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Original research article

Linoleic acid inhibits lipopolysaccharide-induced inflammation by promoting TLR4 regulated autophagy in murine RAW264.7 macrophages

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Abstract
Linoleic acid (LA), an essential fatty acid, has emerged as a pivotal regulator in disorders associated with inflammation in recent years; however, the underlying mechanisms are still not completely understood. We utilized network pharmacology and experimental methodologies to elucidate the mechanisms underlying the anti-inflammatory effects of LA. Our network pharmacology analysis revealed that LA shares common targets with sepsis. These targets are enriched in various pathways comprising C-type signaling pathway, PI3K-Akt signaling pathway, toll-like receptor signaling pathway, neutrophil extracellular trap formation, AMPK signaling pathway, and autophagy-animal. These findings suggest that LA may exert regulatory effects on inflammation and autophagy during sepsis. Subsequently, we established *in vivo* and *ex vivo* models of sepsis using lipopolysaccharide (LPS) in experimental study. Treatment with LA reduced lung damage in mice with LPS-induced lung injury, and reduced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in plasma, bronchoalveolar lavage fluid (BALF), and peritoneal lavage fluid (PLF). LA also decreased the production of TNF-α and IL-6 in RAW264.7 macrophages exposed to LPS. In LPS-induced RAW264.7 macrophages, LA induced an elevation in LC3-II while causing a reduction in p62, which was associated with downregulation of toll-like receptor 4 (TLR4). We utilized 3-methyladenine (3-MA) to inhibit the autophagic activity, which reversed the modulatory effects of LA on LC3-II and p62. 3-MA also prevented the decline in TLR4 expression along with reduction in pro-inflammatory cytokines secretion. Our findings suggest that the activation of autophagy by LA may lead to the downregulation of TLR4, thereby exerting its anti-inflammatory effects.

Keywords: Autophagy; Inflammation; Linoleic acid; Network pharmacology; Toll-like receptor 4

Highlights:
- Linoleic acid may serve as a potential regulator of inflammation in sepsis, as indicated by network pharmacology analysis.
- Linoleic acid exerts anti-inflammatory roles in both mice and RAW264.7 macrophages upon LPS stimulation.
- Linoleic acid activates autophagy in RAW264.7 macrophages with LPS exposure.
- Linoleic acid inhibits inflammation via downregulation of TLR4 by promoting autophagy.

Introduction

The inflammatory response serves as a crucial defense mechanism that safeguards the host against pathogen invasion. Nevertheless, an overactive inflammatory reaction frequently leads to harm in tissues and is heavily linked to the advancement of different disorders associated with inflammation, including rheumatoid arthritis, cardiovascular disease, and sepsis (Delano and Ward, 2016; Komatsu and Takayanagi, 2022; Kong et al., 2022). Thus, limitation of the intensity of inflammation and facilitation of the termination of inflammation might be pivotal for the maintenance of homeostasis.

Over the past few decades, there has been a gradual rise in the consumption of linoleic acid (LA), an indispensable fatty acid that forms part of cellular membranes (Blasbalg et al., 2011; Simopoulos, 2016). In its role as a nutrient, LA has attracted increasing attention due to its advantageous impact

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on inflammation. For example, a study conducted on patients with cardiovascular diseases found that the consumption of LA was associated with reduced levels of pro-inflammatory cytokines (Bersch-Ferreira et al., 2017). Furthermore, the administration of LA displayed anti-inflammatory characteristics in allergic inflammation mediated by mast cells (Kim et al., 2019). Despite the acknowledged beneficial effects of LA on inflammation, the underlying mechanisms remain incompletely elucidated.

