mean ± SD of at least three independent experiments. Differences between two groups were evaluated using Student's *t*-test. $p < 0.05$ was considered to be statistically significant, $p < 0.01$ was considered to be highly statistically significant, $p < 0.001$ was considered to be extremely statistically significant.

3. Result

3.1. Ebosin improved survival and facilitated weight recovery in mice following LPS stimulation

We assayed the activity of Ebosin to inhibit cytokine storm in vivo. After preliminary dose exploration, 4 mg/kg LPS was used for modeling, and Ebosin at 100 mg/kg, 200 mg/kg and 400 mg/kg were used in the low-dose, middle-dose and high-dose treatment groups. Etoricoxib (0.0084 mg/kg) and Lianhuaqingwen (550 mg/kg) were used as positive control. We injected LPS on day 8 after 7 days of prophylactic oral gavage with Ebosin and applied intragastrically for an additional 7 days, followed by continued observation of the mice for 3 weeks, during which time the survival rate and body weight changes were recorded, as shown in Fig. 1. Ebosin was observed to increase the survival rate of mice stimulated by LPS in a dose-dependent manner compared to controls. Higher doses of Ebosin showed the best efficacy with a survival rate of 75 %, which showed equivalent efficacy with positive Etoricoxib. However, the Lianhuaqingwen was noneffective against CRS (Fig. 1B). As indicated by the trend graphs of body weight changes in mice, the weights of all treated groups after LPS stimulation decreased significantly by 2–3 g within three days, and began to recover gradually after three

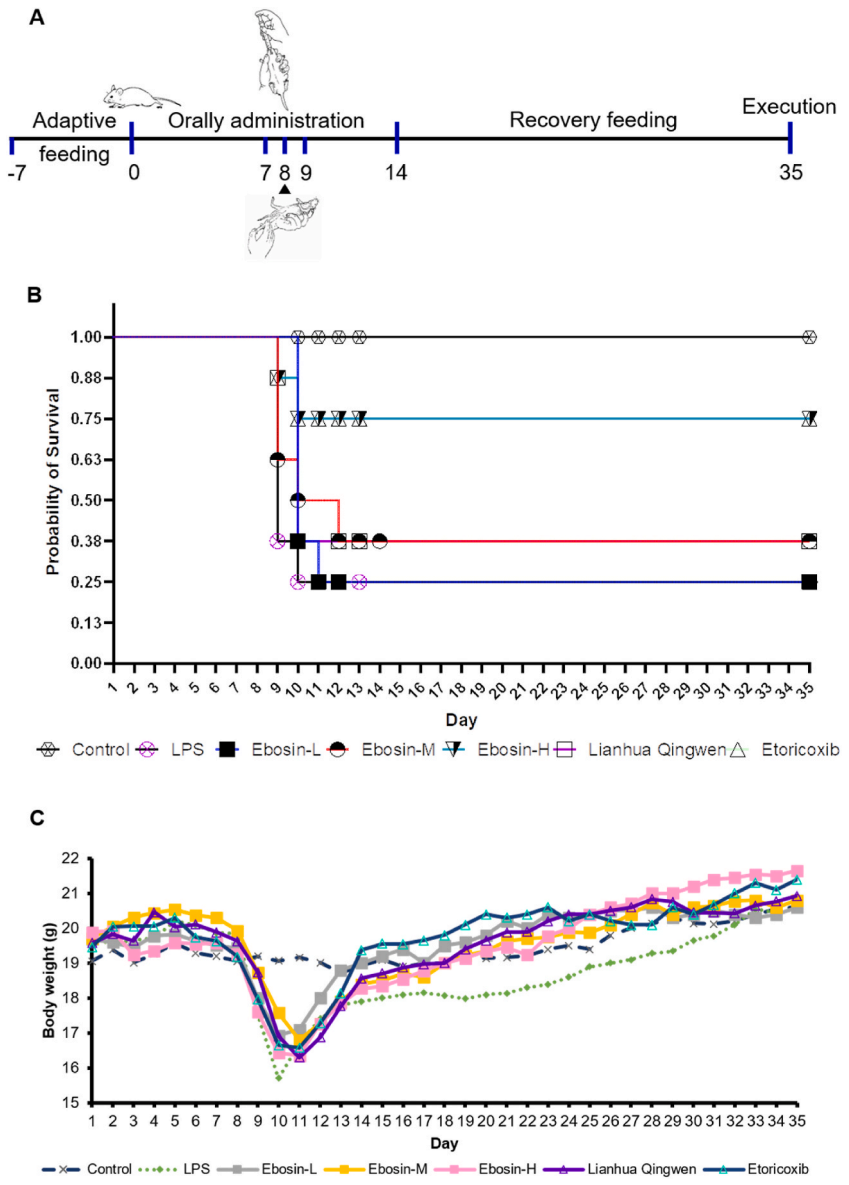

Fig. 1. Ebosin protected mice from LPS challenge. **(A)** Overview of studies investigating the use of Ebosin as a therapeutic for LPS infection. BALB/c mice were challenged with 4 mg/kg of LPS. Mice received a 14-day's intragastrically treatment of Ebosin (Ebosin-L: 100 mg/kg, Ebosin-M: 200 mg/kg, Ebosin-H: 400 mg/kg), Lianhuaqingwen (550 mg/kg), Etoricoxib (0.084 mg/kg) or CMC-Na (control group). (n = 8) **(B)** Survival rate within the 34 days' study. **(C)** Weight changes during the 34 days.

