Original Article

The protective role of kakkalide in sepsis-induced intestinal barrier dysfunction via inhibition of NF-κB pathway activation

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Sepsis, a systemic inflammatory response often triggered by infection, can lead to multi-organ failure, with the intestine being one of the most vulnerable organs. The nuclear factor kappa-B (NF-κB) pathway plays a crucial role in immune responses, inflammation, and cell survival, making it central to sepsis-induced intestinal damage. Kakkalide (KA), a bioactive compound known for its anti-inflammatory, cardiovascular, neuroprotective, and anti-diabetic properties, has potential therapeutic effects. However, its impact on sepsis-induced intestinal injury remains unclear. In this study, murine sepsis models were used both *in vivo* and *in vitro* to evaluate the protective effects of KA on intestinal histopathology, apoptosis, and inflammation. Results showed that KA significantly reduced intestinal damage and apoptosis, as evidenced by hematoxylin-eosin and TUNEL staining. KA also improved intestinal barrier integrity, as indicated by reduced diamine oxidase activity, D-lactic acid content, and fluorescein isothiocyanate intensity, along with increased expression of zonula occludens-1. Furthermore, KA alleviates inflammation by reducing the levels of tumor necrosis factor-α, interleukin-1β, prostaglandin E2, inducible nitric oxide synthase, and cyclooxygenase-2. Immunofluorescence and Western blot analysis revealed that KA inhibited the sepsis-induced phosphorylation of inhibitor-kappaBα and RelA (P65) and prevented P65's translocation to the nucleus. These findings were confirmed in lipopolysaccharide-induced Caco-2 cells, suggesting that KA protected the intestinal barrier during sepsis by suppressing the NF-κB pathway.

***Key Words*: kakkalide, sepsis, NF-κB signaling pathway, intestinal barrier damage**

Sepsis is a systemic inflammatory response syndrome caused by infection, which develops rapidly and complexly, as well as one of the major problems in critical care medicine worldwide. In 2017, global statistics reported 48.9 million cases of sepsis, as well as 11 million sepsis-related deaths, with a mortality rate of 19.7%.[1] With the deepening of medical research, the understanding of the mechanism, diagnosis and treatment of sepsis has been continuously updated in recent years. The pathogenesis of sepsis involves a complex interplay of innate and adaptive immunity. In the early stages of sepsis, excessive inflammation triggers systemic immune activation, which can progress to immunosuppression, reducing the body's ability to respond to pathogens.[2] Its inflammatory response involves not only the release of pro-inflammatory factors interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) but is also accompanied by the production of anti-inflammatory factors interleukin 10 (IL-10).[3,4] Besides immune system, sepsis also involves microcirculatory disturbances,[5] coagulation abnormalities[6] and organ failure,[7] especially damage to the heart, lungs, liver and kidneys.

The nuclear factor kappa-B (NF-κB) transcription factor family consists of five subunits, including RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). The activation of its mechanism can be classified into the classical pathway and the non-canonical pathway. The study of NF-κB in metabolic diseases has gradually increased in recent years, especially in obesity,[8] diabetes,[9] and non-alcoholic fatty liver disease (NAFLD).[10] It has been widely studied in autoimmune diseases such as systemic lupus erythematosus (SLE),[11] multiple sclerosis (MS),[12] and rheumatoid arthritis (RA).[13] In synoviocytes and articular macrophages from RA patients, its activation promotes the expression of pro-inflammatory factors such as interleukin 1 (IL-1), IL-6, and matrix metalloproteinases (MMPs), leading to joint destruction and inflammatory responses.[14] NF-κB is vital not only for disease processes but equally crucial for normal immune defense and tissue repair. The pathway is a key regulator in the innate immune response, sensing pathogens and activating downstream pro-inflammatory gene expression via the TLR (Toll-like receptor)[15] and RLR (RIG-I-like receptor)[16] pathways. In addition, it is involved in adaptive immunity, regulating the activation, differentiation and functional maintenance of T and B cells.[17,18]

Kakkalide (KA), an isoflavone derived from flowers of *Pueraria lobata*, has attracted significant attention due to its wide-ranging biological activities. Presently, research is further exploring its potential value and clinical applications in the treatment of chronic diseases (cardiovascular and neurodegenerative diseases) and oncology. It is well-researched for its cardioprotective effects, particularly in treating myocardial ischemia and hypertension. It relaxes vascular smooth muscle through increasing nitric oxide (NO) production, inhibiting calcium inward flow and other mechanisms, thus achieving the effect of lowering blood pressure.[19] In the animal model of myocardial ischemia/reperfusion injury, it attenuates cardiac damage through antioxidant, anti-inflammatory and reduction of cardiomyocyte apoptosis.[20] It also has an important role in the study of neurodegenerative diseases such as Parkinson's disease.[21] Studies have shown that KA can slow the progression of neurodegenerative diseases by modulating various signaling pathways, such as PI3K/Akt, MAPK, and mTOR, and by reducing neuronal apoptosis.[22] Some studies have also explored the potential role of

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KA in cancer. In human papilloma virus (HPV)-related cancers, KA inhibits cell proliferation and induces cell apoptosis by regulating cell cycle-related proteins and modulating the expression of apoptosis-related proteins, such as B-cell lymphoma-2 (Bcl-2).[23]

In this study, we explored the potential mechanisms by which KA exerts protective effects on the intestinal barrier during sepsis, focusing on its inhibition of NF-κB signaling and the resulting reduction of inflammatory responses. KA has the potential to lower mortality, combat antibiotic resistance, and facilitate the development of future personalized treatments. This could greatly enhance the care of critically ill patients while providing fresh insights and breakthroughs for the wider medical field.

Materials and Methods

***In vivo* model construction of sepsis.** The sepsis model was established using cecal ligation and puncture (CLP) in accordance with the study of Li *et al*.[24] Male C57BL/6 mice of 6–8-week-old were selected and randomly divided into four groups ($n = 6$): 1) Sham; 2) CLP; 3) CLP + KA1 (10 mg/kg); 4) CLP + KA2 (20 mg/kg). The concentration of KA (HY-N4244; MCE, Monmouth Junction, NJ) was determined based on the minimum effective dose suggested by Min *et al*.[25] After a week of adaptive feeding, mice in the Sham and CLP groups were orally garaged with 0.2 ml of 0.5% sodium carboxymethyl cellulose (CMC-Na). Mice in two treatment groups were garaged with 10 and 20 mg/kg KA (dissolved in 0.2 ml of 0.5% CMC-Na), respectively, once a day for three consecutive days. Surgery was performed 1 h after the last dose. Mice were euthanized 24 h after surgery, and the ileum, colon, and serum were collected and prepared for subsequent testing.

Paraffin section. Tissue samples were fixed in 4% paraformaldehyde (PFA) for 24 h at room temperature. After fixation, the tissues were dehydrated through a graded ethanol series (70%, 80%, 95%, and 100%), followed by clearing with xylene to render the sections transparent. Once cleared, the tissues were infiltrated with melted paraffin by immersing them in paraffin wax at 60°C for 1 h. After infiltration, the tissues were embedded in fresh paraffin and allowed to solidify at room temperature. Once the paraffin block hardened, sections were cut to a thickness of 4–6 μm using a microtome. The sections were floated in a warm water bath to flatten them and then mounted onto glass slides. The slides were dried overnight in an oven at 37°C in preparation for staining and further analysis.