Macrophages play a crucial role in the inflammatory response, as they are widely distributed throughout the body's tissues and exhibit sensitivity to various pro-inflammatory stimuli. Lipopolysaccharide (LPS), an essential factor contributing to the pathogenicity of Gram-negative bacteria, is closely associated with numerous clinical disorders and extensively utilized in experimental studies (Raetz and Whitfield, 2002). TLR4, a prototypical pattern recognition receptor (PRR) that binds to LPS, triggers the activation of a pro-inflammatory cascade in macrophages (Iwasaki and Medzhitov, 2004). The dysregulated and excessive pro-inflammatory signaling initiated by TLR4 can lead to severe conditions such as sepsis, septic shock, and even fatality (Poltorak et al., 1998). Increasing studies have investigated that downregulating TLR4 itself and inhibiting TLR4-mediated pathways, such as IRAK1/Erk, TRAF6/NF-κB, and MAPK pathways may function as negative regulators of LPS-induced hyperinflammation and exert protective roles in LPS-induced kidney injuries and septic mice (Ren et al., 2020; Zhang et al., 2023).

The current investigation utilized network pharmacology to explore the potential roles of LA in relation to inflammatory conditions. Furthermore, the LA's ability to reduce LPS-induced inflammation was validated both *in vitro* and *in vivo*. Finally, candidate molecular mechanisms were explored using murine RAW264.7 macrophages challenged with LPS.

Materials and methods

Bioinformatic collection of intracellular targets related to LA and sepsis

The chemical structure and Isomeric SMILES information of LA were downloaded from PubChem database, and LA-related targets were identified using public databases including SEA and SwissTargetPrediction (Daina et al., 2019; Keiser et al., 2007). The targets associated with sepsis were obtained from the GeneCards database using specific criteria (protein coding category and score >0.5), in addition to the DisGeNET database (Piñero et al., 2017, 2020; Stelzer et al., 2016) by searching for "sepsis". The online platform was used to create a Venn diagram by inputting the common targets of LA and sepsis (https://www.bioinformatics.com.cn).

KEGG and GO enrichment analysis

The biological function of shared targets was investigated by utilizing the DAVID database for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analysis. KEGG pathway annotation and KEGG pathway analysis, and GO analysis data were visualized and plotted by the online platform.

PPI Network construction

Shared targets were imported into the STRING database for information of the protein interaction network. Information of protein-protein interaction (PPI) was inputted in Cytoscape3.9.1 software for visualization and network construction. The properties of nodes in the PPI network were deeply indicated by the parameters calculated by Cytoscape such as degree value.

Experiment animals

The male C57BL/6 mice with wild-type characteristics (aged 6–8 weeks and weighing between 20–25 g) were procured from the Laboratory Animal Center of Chongqing Medical University, China. These mice were housed in a specific pathogen-free (SPF) facility, ensuring their health and well-being. The housing conditions maintained a constant temperature of 25 °C and followed a regular light/dark cycle of 12 hours each. The mice had unrestricted access to a standard diet and unlimited water supply.

Reagents and animal treatment

The mice underwent a period of acclimation for at least one week prior to treatment. LA (≥99%, 18:2, n-6, Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (Sangon Biotech, China) and subsequently diluted in olive oil, following the methodology outlined in our previous study (Zhang et al., 2024). The LPS (obtained from *Escherichia coli 055:B5*, Sigma-Aldrich, USA) was dissolved using a sterile solution of phosphate buffer saline (PBS). A widely used *in vivo* sepsis model was established and involved the induction of sepsis through LPS challenge (Chen et al., 2022b; Dos Santos et al., 2022; Li et al., 2022b). Specifically, the mice were divided into four groups in a random manner, namely the control group (intraperitoneal saline injection), LA group (intraperitoneal 50 mg/kg of LA injection), LPS group (intraperitoneal 20 mg/kg of LPS injection), and LA+LPS group (intraperitoneal 50 mg/kg of LA and 20 mg/kg of LPS injection). The mice were intraperitoneally administered with either LA or saline one hour prior to the injection of LPS. After 24 hours, all mice remained alive and were anesthetized using sodium pentobarbital (50 mg/kg) for subsequent treatment, including the collection of blood, bronchoalveolar lavage fluid (BALF), lung tissues, and peritoneal lavage fluid (PLF). The mice were euthanized using cervical dislocation. To obtain plasma, blood samples were gathered and subjected to centrifugation at 8000× g for 10 min at a temperature of 4 °C. The resultant liquid above the sediment was subsequently collected and temporarily stored at –80 °C.