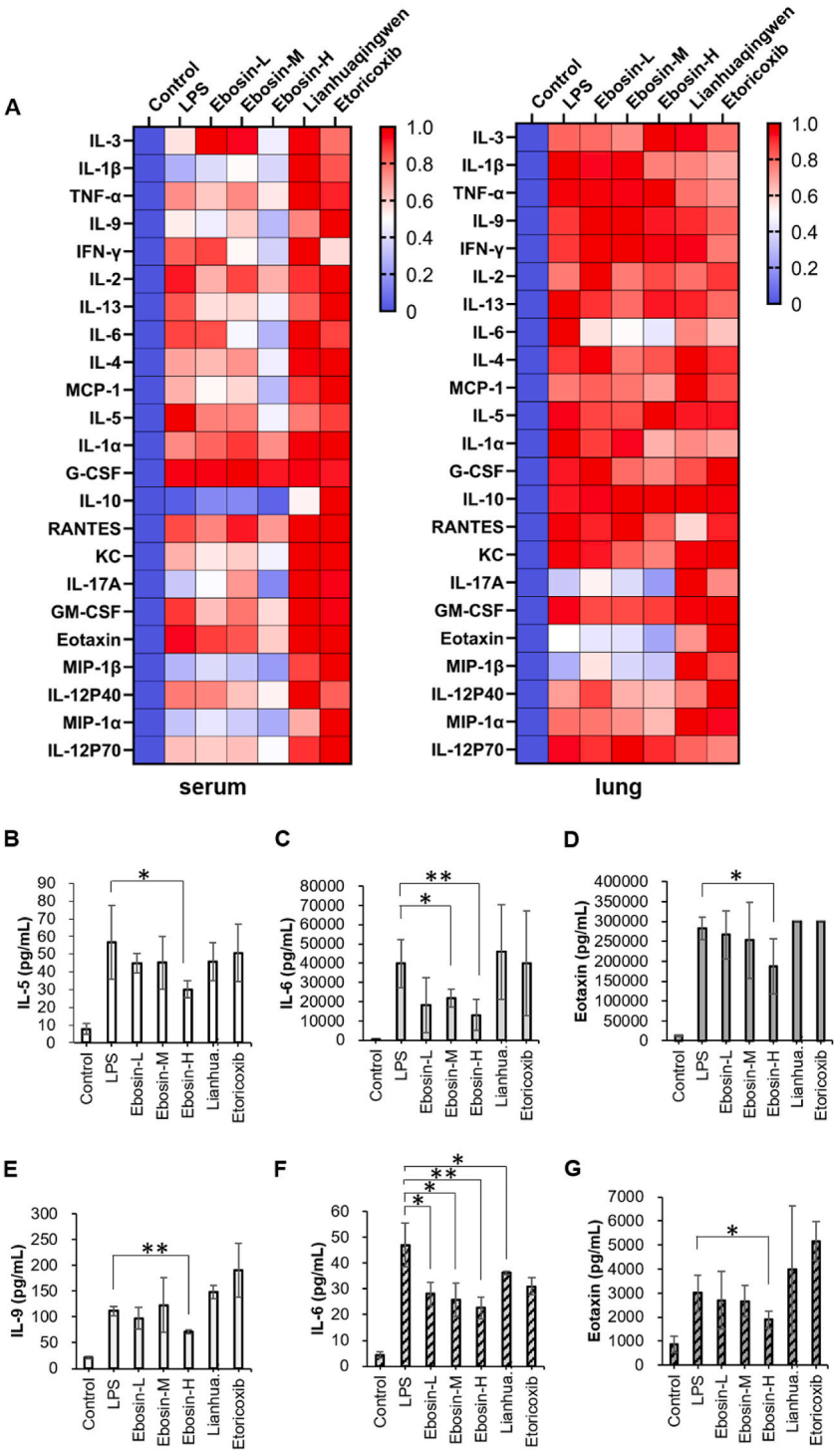

Fig. 2. Ebosin reduces cytokines and chemokines secretion in serum and lung tissues following LPS challenge. Cytokines and chemokines production were sampled from serum and lung tissue and examined by the Bio-Plex Mouse Cytokine 23-plex panel. **(A)** Concentrations of cytokines or chemokines were normalized, and heat maps showed changes in cytokine profiles in serum and lung tissue of different groups of mice. **(B–E)** Secretion of IL-5, IL-6, Eotaxin, and IL-9 in mouse serum. **(F, G)** Secretion of IL-6 and Eotaxin in mouse lung tissues. (mean ± SD, n = 8). $*p < 0.05$, $**p < 0.01$ vs. LPS-induced group.

days of LPS stimulation. After two weeks, the body weight recovered roughly to the same as that of the control group, but the time of recovery in the LPS group only was significantly slower than other groups (Fig. 1C). It was possible that the body's capacity to recuperate solely through its innate immune response was limited, resulting in a prolonged recovery period from the same stimulus.