Hematoxylin-eosin (HE) staining. The sections were stained with hematoxylin for 5 min to color the nuclei blue, followed by rinsing under running tap water. They were then briefly differentiated in 1% acid alcohol for 30 s. After another water rinse, the sections were stained with eosin for 3 min, which colored the cytoplasm and extracellular matrix pink. The sections were then dehydrated through a graded ethanol series (70%, 85%, and 95%), followed by two washes in xylene. Finally, the slides were mounted with a permanent mounting medium, covered with coverslips, and observed under a light microscope. Intestinal injury was assessed according to Chiu's score[26] (0: normal intestinal mucosal villous structure; 1: widening of the subepithelial space at the tip of the villi, often accompanied by mild dilatation and congestion of capillaries; 2: moderate dilatation of the subepithelial space, with mild elevation of the lamina propria; 3: large areas of epithelial elevation, with partial detachment of the apical portion of the villi; 4: detachment of the intestinal villi and lamina propria, accompanied by capillary dilatation; 5: metamorphosis or digestion of the lamina propria, with hemorrhage and/or ulcer formation).

TUNEL staining. Samples were permeabilized with 0.1% Triton X-100 for 8 min at room temperature. After washing with PBS, the In Situ Cell Death Detection Kit (12156792910; Roche, Basel, Switzerland) was applied to fully cover the sections. The samples were then incubated at 37°C for 1 h in a humidified chamber protected from light. Following incubation, the sections were washed three times with PBS to remove any unbound reagents. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, followed by another PBS wash. The slides were mounted with an antifade mounting medium, covered with coverslips, and observed under a fluorescence microscope. Apoptotic cells were identified by red fluorescence in the nuclei.

Kits assay. The activity of diamine oxidase activity (DAO) (BC1285; Solarbio, Beijing, China) and the levels of D-lactic acid (D-LA) (BC5355; Solarbio) were measured according to the kit instructions. The ELISA kits used in this experiment included Mouse TNF-α/IL-1β (EK282/EK201B; MULTI, Hangzhou, China), Human TNF-α/IL-1β (EK182/EK101B; MULTI), and PGE_2 (Prostaglandin E2) (EU2554; Fine, Wuhan, China). Homogenates were prepared by lysing cells or tissues in lysis buffer. For tissue samples, homogenization was performed using a homogenizer to ensure thorough extraction. The lysates were then centrifuged at 12,000–15,000 *g* for 10–20 min at 4°C to collect the supernatant. Subsequent testing was conducted according to the kit manuals.

Intestinal permeability assay. After 21 h post-surgery, fluorescein Isothiocyanate (FITC)-dextran (4 kDa) (F491425; Aladdin, Shanghai, China) was administered to mice by gavage at a dose of 500 mg/kg. Blood samples were collected 3 h later, and the intensity of FITC in the serum was measured using fluorescence to assess intestinal permeability.

Immunohistochemical (IHC) staining. Antigen retrieval was performed by heating the tissue sections in a sodium citrate buffer (pH 6.0) at 100°C for 10 min, followed by cooling of the slides. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide (H_2O_2) for 15 min, and nonspecific binding sites were blocked using 1% bovine serum albumin (BSA) for 15 min. The tissue sections were incubated overnight at 4°C with zonula occludens-1 (ZO-1) antibody (AF5145; Affinity, Changzhou, China) diluted in PBS. The following day, the sections were washed with PBS and incubated with goat anti-rabbit IgG/HRP (SE134; Solarbio) for 45 min at room temperature. After washing, the staining was visualized using a diaminobenzidine (DAB) substrate until the desired color developed. The sections were counterstained with hematoxylin, dehydrated through ethanol and xylene, and mounted with a permanent mounting medium. The staining was observed under a light microscope.

Western blot. Tissues or cells were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. The samples were homogenized using a homogenizer to ensure thorough lysis. The lysates were centrifuged at 15,000 *g* for 15 min at 4°C to remove debris. The supernatant was collected, and the protein concentration was measured using a bicinchoninic acid (BCA) assay. Triple amounts of protein were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein sampling buffer (4×) (PR20003; Proteintech, Wuhan, China) by boiling the samples for 5 min to denature the proteins. The samples were loaded onto an SDS-PAGE gel, and electrophoresis was run to separate the proteins by molecular weight. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a wet transfer system. After transfer, the membrane was blocked with 5% non-fat milk in Tris-buffered saline-tween-20 (TBST) for 1 h at room temperature to prevent nonspecific binding. The membrane was incubated with the primary antibody diluted in TBST overnight at 4°C. The next day, the membrane was washed three times with TBST and incubated with the secondary antibody for 45 min at room temperature. After washing the membrane again with TBST, the protein

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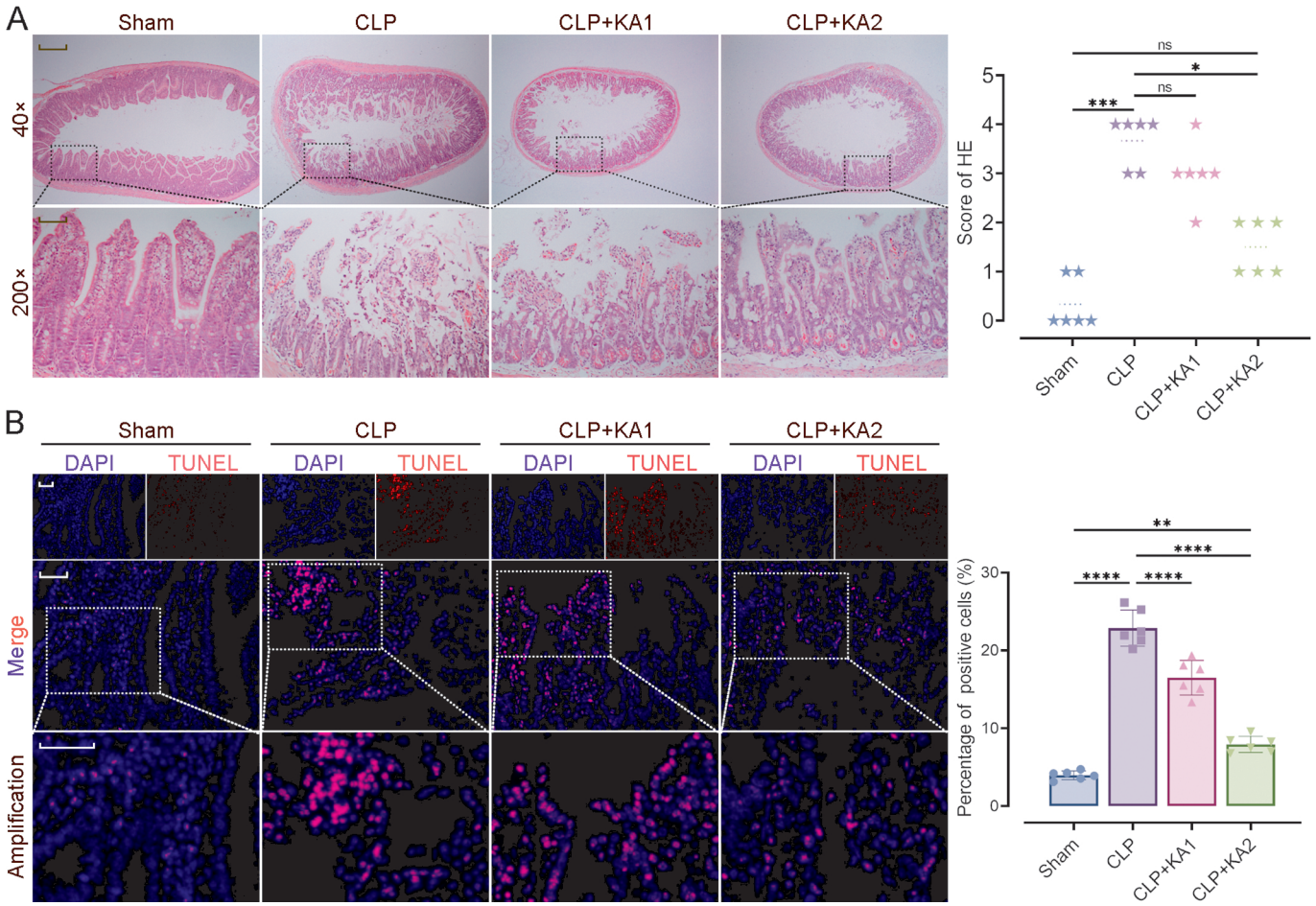