BALF collection

After administering sodium pentobarbital anesthesia, the laryngeal tissue of the mouse was dissected to expose the trachea. A needle was used to puncture the exposed trachea, followed by two rinses with 0.5 ml of cold PBS solution. The collected samples in PBS were then subjected to centrifugation at a speed of 3,000 revolutions per minute for 10 min at a temperature of 4 °C. Subsequently, the resulting supernatant was obtained and temporarily stored at a temperature of 4 °C.

PLF collection

The anaesthetized mice were intraperitoneally injected with 10 ml of cold PBS. Gentle manipulation of the abdomen for 2 min facilitated proper distribution of PBS within the peritoneal cavity. The incubation period of 5 min was observed prior to the removal of fluid from the abdominal cavity. The collected samples in PBS were then subjected to centrifugation at a speed of 3,000 revolutions per minute for 10 min at a temperature of 4 °C. Subsequently, the resulting supernatant was obtained and temporarily stored at a temperature of 4 °C.

H&E staining

The left lung tissues of mice were preserved in a solution containing 4% formaldehyde. After undergoing sectioning, the lung tissue samples were stained using haematoxylin and eosin (H&E). An imaging system (Leica, Wetzlar, Germany) was employed for morphological examination. The Smith lung injury pathology scoring method was used to quantify histological changes associated with lung injury, assessing pulmonary edema, inflammatory cell infiltration in alveolar and interstitial spaces, hemorrhage in alveolar and interstitial areas, and the occurrence of atelectasis and hyaline membrane formation (Eltzschig et al., 2014). Scores are on a scale of 0–4 points: 0 is normal, 1 is mild, and 2 is moderate, 3 is severe, 4 is extremely severe.

Cell culture

The RAW264.7 macrophage cell line was obtained from Procell Life Science & Technology. The cells were cultured in a humidified air environment at 37 °C with 5% CO_2, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution (containing 100 units/ml of penicillin and 0.1 mg/ml of streptomycin), all purchased from Gibco, USA.

Reagents and cell treatment

The LA was dissolved in ethanol and further diluted in DMEM supplemented with 2% Bovine Serum Albumin (BSA, Beyotime, China) to achieve the final concentration as previously documented (Reyes et al., 2020). 3-MA (MedChemExpress, USA) was dissolved in sterile PBS. Cells were seeded overnight in 6-well plates and randomly assigned to different groups including the control group (cultured with serum-free DMEM containing 2% BSA), LA group (administered LA 10 μM), LPS group (administered LPS 1 μg/ml), and LA+LPS group (administered LA 10 μM and LPS 1 μg/ml). RAW264.7 cells in all groups were incubated for 24 h. For autophagy inhibition, cells were pre-treated with 3-MA (5 mM) for 2 h.

Cell viability

The impact of LA on the viability of cells was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to established protocols. RAW264.7 cells were cultured overnight in a 96-well plate with a cell density of 5×10^3 cells per well. Subsequently, different concentrations of LA (0, 5, 10, 20, and 50 μM) were administered to the cells for a duration of 24 h. Subsequently, all wells were treated with CCK-8 reagent for a period of 1.5 h before measuring absorbance at a wavelength of 450 nm.

Monodansylcadaverine (MDC) staining

The MDC dye is commonly used to detect autophagy and specifically stains autophagosomes, which exhibit a fluorescent green color when examined using a fluorescence microscope (Leica, Wetzlar, Germany) after being excited by ultraviolet light. The fluorescent probe MDC stains acidic membranous structures, resulting in background staining in normal cells. The MDC staining in this study was conducted using a commercial assay kit (Biosharp, China).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA kits were employed to measure the concentrations of TNF-α and IL-6 in the supernatant obtained from centrifugation of the aforementioned groups (Neobioscience, China).