3.2. Ebosin reduces cytokine secretion in serum and lung tissues following LPS stimulation

After 24 h by LPS stimulation, the content of cytokines and chemokines in serum and lung were examined by means of the Bio-Plex Mouse Cytokine 23-plex panel (Fig. 2A). Of the 23 cytokines and chemokines initially screened, the expression levels of all cytokines were significantly increased by LPS stimulation. After treatment with Ebosin, a decreasing trend was detected for 14 cytokines in the serum, and a decrease in the expression of 7 factors in the lung tissue. Compared with the LPS model group, serum secretion of IL-5 was 0.5-fold lower ($p < 0.05$), IL-6 was 0.7-fold lower ($p < 0.01$), Eotaxin was 0.3-fold lower ($p < 0.05$), and IL-9 was 0.4-fold lower ($p < 0.01$) in the Ebosin-H group (Fig. 2B–E). Moreover, the secretion of IL-6 and Eotaxin in lung tissue was also reduced by 0.5-fold ($p < 0.01$) and 0.4-fold ($p < 0.05$) in the Ebosin-H group (Fig. 2F–G), respectively. In addition, moderate doses of Ebosin significantly reduced IL-6 secretion in serum and lung tissue (Fig. 2C–F $p < 0.05$). Whereas, Etoricoxib and Lianhuaqingwen could hardly inhibit cytokines and chemokines secretion except for IL-6 secretion in lung tissue by Lianhuaqingwen ($p < 0.05$). We suggested that Ebosin was able to significantly reduce the release of various cytokines and chemokines after stimulation by LPS, thereby alleviate the resulting cytokine storm.

3.3. Treatment with ebosin attenuates lung, liver and spleen injury

As shown in Fig. 3, histopathological analysis after H&E staining showed necrosis of some epithelial cells in the bronchial mucosa, local alveolar enlargement or lung atrophy, cuboidalisation of alveolar epithelial cells, thickening of alveolar septa, local alveolar hemorrhage and alveolar pneumonitis in lung tissues stimulated by LPS. There was improvement in some epithelial cell necrosis of the bronchial mucosa after Ebosin treatment, and alveolar hemorrhage and alveolar pneumonia were improved. Lianhuaqingwen demonstrated some improvement in alveolar pneumonia, but not significantly. Etoricoxib showed significant improvement in bronchial mucosal cells and effectively reduced the severity of alveolar pneumonia, as well as not causing alveolar hemorrhage (Fig. 3A and B). Liver tissue showed normal hepatic lobular structure after LPS stimulation, some hepatocyte necrosis and neutrophilic infiltration in the confluent region, and mild hepatic fibrosis. And partial hepatocyte necrosis in the confluent region were ameliorated after Ebosin treatment. Lianhuaqingwen treatment also improved some instances of hepatocellular necrosis, however, there was an increase