Fig. 1. KA mitigates sepsis-induced pathological damage and apoptosis in ileum. (A) HE staining and score of ileums, scale bar (40×/200×) = 500/100 μm. (B) TUNEL staining and quantization of ileums (400×), scale bar = 50 μm. The results are shown as the mean ± SD, $n = 6$.

bands were detected using an enhanced chemiluminescence (ECL) substrate and visualized using a chemiluminescence imaging system. Information on antibodies was provided in Supplemental Table 1*.

Immunofluorescence (IF) staining. Sections were washed in PBS and dried with absorbent paper. An immunohistochemistry pen was used to draw a circle around the tissue to prevent liquid spillage during reagent application. Nonspecific binding sites were blocked by incubating the samples in 1% BSA in PBS for 15 min. After blocking, the samples were incubated overnight at 4°C with the primary antibody diluted in PBS containing 1% BSA. The following day, the sections were washed three times with PBS and incubated with a fluorescently labeled secondary antibody for 45 min at room temperature, protected from light. After another wash with PBS, the nuclei were counterstained with DAPI, and the sections were mounted with antifade mounting medium. Finally, the slides were covered with coverslips and visualized using a fluorescence microscope.

Cell culture and treatment. The Caco-2 cells were divided into three groups: 1) Control; 2) LPS; 3) LPS+KA 20 μM. Cells were initially cultured in MEM (41500-067; Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator. When the cells reached 90% confluency, the culture medium was removed, and the cells were washed twice with PBS to eliminate any residual serum. After allowing the cells to adhere to the plate, they were treated with 0, 2, 5, 10, 20, and 40 μM of KA for 24 h. The optimal concentration of KA was determined using the Thiazolyl blue (MTT) assay based on cell viability results. After identifying the optimal KA concentration, the cells were co-treated with 100 μg/ml LPS (L2880; Sigma, St. Louis, MO) following KA treatment. Concentration of LPS was referenced to previous studies.[27]

Statistical data analysis. All statistical data was analyzed by GraphPad Prism 9.0. Data were expressed as the mean ± SD for normally distributed variables. For comparisons among more than two groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for normally distributed data. $P<0.05$ was considered statistically significant. For visualization purposes, error bars in figures represent the SD unless otherwise stated. All experiments were conducted in triplicate, and the statistical analyses were repeated independently to ensure the robustness of the results.

Results

Effects of KA on intestinal damage in septic mice. The HE staining results of the ileum revealed local intestinal tissue necrosis and thinning of the intestinal mucosal layer induced by sepsis, characterized by cell loss, tissue disintegration, and destruction of the extracellular matrix (Fig. 1A). The cells exhibited red fluorescence in the CLP group, indicating DNA fragmentation and suggesting that they were in a state of apoptosis. Cells in the Sham group that had not undergone apoptosis showed no significant red signal, only background staining (Fig. 1B). After KA treatment, a reduction in intestinal pathological

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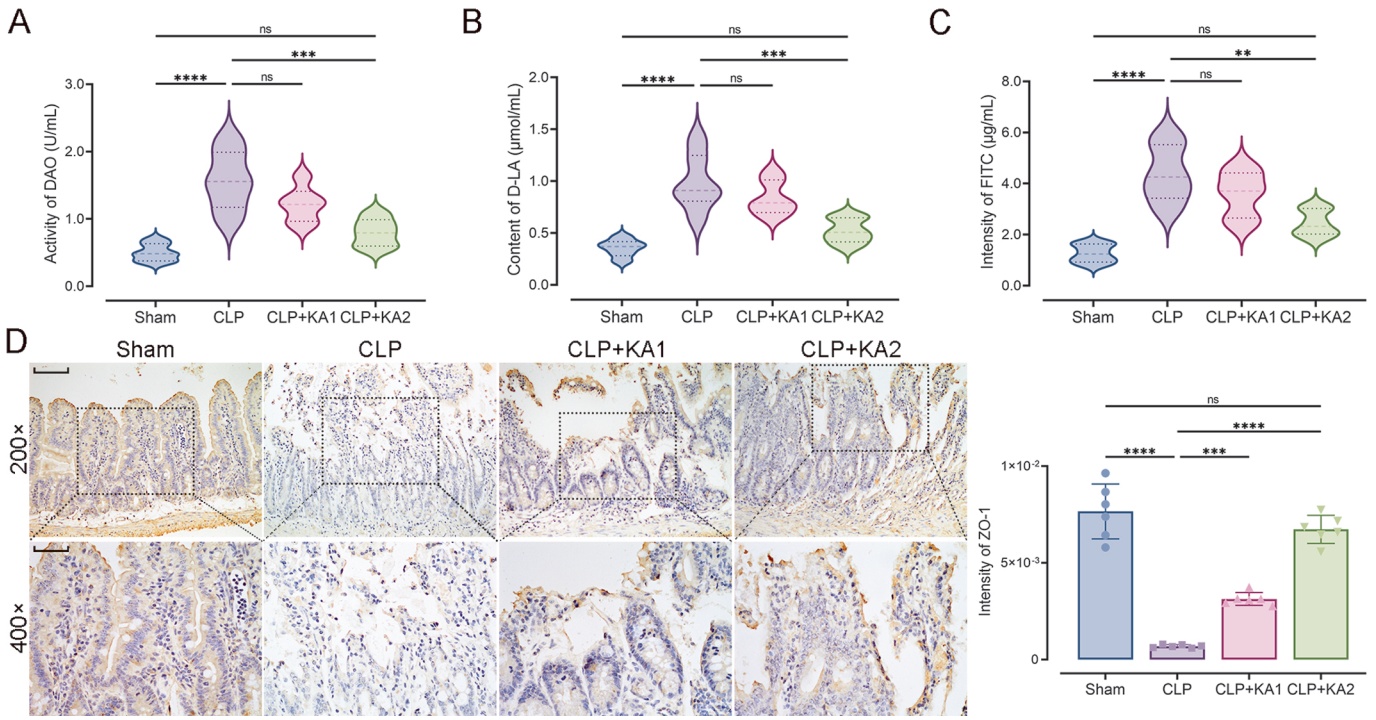