Western blot analysis

The RAW264.7 cells were harvested from 6-well plates and subjected to RIPA lysis buffer (Beyotime, China) supplemented with phosphatase inhibitors (Beyotime, China) for complete protein extraction. The quantification of total protein was performed using the BCA assay kit (Beyotime, China). Subsequently, the whole proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels with varying concentrations of 7.5% or 12.5% (Epizyme, China), followed by transfer onto polyvinylidene fluoride membranes (PVDF, Millipore, USA). TBS-T buffer solution supplemented with 0.1% Tween-20 and 5% skimmed milk powder (Sangong Biotech, China) was employed to obstruct the PVDF membranes at room temperature for a duration of 1.5 h. After being blocked, the membranes were incubated with appropriate primary antibodies at 4 °C overnight. The primary antibodies against LC3A/B (1:1500, Cell Signaling Technology, USA), SQSTM1/p62 (1:1500, Cell Signaling Technology, USA), AMPKα (1:1500, Cell Signaling Technology, USA), Phospho-AMPKα (1:1500, Cell Signaling Technology, USA), TLR4 (1:500, Beyotime, China), GAPDH (1:20000, ABclonal, China), and β-actin (1:1000, Bioss, China) were used. After incubating with primary antibodies overnight, the membranes underwent five washes using TBS-T. Afterwards, secondary antibodies against rabbits (1:5000, Bioss, China) were added and allowed to incubate at room temperature for 1.5 h. Subsequently, the membranes underwent an additional round of washing. ECL FemtoLight oxidant and substrate (Epizyme, China) were mixed in a 1:1 ratio to apply to the membranes. Then the band signals were captured using the FUSION SOLOS Imaging System (VILBER BIO IMAGING, FRANCE) and analyzed by ImageJ-win64 software.

Statistical analysis

The experimental data was analyzed and processed utilizing the software GraphPad Prism 8. Multiple comparisons between groups were performed using one-way ANOVA Tukey's test. The experimental data was presented using mean values along with their corresponding standard deviations (SD). Statistical significance was considered at a significance level of $P < 0.05$.

Results

KEGG pathway and GO enrichment analysis of shared targets

Due to the pivotal roles of LPS-induced excessive inflammation in sepsis pathogenesis (Dickson and Lehmann, 2019; Raetz and Whitfield, 2002), we utilized network pharmacology analysis to investigate the underlying mechanisms by which LA exerts its regulatory effects on sepsis. The chemical structure of LA was used to predict a total of 190 potential targets for LA (Fig. 1A).

For KEGG pathway and GO enrichment analysis, a total of 92 shared targets of LA and sepsis (Fig. 1B) were inputted to the DAVID database. As shown in Fig. 1C, the shared targets exhibited significant enrichment in signal transduction, immune system, viral infection-associated disease, and overview cancer categories. The top 30 pathways not in the human disease category were listed, including C-type signaling pathway, PI3K-Akt signaling pathway, toll-like receptor signaling pathway, neutrophil extracellular trap formation, AMPK signaling pathway, and autophagy-animal (Fig. 1D). These pathways

demonstrated close associations with inflammation. Similarly, the GO enrichment analysis indicated a significant enrichment of shared targets in signal transduction and the inflammatory response (Fig. 1E). Taken together, these discoveries offer significant insights into the potential impact of LA on inflammation modulation in septic conditions.