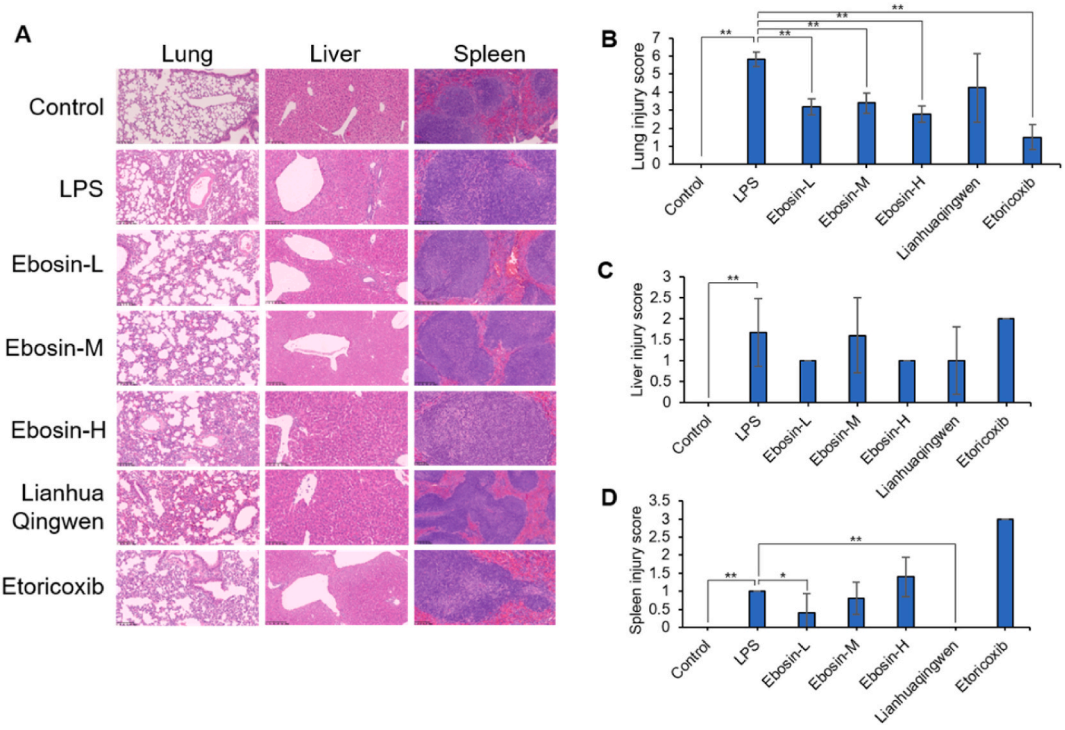

Fig. 3. Treatment with Ebosin attenuates lung, liver and spleen injury. The lung, liver and spleen followed by isolation, fixation, embedding, and tissue sectioning, the sections were stained with hematoxylin and eosin (H&E). **(A)** Pathological sections of lung, liver and spleen tissues stained by H&E. **(B)** Scoring of lung injury indicators in each group. **(C)** Scoring of liver injury indicators in each group. **(D)** Scoring of spleen injury indicators in each group. (mean ± SD, n = 8). *$p < 0.05$, **$p < 0.01$ vs. LPS-induced group.

in neutrophils observed in the central vein and hepatic blood sinusoids. Etoricoxib resulted in neutrophil still aggregation while mild hepatic fibrosis with more eosinophilic collagen fibres occurred in the confluent area (Fig. 3A–C). In the spleen, no structural abnormality was seen after LPS stimulation, but the splenic vesicles were enlarged, lymphocytes were proliferated, and some lymphocyte apoptosis could be observed, whereas lymphocyte apoptosis was improved after Ebosin treatment. There was no notable increase in the size of splenic nodules post Lianhuaqingwen intervention, while notable improvement was seen in lymphocyte and macrophage hyperplasia. On the contrary, Etoricoxib therapy led to an increase in lymphocyte hyperplasia and enlargement of splenic tubercles (Fig. 3A–D). Combined with histopathological changes scores, Ebosin treatment reduced lung injury scores by 0.42–0.52-fold ($p < 0.01$), liver injury scores by about 0.4-fold, and spleens by 0.6–0.8-fold ($p < 0.05$) compared to the LPS group.

3.4. Ebosin inhibits the functional response of macrophages and neutrophils to pro-inflammatory stimuli

The inflammatory response also includes production and release of pro-inflammatory cytokines (IL-5, IL-6, and IL-9) and chemokines (Eotaxin), and the levels of cytokines could be representative of lymphocyte infiltration. The inflammatory infiltrates are characterized by an increased number of lymphocytes as well as macrophages such as CD11b. Subsequently, we selected antiCD11b as primary monoclonal for immunohistochemical analysis of mouse lung tissue. As shown in Fig. 4, the content of antiCD11b in mouse lungs doubled after LPS stimulation. It was significantly reduced after Ebosin or positive control treatment, and all of them had significant difference ($p < 0.05$), the Ebosin-L, Ebosin-M and positive control groups also had highly significant differences compared with the LPS group ($p < 0.01$).

3.5. Ebosin downregulates NF-κB pathway and blocks Stat3 phosphorylation in macrophages following LPS stimulation

Macrophages have historically been considered to be the main source of cytokines and chemokines. To further elucidate the role of Ebosin in the CRS, we evaluated signaling transduction pathways associated with cytokines expression in macrophages by immunoblotting. Due to the fact that Ebosin inhibited the expression of cytokines in mouse serum and lung tissue significantly, we decided to use RAW264.7 mouse cells in this assay. Followed by a 12 h's stimulation with LPS, macrophages were preincubated with various concentrations of Ebosin for 30 min, and then the phosphorylation of NF-κB p65, IKKα/β, IκBα and Stat3 were analyzed. The results showed that LPS stimulation significantly increased the phosphorylation of IKKα/β, NF-κB p65 and Stat3 ($p < 0.05$), and their phosphorylation levels were inhibited by Ebosin in a dose dependent manner, especially 160 and 400 ng/mL Ebosin treatment ($p < 0.05$) (Fig. 5). It can be concluded that Ebosin inhibited the NF-κB and Stat3 pathways, and then inhibited the response of macrophages to pro-inflammatory stimuli.