Fig. 2. KA alleviates sepsis-induced intestinal barrier damage in ileum. (A) Activity of DAO detected by ELISA in serum. (B) Concentration of D-LA detected by ELISA in serum. (C) Fluorescence intensity of FITC in serum. (D) IHC staining and quantization of ZO-1in ileums, scale bar (200×/400×) = 100/50 μm. The results are shown as the mean ± SD, $n = 6$.

damage and a decrease in apoptotic cells were observed markedly.

Effects of KA on sepsis-induced intestinal barrier dysfunction. Sepsis induced enhanced DAO activity and increased D-LA levels in blood, whereas KA restored their levels to a level indistinguishable from that of control (Fig. 2A and B). The DAO activity was used to assess the integrity of the intestinal mucosa and its degree of damage, while D-LA levels could reflect barrier dysfunction of the intestinal flora. The combination of the two might provide a more comprehensive assessment of intestinal health. The fluorescence intensity of FITC in the blood, measured by a Microplate reader, was found to be higher in the CLP group, indicating a greater leakage of FITC-dextran into the bloodstream. This suggested increased intestinal permeability and damage of the intestinal epithelial barrier. While KA could alleviate intestinal damage and maintain the integrity of the intestinal epithelial barrier (Fig. 2C). The IHC results showed the positive staining of ZO-1 was concentrated at the cell membrane boundaries, appearing as brown at the intercellular junctions, reflecting the presence and integrity of tight junction in the Sham group. In the CLP group, the staining is weaker, unevenly distributed, and absent at the cell membrane, indicating damage to the intercellular tight junctions. In contrast, KA was able to increase the expression level of ZO-1 and restore the intestinal barrier function to a certain extent (Fig. 2D).

Effects of KA on inflammatory response in septic mice. The results of inflammatory factor levels indicated that sepsis increased the levels of TNF-α, IL-1β and PGE_2 in serum. While, KA could significantly reduce the expression levels of the above factors and effectively reduce the inflammatory response (Fig. 3A and C). Elevated serum level of IL-1β is indicative of an intense inflammatory response in the gut or systemically. It acts synergistically with TNF-α to exacerbate sepsis-induced multi-organ dysfunction.[28] And high level of PGE_2 is associated with damage to the intestinal barrier and increased intestinal permeability.[29] Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) are involved in the production of NO and prostaglandins, respectively, during inflammation. Their up-regulated expression often implies that cells or tissues are in a state of inflammatory activation. The high fluorescence intensity of iNOS might lead to the production of large amounts of NO, which could further result in cytotoxicity, tissue damage, and functional impairment. The high fluorescence intensity of COX2 might trigger a strong inflammatory response accompanied by an increase in PGE_2 in the CLP group. After KA treatment, the expression levels of iNOS and COX2 decreased and the inflammatory response was alleviated (Fig. 3D).

Effects of KA on NF-κB pathway in septic mice. To elucidate the mechanism by which KA improved sepsis, we examined the protein expression levels of factors associated with the NF-κB pathway. Normally, NF-κB binds to the inhibitory protein IκB and stays in the cytoplasm, unable to enter the nucleus. However, in sepsis, the IκB was phosphorylated and degraded, releasing NF-κB, allowing it to enter the nucleus and initiate the expression of specific genes. Increased phosphorylation of P65 is one of the hallmarks of NF-κB pathway activation. Enhanced phosphorylation of P65 implied that it had been activated and was able to translocate to the nucleus and act as a transcription factor group in the CLP group (Fig. 4A). As shown in Fig. 4B the results suggested that sepsis induced a shift in the fluorescent signal of P65 from the cytoplasm to the nucleus, implying that the NF-κB pathway was activated. In the Sham group, the fluorescence signal of P65 was mainly confined to the cytoplasm, indicating that the NF-κB pathway was not activated, and P65 failed to translocate to the nucleus to initiate transcription. Additionally, it was found that KA significantly inhibited the nuclear entry of P65, thereby suppressing the transcription of downstream genes.

Effects of KA on LPS-induced inflammatory response and NF-κB activation in Caco-2 cells. In our *in vitro* experiments, LPS was used to stimulate cells to mimic the inflammatory response in sepsis. Based on the MTT results, the highest non-toxic concentration of KA for the cells, 20 μM, was selected for

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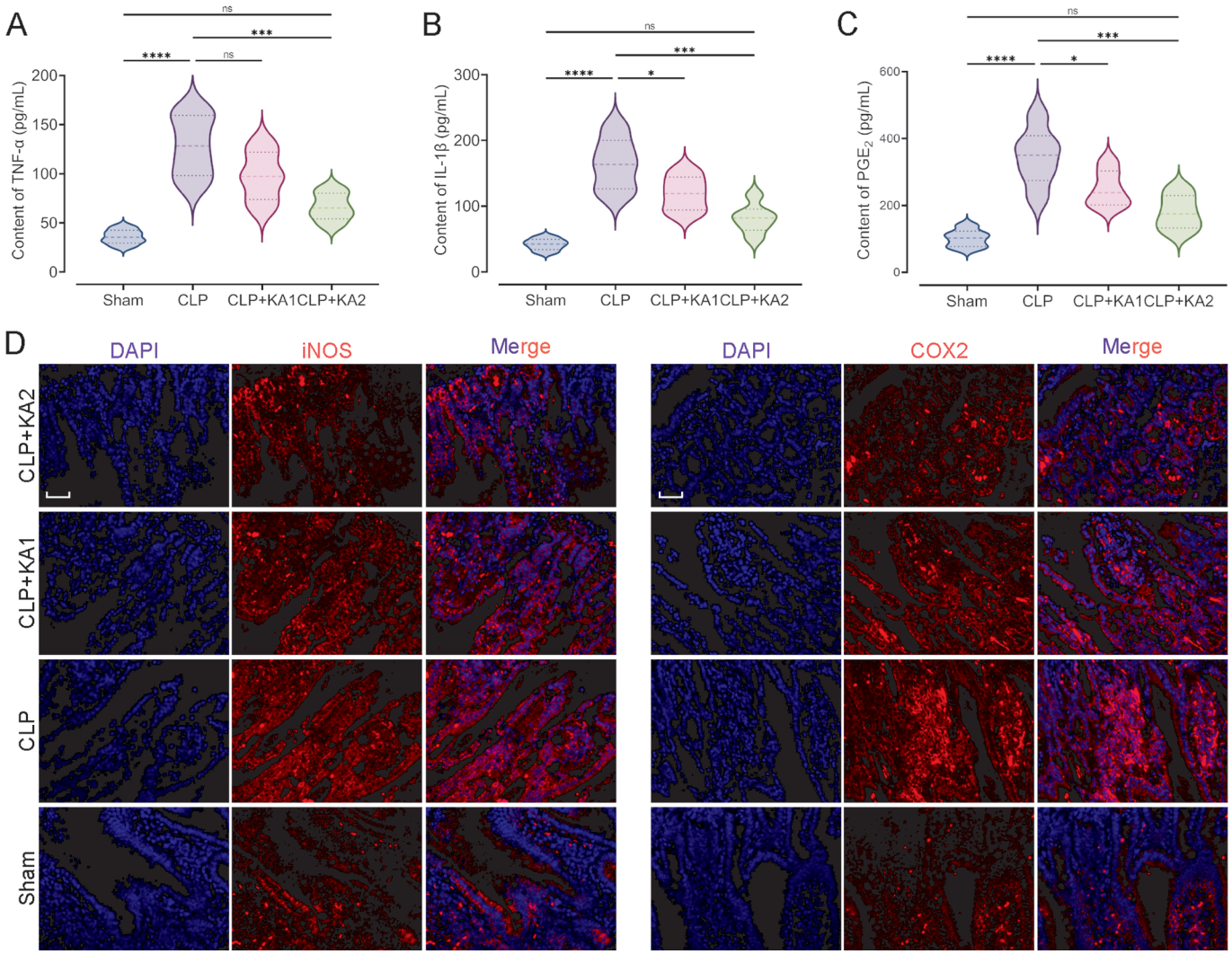