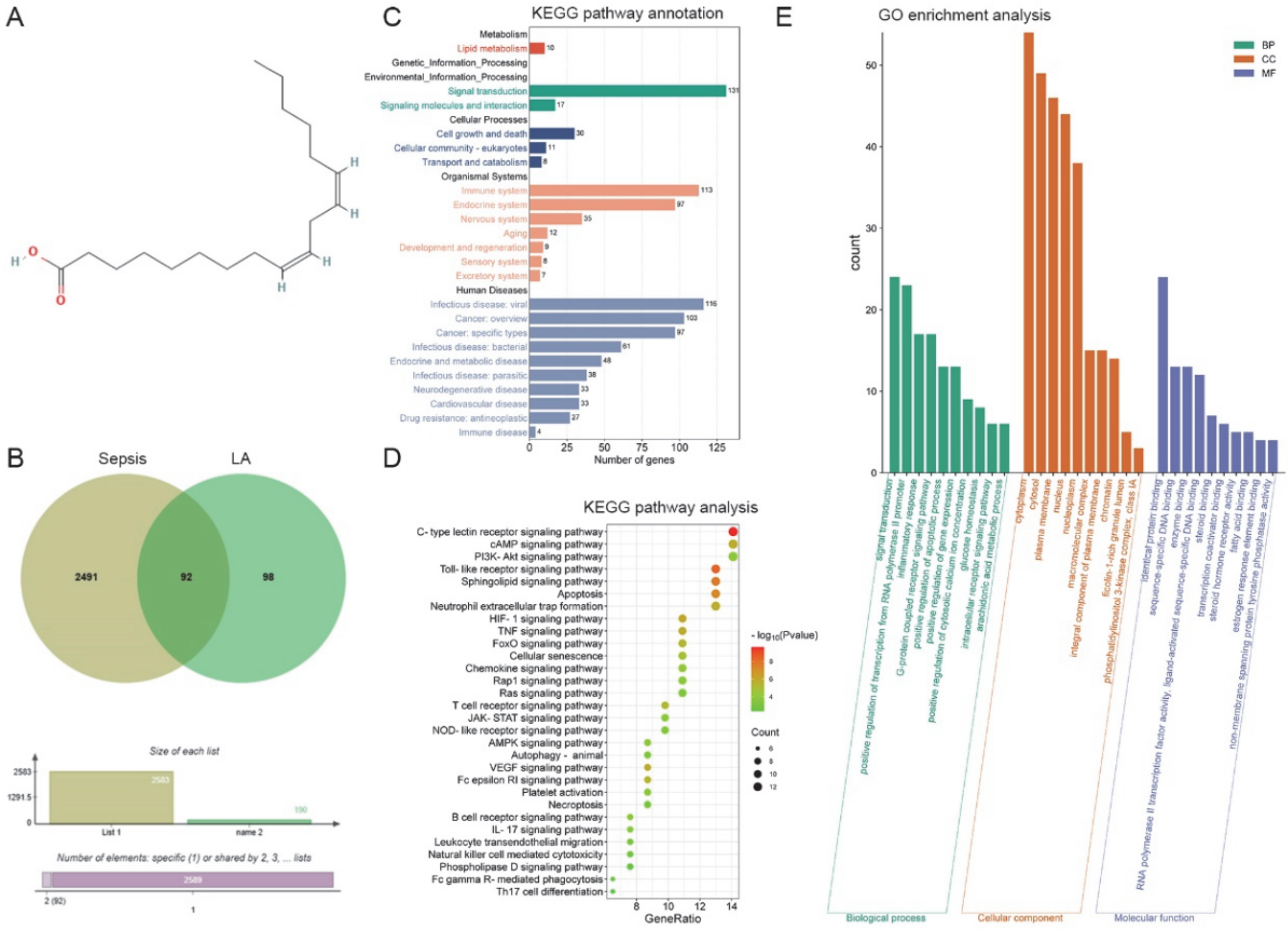

Fig. 1. Chemical structure of LA, Venn diagram, functional and pathway analysis of shared targets. (**A**) 2D chemical structure of LA downloaded from PubChem (CAS: 60-33-3); (**B**) Venn diagram of overlapped targets between sepsis- and LA-related targets; (**C**) Four categories of KEGG pathway annotation, namely metabolism, cellular processes, organismal systems, and human diseases. (**D**) Top 30 pathways not in the human disease category among KEGG pathway analysis with dot plot. (**E**) The GO enrichment analysis including top 10 biological processes (BP), top 10 cellular components (CC), and top 10 molecular functions (MF).