4. Discussion

In many cases, cytokine storm, cytokine release syndrome (CRS), cytokine cascade, or hypercytokinemia refer to the same concept, but are expressed differently based on various perspectives. The concept of cytokine release syndrome was first introduced in 1989 by Chatenoud L. Fourteen patients who underwent kidney transplantation and were injected with standard doses of CD3 monoclonal antibody (OKT3), azathioprine, and methylprednisolone 6 h prior to the operation developed postoperative symptoms such as elevated temperature, headache, and the release of large quantities of cytokines, which were relieved by glucocorticoid treatment [25]. In 1993, FERRARA et al. first proposed cytokine storms [26]. They were subsequently observed in patients with severe acute respiratory syndrome (SARS) in 2003 and in patients infected with the H5N1 subtype of the influenza A virus in 2005, resulting in multiple organ failure, high mortality rates, and gaining significant interest in medical research [27]. In 2005, HUANG et al. also noted that Acute Respiratory Distress Syndrome (ARDS) can be triggered by SARS coronavirus [28]. With the rapid advancement of immunotherapy in

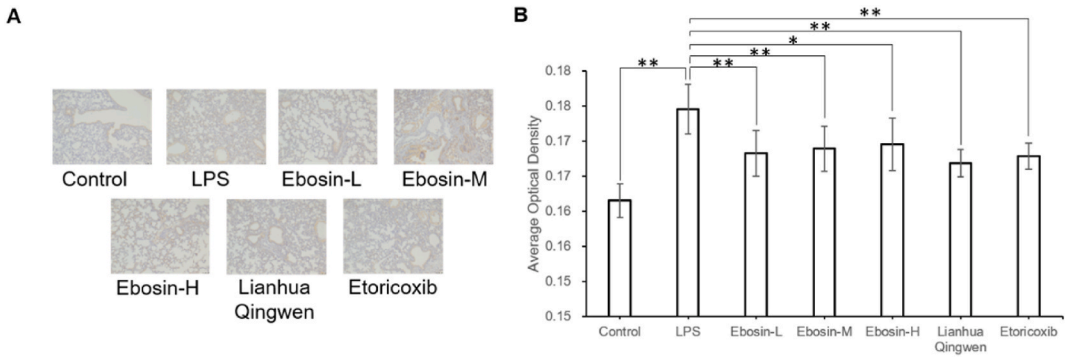

Fig. 4. Ebosin reduces CD11b expression by immunohistochemical assay. The lung slices were incubated with antiCD11b antibody at 1: 2000 at 4 °C overnight, then with secondary antibody HPR-anti-Rabbit IgG. (A) Lung tissue sections imaged at a magnification of 200 × . (B) Statistical graph of immunohistochemical results after quantitative analysis by Image J software. (mean ± SD, n = 8). *$p < 0.05$, **$p < 0.01$ vs. LPS-induced group.