Fig. 3. KA improves sepsis-induced inflammatory injury in ileum. (A) Concentration of TNF-α detected by ELISA in serum. (B) Concentration of IL-1β detected by ELISA in serum. (C) Concentration of PGE_2 detected by ELISA in serum. (D) IF staining of iNOS and COX2 (400×), scale bar = 50 μm. The results are shown as the mean ± SD, $n = 6$.

treatment (Fig. 5A). Consistent with *in vivo* results, KA significantly inhibited the activation of NF-κB signaling pathway (Fig. 5B). And KA reduced the LPS-induced elevated content levels of TNF-α, IL-1β, and PGE_2 in the supernatant to alleviate the inflammatory response (Fig. 5C–E).

Discussion

In recent years, the link between sepsis and intestinal injury has attracted much attention, particularly the role of intestinal barrier dysfunction in the pathological progression of sepsis. The intestinal barrier consists of intestinal epithelial cells, mucosal layers, and tight junctions that prevent harmful substances from entering the organism. During sepsis, factors such as inflammatory response,[30] hypotension,[31] and hypoxia[32] lead to intestinal ischemia and hypoxia, which disrupt the tight junction structures and increase intestinal permeability. This allows intestinal bacteria and their toxins to enter the bloodstream, exacerbating the systemic inflammatory response and creating a vicious cycle. In addition, it triggers an excessive cytokine storm that further impairs intestinal barrier function and increases the risk of systemic infection.[33] It not only exacerbates the inflammatory response, but may also lead to septic shock and multiple organ dysfunction syndrome (MODS).[34] We demonstrated by *in vivo* and *in vitro* experiments that sepsis truly induced elevated expression levels of inflammatory markers TNF-α, IL-1β, and PGE_2.

NF-κB is a pivotal regulator of immune and inflammatory responses, activated in response to various stress signals, including bacterial endotoxins,[35] cytokines,[36] and reactive oxygen species (ROS),[37] all of which are significantly elevated during sepsis. Upon activation, NF-κB translocates to the nucleus, where it binds to DNA and promotes the transcription of pro-inflammatory genes, including TNF-α, IL-6, and IL-1β, as well as genes involved in leukocyte recruitment and adhesion.[38,39] Tight junction proteins, such as occludin, claudin, and ZO-1, play a critical role in maintaining epithelial barrier integrity. Under normal conditions, these proteins form a seal between adjacent epithelial cells, preventing paracellular leakage. However, during sepsis, the activation of NF-κB leads to down-regulation of these proteins' expression, which increases intestinal permeability and contributes to the phenomenon of 'leaky gut'.[40]