PPI network of shared targets

The PPI network was constructed to visually represent the shared targets, as shown in Fig. 2A. Node size and color intensity were scaled based on their degree value, with larger nodes indicating a higher probability of being core targets. As illustrated in Fig. 2B, the top 10 targets were identified and considered as putative core targets for LA against sepsis, based on their degree values.

LA suppressed LPS-induced inflammation in vivo *and* in vitro

The network pharmacology revealed the potential anti-inflammatory effects of LA. To investigate this, mice and murine RAW264.7 macrophages were challenged with LPS to establish *in vivo* and *in vitro* sepsis models, respectively. According to our previous study (Zhang et al., 2024), a concentration of 50 mg/kg of LA was determined for treating mice. The treatment with LA significantly ameliorated LPS-induced lung injury, as evidenced by the mitigation of pulmonary edema, reduction in alveolar and interstitial inflammatory cell infiltration, and attenuation of alveolar and interstitial hemorrhage (Fig. 3A and B). The levels of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were assessed using ELISA to measure the extent of inflammatory response (Facchin et al., 2022). Treatment involving LA exhibited a significant decrease in the concentrations of TNF-α and IL-6 observed in plasma, BALF, and PLF (Fig. 3C–H), indicating its anti-inflammatory properties. The effect of LA on the viability of RAW264.7 cells was evaluated using the CCK-8 assay to establish an optimal concentration for subsequent studies. A concentration of 10 μM was chosen due to its minimal impact on cell viability (Fig. 3I). Consistent with the results from the *in vivo* experiment, LA also decreased LPS-induced production of TNF-α and IL-6 *in vitro* (Fig. 3J and K). These findings demonstrate that LA possesses a negative regulatory effect on inflammation.