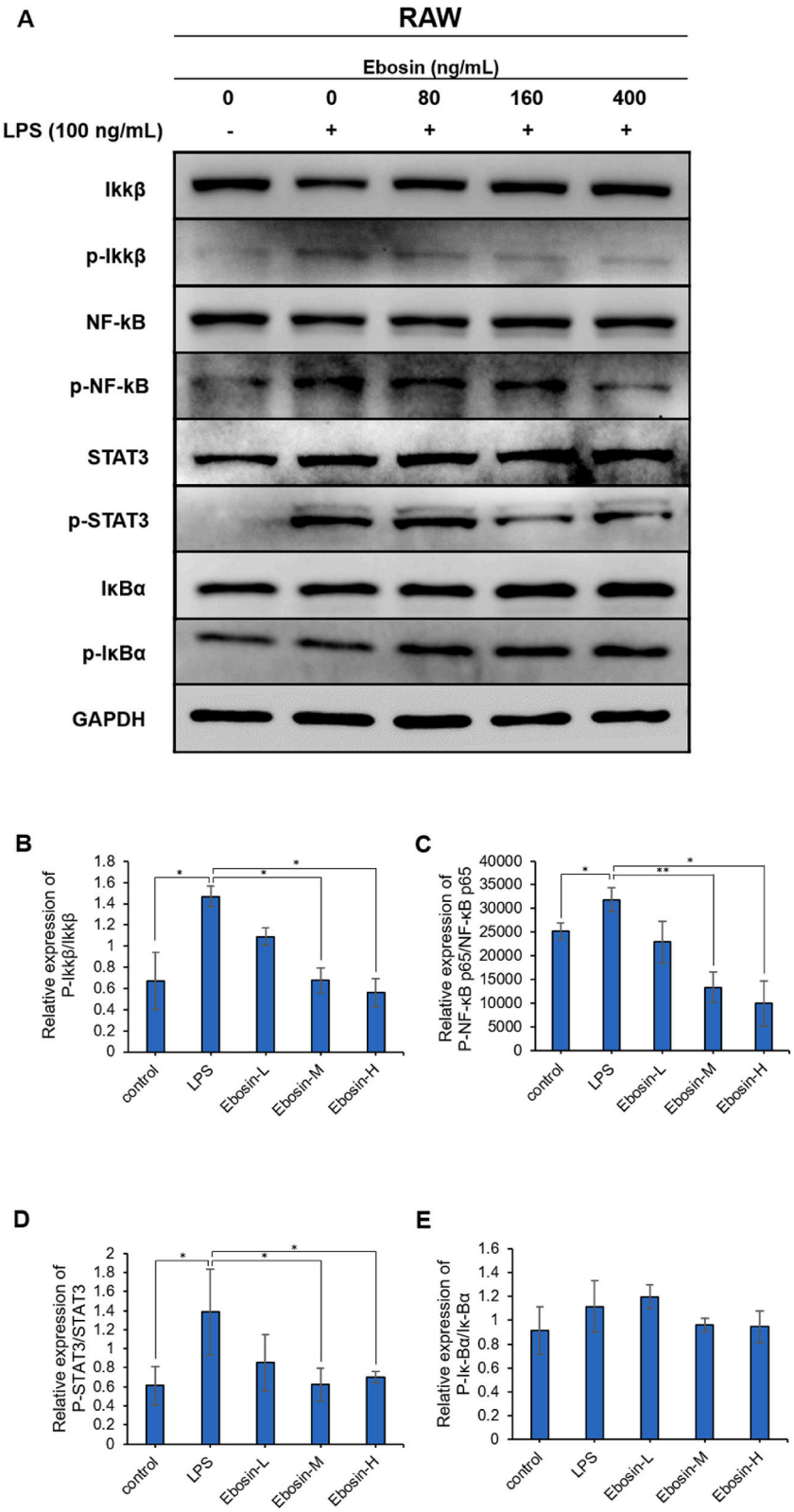

Fig. 5. Western blot analysis of LPS-induced NF-κB p65, IKKα/β, IκBα and STAT3 phosphorylation in RAW264.7 cells treated with Ebosin. (mean ± SD, n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS-induced group.

recent years, cytokine storm has become a widely recognized adverse effect of immunotherapy. The emergence of COVID-19 has once again focused global attention on it.

During pathogen invasion the body first activates the intrinsic immune system to fight against the infection, pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) are recognized by pattern recognition receptors (PRRs), such as Toll-like recep-tors (TLR), *C*-type lectin receptors (CLR), RIG-I-like receptors (RLR), and NOD-like receptors (NLR). Among them, TLR has been most widely studied in relation to cytokine storm [29,30]. When lipopolysaccharide (LPS), the bacterial PAMP, is recognized by the corresponding TLR4 receptor, it recruits and activates the associated kinases through the myeloid differentiation factor 88 (MyD88)-dependent pathway. This activation results in the activation of Mitogen-Activated Protein Kinase (MAPK) and Nuclear Factor-κB (NF-κB) signaling pathways, which induce the production of IL-1, IL-6, TNF-α and other pro-inflammatory cytokines and chemokines are secreted. As a result, more immune cells are recruited to phagocytose pathogens in infected foci, and the immune system is constantly activated, resulting in the release of a multitude of cytokines. This establishes a positive feedback pathway of inflammation [31]. Under normal circumstances, inflammation leads to an increase in anti-inflammatory cytokines, including IL-4, IL-10, transforming growth factor-β (TGF-β), IL-13 and other similar cytokines. The production and release of pro-inflammatory mediators, such as TNF-α, IL-1, and IL-6, is negatively inhibited leading to regulated positive feedback. At this stage, the body's natural inflammatory and anti-inflammatory factors are in balance, resulting in the elimination of invading pathogenic microorganisms from the body [32]. However, when this regulatory mechanism fails, the immune cells in the body become highly activated, resulting in a cytokine storm. The balance of endogenous inflammatory/anti-inflammatory factors is disrupted, and although the immune system eliminates disease-causing microorganisms, it also kills a large number of normal cells, causing endothelial damage, leading to multi-organ dysfunction and exacerbation of the disease [33]. The lungs, which have a high density of blood vessels, are more susceptible to damage [34].