Research continues to emphasize the distinction between the

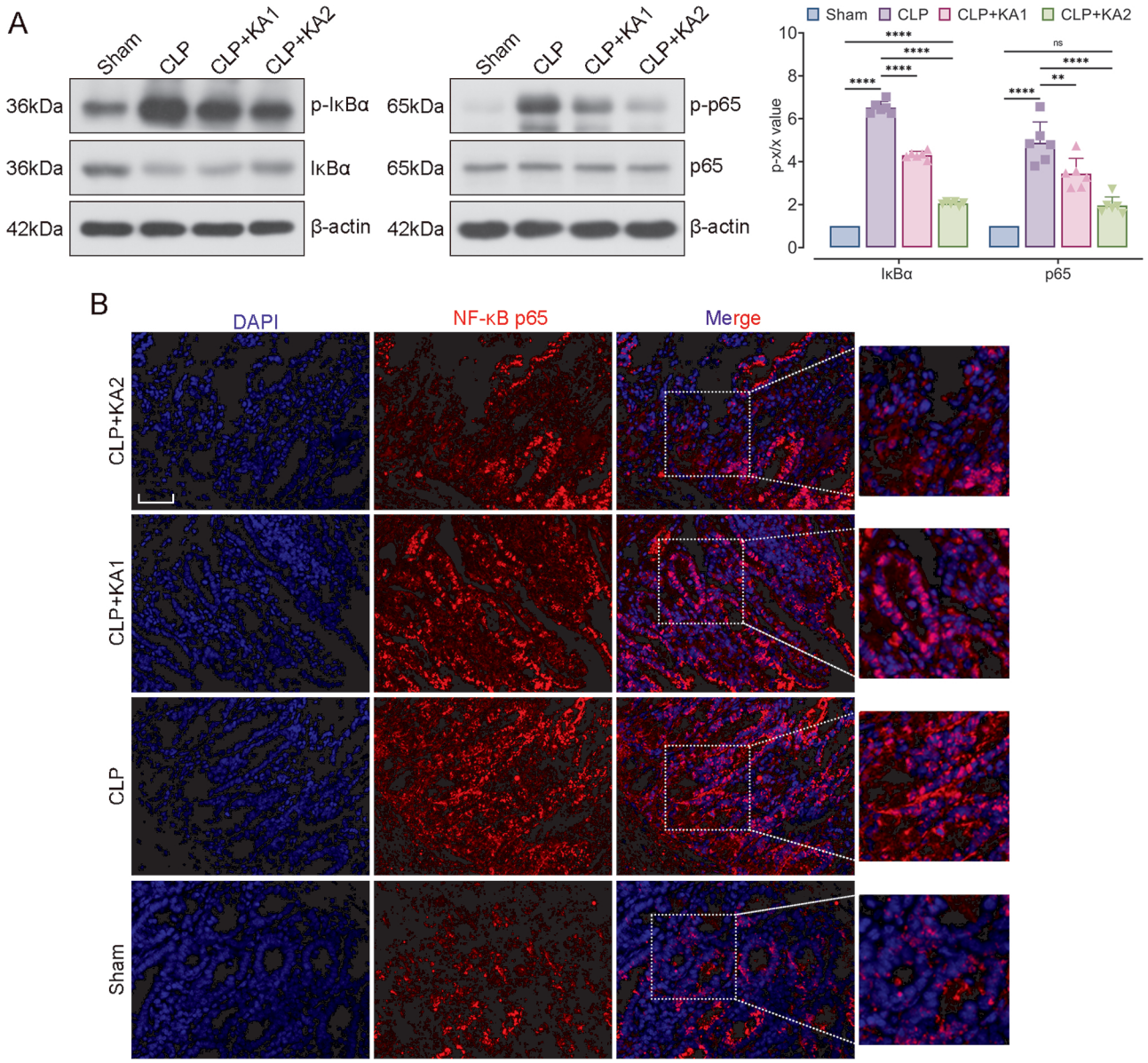

Fig. 4. KA inhibits the activation of NF-κB pathway in ileum. (A) WB and quantization of NF-κB pathway factors (p-IκBα, IκBα, p-p65, and p65). (B) IF staining of p65 (400×), scale bar = 50 μm. The results are shown as the mean ± SD, *n* = 6.

canonical and non-canonical NF-κB pathways. The non-canonical pathway is slower and is triggered by specific signals, such as CD40 ligand, lymphotoxin-β, and B-cell activating factor of the TNF family (BAFF), resulting in the activation of the RelB/p52 dimer.[41,42] This pathway is essential for lymphoid organ development and adaptive immunity. In contrast, the canonical pathway is primarily activated by pro-inflammatory signals like TNF-α, IL-1, and LPS, leading to the activation of the p65/p50 complex.[43,44] This pathway is critical for regulating inflammatory responses, innate immunity, and acute stress reactions. In our study, external stimuli induced an increase on the expression levels of pro-inflammatory factors TNF-α, IL-1β, and PEG_2, activating the IκB kinase complex (IKK). The activation of IKK promotes the phosphorylation of IκB proteins, particularly inhibitor-kappaBα (IκBα). Subsequently, IκBα is ubiquitinated and degraded, leading to the release of the NF-κB dimers (p65/p50). The released p65/p50 rapidly translocates into the nucleus, binds to specific DNA sequences, and initiates the transcription of pro-inflammatory genes, thereby driving the inflammatory response.

Currently, the treatment of sepsis mainly relies on antibiotics[45] and fluid resuscitation,[46] but the effect is limited. The extensive use of antibiotics may trigger resistance.[47] KA is an isoflavone glycoside in the traditional Chinese medicine *Pueraria lobata*. Its main metabolite in the organism is *Puerarin*. It has shown strong antioxidant capacity, reducing oxidative stress-induced cellular damage by scavenging free radicals and modulating the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx).[48] In inflammatory bowel disease (IBD), it also attenuates oxidative stress and inflammatory responses (TNF-α, IL-1β, and IL-6) by inhibiting the MAPK signaling pathway.[49] The findings in our study suggest that KA holds significant therapeutic potential in the treatment of sepsis-induced intestinal injury. One of the most striking effects on intestinal barrier function is its ability to restore the expression and organization of tight junction proteins. In sepsis model, KA treatment reverses the loss of tight junction

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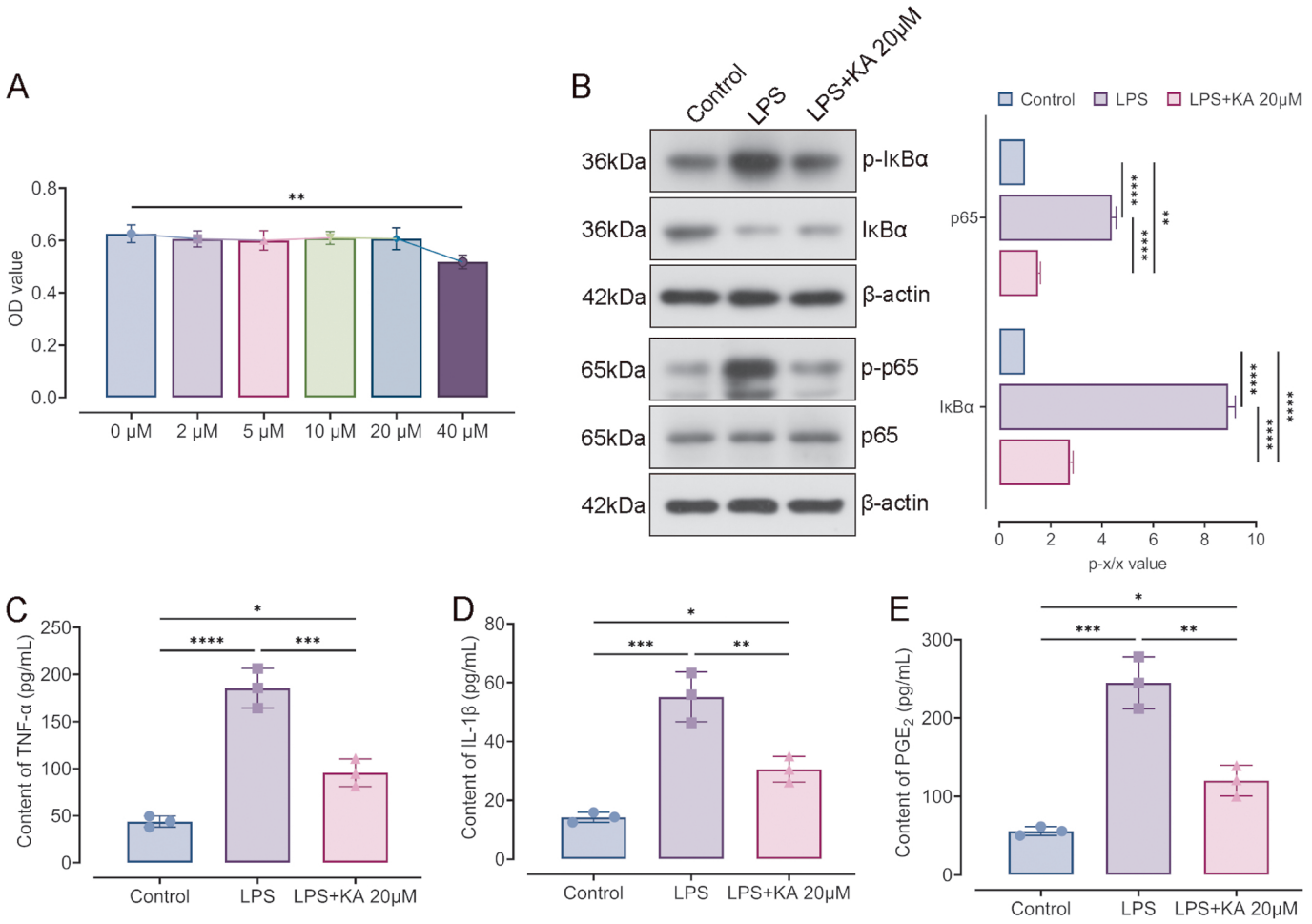

Fig. 5. KA inhibits the activation of NF-κB pathway and alleviates inflammatory injury induced by LPS in Caco-2 cells. (A) Levels of cell viability detected by MTT. (B) WB and quantization of NF-κB pathway factors (p-IκBα, IκBα, p-p65, and p65). (C) Concentration of TNF-α detected by ELISA in cellular supernatant. (D) Concentration of IL-1β detected by ELISA in cellular supernatant. (E) Concentration of PGE_2 detected by ELISA in cellular supernatant. The results are shown as the mean ± SD, $n = 3$.

proteins. It maintains the barrier between epithelial cells by inhibiting the phosphorylation of IκBα, preventing the entry of the NF-κB complex into the nucleus, and restoring the expression level of ZO-1. This restoration of tight junction integrity prevents the paracellular leakage of bacteria and endotoxins, reducing the risk of bacterial translocation and systemic inflammation. Also, it attenuates the systemic inflammatory response by reducing the expression of inflammatory factors (iNOS and COX2).