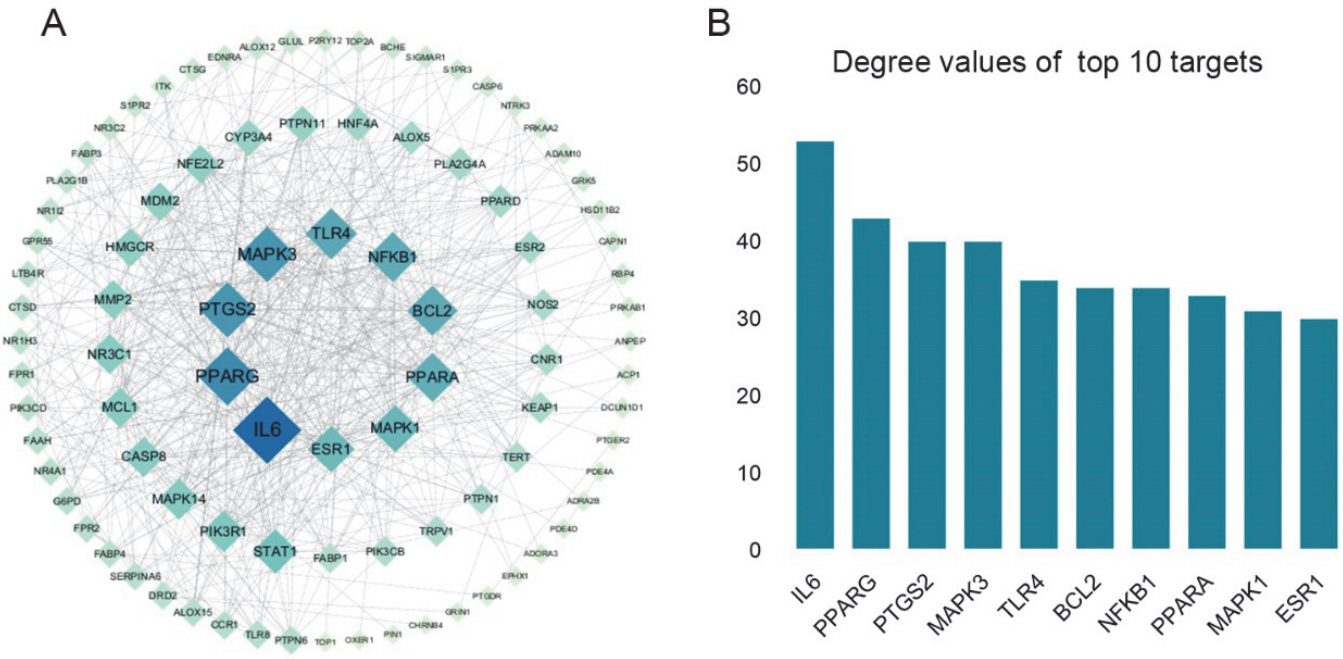

Fig. 2. PPI network and putative core targets. (**A**) The node-size mapping of the 92 shared targets between LA- and sepsis-related targets. (**B**) Putative core targets among the 92 shared targets according to degree value.

LA promoted autophagy and enhanced phosphorylation of AMPK

Autophagic vesicles can be identified by the specific *in vitro* marker, MDC. The MDC staining in our study revealed that LA treatment enhanced the intensity of green fluorescence, indicating a potential activation of autophagy in the presence of LPS (Fig. 4A). Furthermore, western blot analysis showed that LA increased LC3-II levels while decreasing p62 levels, providing evidence for augmented autophagy (Fig. 4B and C). Additionally, LA treatment promoted AMP-activated protein kinase (AMPK) phosphorylation (Fig. 4D). Therefore, it is plausible to propose that LA promotes autophagy in RAW264.7 cells challenged by LPS through AMPK activation.

LA reduced TLR4

The Toll-like receptor 4 (TLR4), which serves as the receptor for LPS (da Silva Correia and Ulevitch, 2002; Lu et al., 2008), plays crucial roles in mediating inflammatory responses (Ciesielska et al., 2021; Yu et al., 2022). The treatment of LA resulted in a significant downregulation of TLR4 in LPS-stimulated RAW264.7 macrophages, as depicted in Fig. 5, suggesting that this effect may contribute to the anti-inflammatory properties of LA.

LA inhibited inflammation through reduction of TLR4 via promotion of autophagy

The inhibitory effects on autophagy were induced by the administration of 3-MA, a widely recognized autophagy inhibitor (Miller et al., 2010). This was evidenced by the observed decrease in LC3-II levels and increase in p62 levels (Fig. 6A and B). Furthermore, treatment with 3-MA restored the level of TLR4 (Fig. 6C), indicating that LA may downregulate TLR4 through promoting autophagy. Co-incubation with 3-MA rescued the LPS-induced production of TNF-α and IL-6 compared to LA alone (Fig. 6D and E). In conclusion, these findings suggest that LA exerts a potent anti-inflammatory effect by reducing TLR4 via promotion of autophagy.