A significant increase in cytokines has been reported in COVID-19 patients, including IL-6, IL-10, IL-1β, IL-7, IL-8, IL-9, granulocyte colony-stimulating factor (G-CSF), IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon γ inducible protein-10 (IP-10) and macrophage inflammatory protein-1α (MIP-1α) [34–37]. Cytokines such as IL-6 are significantly higher in critically ill patients compared to non-critically ill patients. In this study, we modeled 4 mg/kg LPS after pre-dose mapping. Three dose groups of Ebosin (low, medium, and high) were used, along with two positive control groups of Lianhuaqingwen and Etoricoxib. Lianhuaqingwen, as one of the representatives of traditional Chinese medicine, is widely used in China to treat mild COVID-19 patients [38–44], and Etoricoxib is also a small molecule drug that has been used in cytokine storm-related animal experiments and clinical studies [45–49]. The survival rate and body weight changes of the animal experiments are shown in Fig. 1. Compared with the control group, medium and high Ebosin-treated groups could reduce the mortality rate of mice, and the high-dose group was more effective, which suggests that Ebosin could improve the survival rate of mice after LPS stimulation. In view of anti-interleukin drugs such as anakinra and tocilizumab did not shorten the time to clinical improvement patients with COVID-19 associated cytokine release syndrome [50,51], it is promising to develop Ebosin to a kind of novel anti-cytokine storm drug. We further collected mouse serum and lung tissues for Luminex multiplex cytokine analysis 24 h after LPS modeling. The results demonstrate a significant increase in the expression of nearly all cytokines after stimulation with LPS. Ebosin effectively inhibits the expression of IL-5, IL-6, IL-9 cytokines, and Eotaxin chemokines. It is suggested that we can alleviate cytokine storm by inhibiting the aforementioned four factors in vivo. Compared with Ebosin, positive Etoricoxib showed equivalent efficacy with CRS. However, it didn't inhibit 23 cytokines and chemokines in serum and lung tissues, and which was inconsistent with the previous report to inhibit IL-6 and IL-1β in patients with rheumatoid arthritis [45]. We speculate Etoricoxib may be involved to other mechanism for CRS, but it still needs further research.

Inflammation linked with cytokine storms begins locally and disseminates via the bloodstream throughout the body. Redness, swelling, heat, pain, and loss of function are the main features of acute inflammation. When these reactions occur in the skin or other tissues, they increase blood flow, allowing leukocytes and plasma proteins to reach the outside of the injured blood vessel, raise the local temperature, which facilitates the host's ability to fight off the bacterial infection, and at the same time produce a sensation of pain that alerts the host to respond [52]. Reparative repair usually takes place shortly after the start of inflammation. In many instances, repair can entirely reestablish tissue and organ function. Fibrosis develops when chronic inflammation or significant etiologic triggers damage the local tissue structure, resulting in persistent organ dysfunction [53]. In this work, pathological analysis was also performed on each group of LPS-treated mice, and the results of H&E-stained pathological sections of lungs, livers, and spleens showed significant differences between the LPS group and the control group, while Ebosin treatment was able to alleviate the pathological damage caused by LPS to a certain degree. Compared with Etoricoxib, Ebosin took advantage of hepatocyte necrosis in the confluent region and lymphocyte proliferation in the spleen.

Organ failure in COVID-19 patients may result from the overactivation of macrophages and the release of inflammatory factors in large quantities, which can damage tissue sites [54,55]. Moreover, neutrophils attract other white blood cells and lymphocytes by releasing cytokines. The particles of neutrophils contain a variety of toxins that kill or inhibit the growth of bacteria and fungi. When there is severe inflammation, it is typically defined by the infiltration of monocytes/macrophages into the lungs, heart, spleen, lymph nodes, and kidneys, a significant increase in cytokines, a massive decrease in lymphocytes, and abnormalities in coagulation [7,8]. Upon bacterial stimulation, CD11b expression on the surface of macrophages and neutrophils increases significantly [56–60]. Liu et al. explored the value of neutrophil CD64, CD11b combined with systemic immune inflammation index (SII) in predicting abdominal infection after colorectal cancer surgery [61]. Thus, CD11b expression indicates the extent of neutrophil infiltration in tissues, serving as a sensitive indicator for evaluating infection. We performed immunohistochemical analysis of lung tissues, which showed that the fluorescence intensity of CD11b antibody binding was significantly elevated in the LPS modeling group compared with the control group, while Ebosin treatment significantly inhibited CD11b expression. It is therefore thought that Ebosin may reduce tissue damage by decreasing neutrophil infiltration. In addition, Lianhuaqingwen and Etoricoxib also inhibited CD11b expression, the result

indicated the two drugs could attenuate inflammatory cell infiltration and improve pulmonary function.

LPS binds to TLRs receptors on the membranes of immune cells such as dendritic cells (DCs), neutrophils, macrophages, and natural killer cells and enters the cells, activating the NF-κB pathway through a series of reactions and increasing the transcription and secretion of downstream inflammatory factors [62]. The Stat3 pathway is activated by cytokines like IL-6, which leads to increased release of cytokines and chemokines from macrophages [63]. To investigate the specific mechanism by which Ebosin achieves inhibition of cytokine storm, we explored it in Raw264.7 cells. Western blot results showed that phosphorylation of IKKα/β, NF-κB p65 and Stat3 was significantly increased after LPS stimulation, whereas Ebosin treatment decreased the level of their phosphorylation. We hypothesized that Ebosin may reduce cytokine and chemokine release by inhibiting Stat3 and NF-κB pathways. It was reported that curcumin also inhibited inflammatory cytokines and production and reduced the severity of viral pneumonia by inhibiting NF-κB signaling in macrophages [64,65]. Specifically, curcumin inhibits IKKβ activation and blocks cytokine-mediated NF-κB activation, thereby blocking pro-inflammatory gene expression [66,67]. On the other hand, activation of the JAK-STAT pathway is induced by cytokine binding to the surface receptor, which regulates transcription through STAT. Baricitinib is an ATP-competitive kinase inhibitor that selectively inhibits JAK1/JAK2, thereby suppressing pro-inflammatory signaling by a variety of cytokines such as IL-6, IL-12, IL-23, and IFN-γ. In COVID-19 outbreaks, Baricitinib was shown to be effective in controlling cytokine storms [68,69]. The above findings corroborate the findings of this paper that downstream phosphorylation of IKKα/β, Stat3, and NF-κB p65 after LPS stimulation can be reduced by Ebosin, which ultimately inhibits CRS (Fig. 6).