Despite promising results, there are challenges in the clinical translation of KA, particularly regarding its bioavailability and pharmacokinetics. It has poor oral absorption and rapid clearance in the intestine, which limits its effectiveness.[50] Researchers are focusing on developing drug delivery systems, such as nano formulations[51,52] and liposomal preparations,[53] to enhance its therapeutic potential. Besides, the LPS-induced inflammatory response is mainly through the TLR4 pathway, whereas clinical sepsis may involve multiple pathogens and complex immune and metabolic responses.[54] Therefore, the LPS model cannot fully simulate the complexity of sepsis *in vivo*.

In conclusion, KA's inhibition of NF-κB activation presents a compelling strategy for improving intestinal barrier function and reducing the systemic complications of sepsis. The therapeutic potential of KA in this context warrants further exploration, with the aim of developing effective treatments to improve outcomes in patients with sepsis.

Author Contributions

TX and XG contributed to the conceptualization of the study. TX, JH, NW, ZH, and HY contributed to data analysis and interpretation. TX and JH contributed to drafting the manuscript. XG supervised the study and revised the manuscript. All authors have approved the submitted version of the manuscript.

Data Availability Statement

The data and materials in the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Rudd KE, Johnson SC, Agesa KM, *et al*. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. *Lancet* 2020; **395**: 200–211.

2 Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013; **13**: 862–874.

3 Camus G, Deby-Dupont G, Duchateau J, Deby C, Pincemail J, Lamy M. Are similar inflammatory factors involved in strenuous exercise and sepsis? *Intensive Care Med* 1994; **20**: 602–610.

4 Bone RC. The pathogenesis of sepsis. *Ann Intern Med* 1991; **115**: 457–469.

5 Mohammed I, Nonas SA. Mechanisms, detection, and potential management of microcirculatory disturbances in sepsis. *Crit Care Clin* 2010; **26**: 393–408.

6 Iba T, Umemura Y, Wada H, Levy JH. Roles of coagulation abnormalities and microthrombosis in sepsis: pathophysiology, diagnosis, and treatment. *Arch Med Res* 2021; **52**: 788–797.

7 Lelubre C, Vincent JL. Mechanisms and treatment of organ failure in sepsis. *Nat Rev Nephrol* 2018; **14**: 417–427.

8 Douglass JD, Ness KM, Valdearcos M, *et al*. Obesity-associated microglial inflammatory activation paradoxically improves glucose tolerance. *Cell Metab* 2023; **35**: 1613–1629.e8.

9 Zhou C, She X, Gu C, *et al*. FTO fuels diabetes-induced vascular endothelial dysfunction associated with inflammation by erasing m6A methylation of TNIP1. *J Clin Invest* 2023; **133**: e160517.

10 Lee SM, Koh DH, Jun DW, *et al*. Auranofin attenuates hepatic steatosis and fibrosis in nonalcoholic fatty liver disease via NRF2 and NF-κB signaling pathways. *Clin Mol Hepatol* 2022; **28**: 827–840.

11 Al-Azab M, Idiiatullina E, Liu Z, *et al*. Genetic variants in UNC93B1 predispose to childhood-onset systemic lupus erythematosus. *Nat Immunol* 2024; **25**: 969–980.

12 Mc Guire C, Prinz M, Beyaert R, van Loo G. Nuclear factor kappa B (NF-κB) in multiple sclerosis pathology. *Trends Mol Med* 2013; **19**: 604–613.

13 Fearon U, Canavan M, Biniecka M, Veale DJ. Hypoxia, mitochondrial dysfunction and synovial invasiveness in rheumatoid arthritis. *Nat Rev Rheumatol* 2016; **12**: 385–397.

14 Kim KS, Oh DH, Choi HM, *et al*. Pyrrolidine dithiocarbamate, a NF-κB inhibitor, upregulates MMP-1 and MMP-13 in IL-1β-stimulated rheumatoid arthritis fibroblast-like synoviocytes. *Eur J Pharmacol* 2009; **613**: 167–175.

15 Brennan JJ, Gilmore TD. Evolutionary origins of Toll-like receptor signaling. *Mol Biol Evol* 2018; **35**: 1576–1587.

16 Lee Y, Wessel AW, Xu J, *et al*. Genetically programmed alternative splicing of NEMO mediates an autoinflammatory disease phenotype. *J Clin Invest* 2022; **132**: e128808.

17 Itahashi K, Irie T, Yuda J, *et al*. BATF epigenetically and transcriptionally controls the activation program of regulatory T cells in human tumors. *Sci Immunol* 2022; **7**: eabk0957.

18 Alankus B, Ecker V, Vahl N, *et al*. Pathological RANK signaling in B cells drives autoimmunity and chronic lymphocytic leukemia. *J Exp Med* 2021; **218**: e20200517.

19 Zhou T, Wang Z, Guo M, *et al*. Puerarin induces mouse mesenteric vasodilation and ameliorates hypertension involving endothelial TRPV4 channels. *Food Funct* 2020; **11**: 10137–10148.

20 Chiu PY, Wong SM, Leung HY, *et al*. Acute treatment with Danshen-Gegen decoction protects the myocardium against ischemia/reperfusion injury via the redox-sensitive PKCε/mK_{ATP} pathway in rats. *Phytomedicine* 2011; **18**: 916–925.

21 Lin CM, Lin RD, Chen ST, *et al*. Neurocytoprotective effects of the bioactive constituents of *Pueraria thomsonii* in 6-hydroxydopamine (6-OHDA)-treated nerve growth factor (NGF)-differentiated PC12 cells. *Phytochemistry* 2010; **71**: 2147–2156.

22 Chauhan P, Wadhwa K, Mishra R, *et al*. Investigating the potential therapeutic mechanisms of puerarin in neurological diseases. *Mol Neurobiol* 2024; **61**: 10747–10769.

23 Cardona-Mendoza A, Fonseca-Benitez A, Buitrago DM, Coy-Barrera E, Perdomo SJ. Down-regulation of human papillomavirus E6 oncogene and antiproliferative effect of *Schisandra chinensis* and *Pueraria lobata* natural extracts on Hela cell line. *J Ethnopharmacol* 2024; **319(Pt 3)**: 117225.