In summary, we investigated the effect of Ebosin on the survival rate of LPS-stimulated mice in in vivo experiments, and Ebosin-treated groups reduced the mortality rate of mice compared with the control group, and the high-dose group was more effective. Luminex multiplex cytokine assay on serum and lung tissue showed that Ebosin significantly inhibited the expression of IL-5, IL-6, IL-9 cytokines and Eotaxin chemokine. Ebosin was also found to alleviate LPS-induced pathological damage to various organs and significantly inhibited the expression of CD11b in lung tissue. In the subsequent cellular experiments, it was observed that Ebosin inhibited the phosphorylation of IKKα/β, Stat3, and NF-κB p65 downstream after LPS stimulation. The reduction of cytokine release was speculated to be accomplished by impeding the Stat3 and NF-κB pathways. Based on the result, Ebosin may be used to treat or prevent CRS and has the potential to be developed as a treatment for COVID-19.

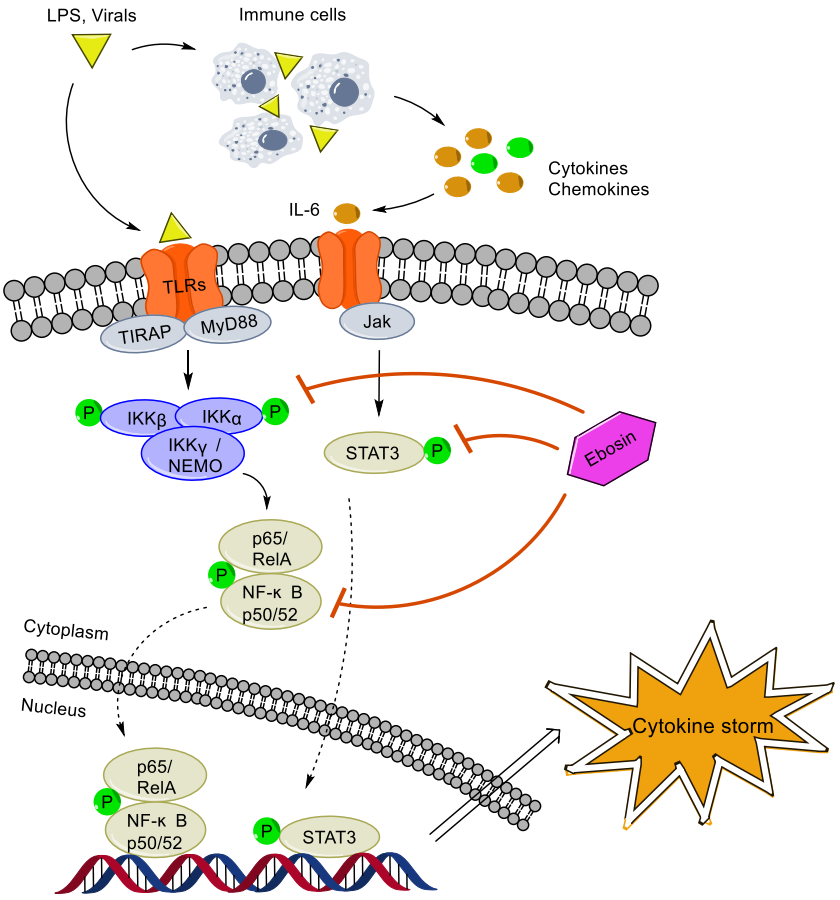

Fig. 6. The possible mechanisms of Ebosin on cytokine storm.

Ethics statement

The animal study was reviewed and approved by Animal Experimental Ethics Committee of Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College (Approval Number: IMB-20210929D$_1$02). All animal experimental protocols were performed according to the Chinese National Guidelines for the Care and Use of Laboratory Animals.

Data availability

All available data generated by experiments mentioned in this article. Raw datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

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CRediT authorship contribution statement

Zhuochen Zhuang: Writing – original draft, Investigation. **Yu Chen:** Investigation. **Zhe Liu:** Investigation. **Yu Fu:** Investigation. **Fei Wang:** Methodology, Conceptualization. **Liping Bai:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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