24 Li Z, Gao M, Yang B, *et al*. Naringin attenuates MLC phosphorylation and NF-κB activation to protect sepsis-induced intestinal injury via RhoA/ROCK pathway. *Biomed Pharmacother* 2018; **103**: 50–58.

25 Min SW, Park YJ, Kim DH. Kakkalide and its metabolite irisolidone ameliorate carrageenan-induced inflammation in mice by inhibiting NF-κB pathway. *Inflammation* 2011; **34**: 344–351.

26 Zhu Y, Wang Y, Teng W, *et al*. Role of aquaporin-3 in intestinal injury induced by sepsis. *Biol Pharm Bull* 2019; **42**: 1641–1650.

27 Xie S, Yang T, Wang Z, *et al*. Astragaloside IV attenuates sepsis-induced intestinal barrier dysfunction via suppressing RhoA/NLRP3 inflammasome signaling. *Int Immunopharmacol* 2020; **78**: 106066.

28 Boutanquoi PM, Khan AS, Cabeza L, *et al*. A novel fatty acid analogue triggers CD36-GPR120 interaction and exerts anti-inflammatory action in endotoxemia. *Cell Mol Life Sci* 2024; **81**: 176.

29 Polese B, Thurairajah B, Zhang H, *et al*. Prostaglandin E_2 amplifies IL-17 production by γδ T cells during barrier inflammation. *Cell Rep* 2021; **36**: 109456.

30 Bosmann M, Ward PA. The inflammatory response in sepsis. *Trends Immunol* 2013; **34**: 129–136.

31 Shapiro NI, Douglas IS, Brower RG, *et al*. National Heart, Lung, and Blood Institute Prevention and Early Treatment of Acute Lung Injury Clinical Trials Network. Early restrictive or liberal fluid management for sepsis-induced hypotension. *N Engl J Med* 2023; **388**: 499–510.

32 Mallat J, Rahman N, Hamed F, Hernandez G, Fischer MO. Pathophysiology, mechanisms, and managements of tissue hypoxia. *Anaesth Crit Care Pain Med* 2022; **41**: 101087.

33 Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. *Semin Immunopathol* 2017; **39**: 517–528.

34 Silva CMS, Wanderley CWS, Veras FP, *et al*. Gasdermin D inhibition prevents multiple organ dysfunction during sepsis by blocking NET formation. *Blood* 2021; **138**: 2702–2713.

35 Kim WG, Kim HI, Kwon EK, Han MJ, Kim DH. *Lactobacillus plantarum* LC27 and *Bifidobacterium longum* LC67 mitigate alcoholic steatosis in mice by inhibiting LPS-mediated NF-κB activation through restoration of the disturbed gut microbiota. *Food Funct* 2018; **9**: 4255–4265.

36 Efferth T, Oesch F. The immunosuppressive activity of artemisinin-type drugs towards inflammatory and autoimmune diseases. *Med Res Rev* 2021; **41**: 3023–3061.

37 Sá-Pessoa J, López-Montesino S, Przybyszewska K, *et al*. A trans-kingdom T6SS effector induces the fragmentation of the mitochondrial network and activates innate immune receptor NLRX1 to promote infection. *Nat Commun* 2023; **14**: 871.

38 Sakai J, Cammarota E, Wright JA, *et al*. Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Sci Rep* 2017; **7**: 1428.

39 Yoshioka Y, Sugino Y, Shibagaki F, Yamamuro A, Ishimaru Y, Maeda S. Dopamine attenuates lipopolysaccharide-induced expression of proinflammatory cytokines by inhibiting the nuclear translocation of NF-κB p65 through the formation of dopamine quinone in microglia. *Eur J Pharmacol* 2020; **866**: 172826.

40 Xu Y, An X, Liu L, *et al*. Self-cascade redox modulator trilogically renovates intestinal microenvironment for mitigating endotoxemia. *ACS Nano* 2024; **18**: 2131–2148.

41 Haselager M, Thijssen R, West C, *et al*. Regulation of Bcl-XL by non-canonical NF-κB in the context of CD40-induced drug resistance in CLL. *Cell Death Differ* 2021; **28**: 1658–1668.

42 Gardam S, Brink R. Non-canonical NF-κB signaling initiated by BAFF influences B cell biology at multiple junctures. *Front Immunol* 2014; **4**: 509.

43 Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α modulation of intestinal tight junction permeability is mediated by NIK/IKK-α axis activation of the canonical NF-κB pathway. *Am J Pathol* 2016; **186**: 1151–1165.

44 Wan CK, Li P, Spolski R, *et al*. IL-21-mediated non-canonical pathway for IL-1β production in conventional dendritic cells. *Nat Commun* 2015; **6**: 7988.

45 Donnelly JP, Seelye SM, Kipnis P, *et al*. Impact of reducing time-to-antibiotics on sepsis mortality, antibiotic use, and adverse vents. *Ann Am Thorac Soc* 2024; **21**: 94–101.

doi: 10.3164/jcbn.24-182

46 Tseng CH, Chen TT, Wu MY, Chan MC, Shih MC, Tu YK. Resuscitation fluid types in sepsis, surgical, and trauma patients: a systematic review and sequential network meta-analyses. *Crit Care* 2020; **24**: 693.
47 de la Fuente-Nunez C, Cesaro A, Hancock REW. Antibiotic failure: beyond antimicrobial resistance. *Drug Resist Updat* 2023; **71**: 101012.
48 Jang HM, Park KT, Noh HD, Lee SH, Kim DH. Kakkalide and irisolidone alleviate 2,4,6-trinitrobenzenesulfonic acid-induced colitis in mice by inhibiting lipopolysaccharide binding to toll-like receptor-4 and proteobacteria population. *Int Immunopharmacol* 2019; **73**: 246–253.
49 Chen X, Zhang J, Li R, *et al*. *Flos Puerariae-Semen Hoveniae* medicinal pair extract ameliorates DSS-induced inflammatory bowel disease through regulating MAPK signaling and modulating gut microbiota composition. *Front Pharmacol* 2022; **13**: 1034031.
50 Li H, Dong L, Liu Y, Wang G, Wang G, Qiao Y. Biopharmaceutics classification of puerarin and comparison of perfusion approaches in rats. *Int J Pharm* 2014; **466**: 133–138.
51 Luo CF, Yuan M, Chen MS, *et al*. Pharmacokinetics, tissue distribution and relative bioavailability of puerarin solid lipid nanoparticles following oral administration. *Int J Pharm* 2011; **410**: 138–144.
52 Xu H, Hu M, Liu M, *et al*. Nano-puerarin regulates tumor microenvironment and facilitates chemo- and immunotherapy in murine triple negative breast cancer model. *Biomaterials* 2020; **235**: 119769.
53 Barro L, Hsiao JT, Chen CY, Chang YL, Hsieh MF. Cytoprotective effect of liposomal puerarin on high glucose-induced injury in rat mesangial cells. *Antioxidants (Basel)* 2021; **10**: 1177.
54 Kayagaki N, Wong MT, Stowe IB, *et al*. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 2013; **341**: 1246–1249.