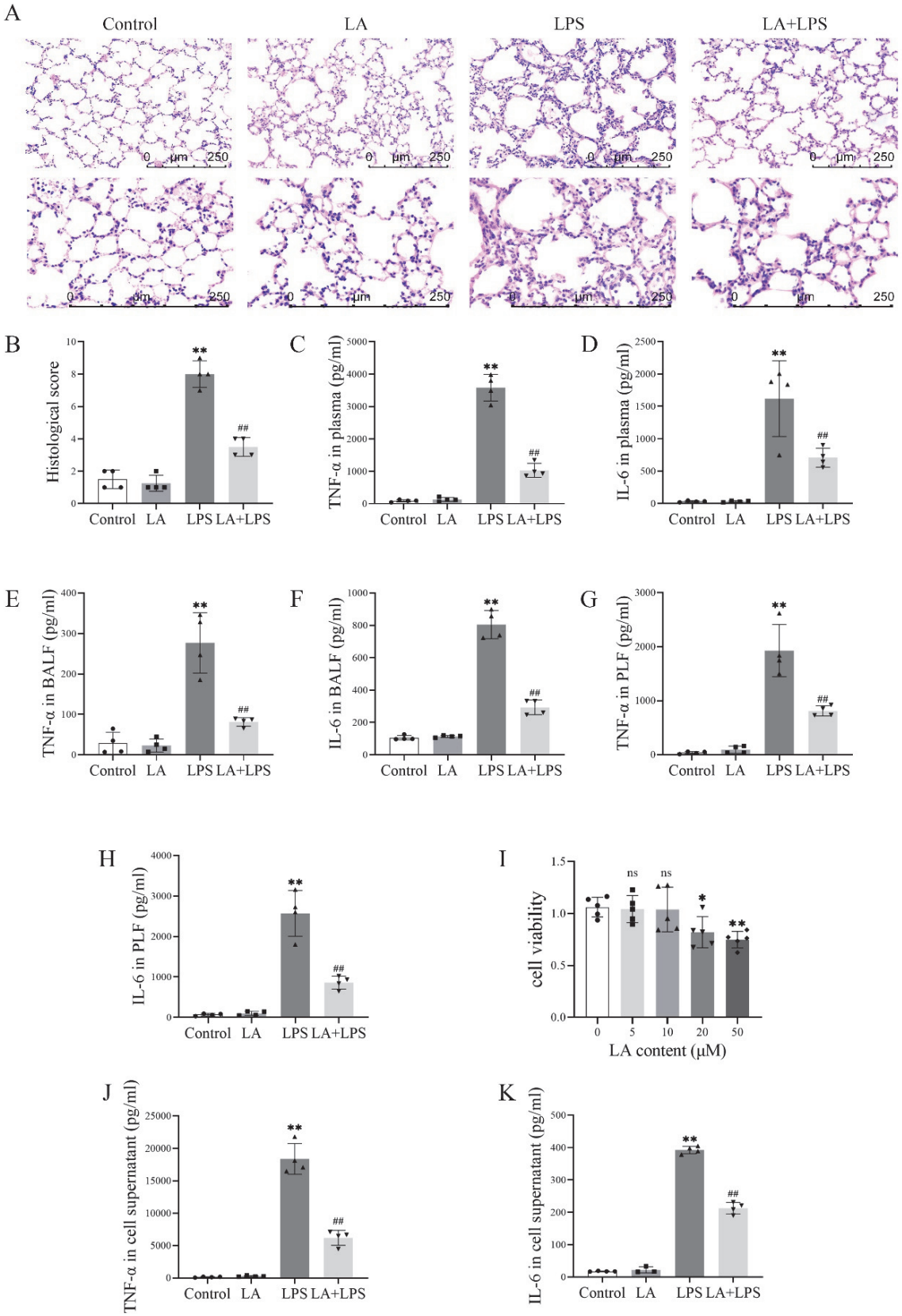

Fig. 3. Effect of LA on lung injury, cell viability, and production of pro-inflammatory cytokines. (**A**) Histological images stained with H&E. (**B**) Histological score assessed based on pulmonary edema, inflammatory cell infiltration, hemorrhage alveolar and interstitial hemorrhage, atelectasis and hyaline membrane formation. (**C–H**) TNF-α and IL-6 in plasma, BALF, and PLF determined by ELISA. (**I**) RAW264.7 cells viability evaluated by CCK-8 assay with exposure to various concentrations of LA (0, 5, 10, 20, and 50 μM) for 24 h. (**J**) TNF-α and (**K**) IL-6 in a culture of medium supernatant of RAW264.7 macrophages were determined by ELISA; ns = not significance, ** $p < 0.01$ versus Control; ## $p < 0.01$ versus LPS.

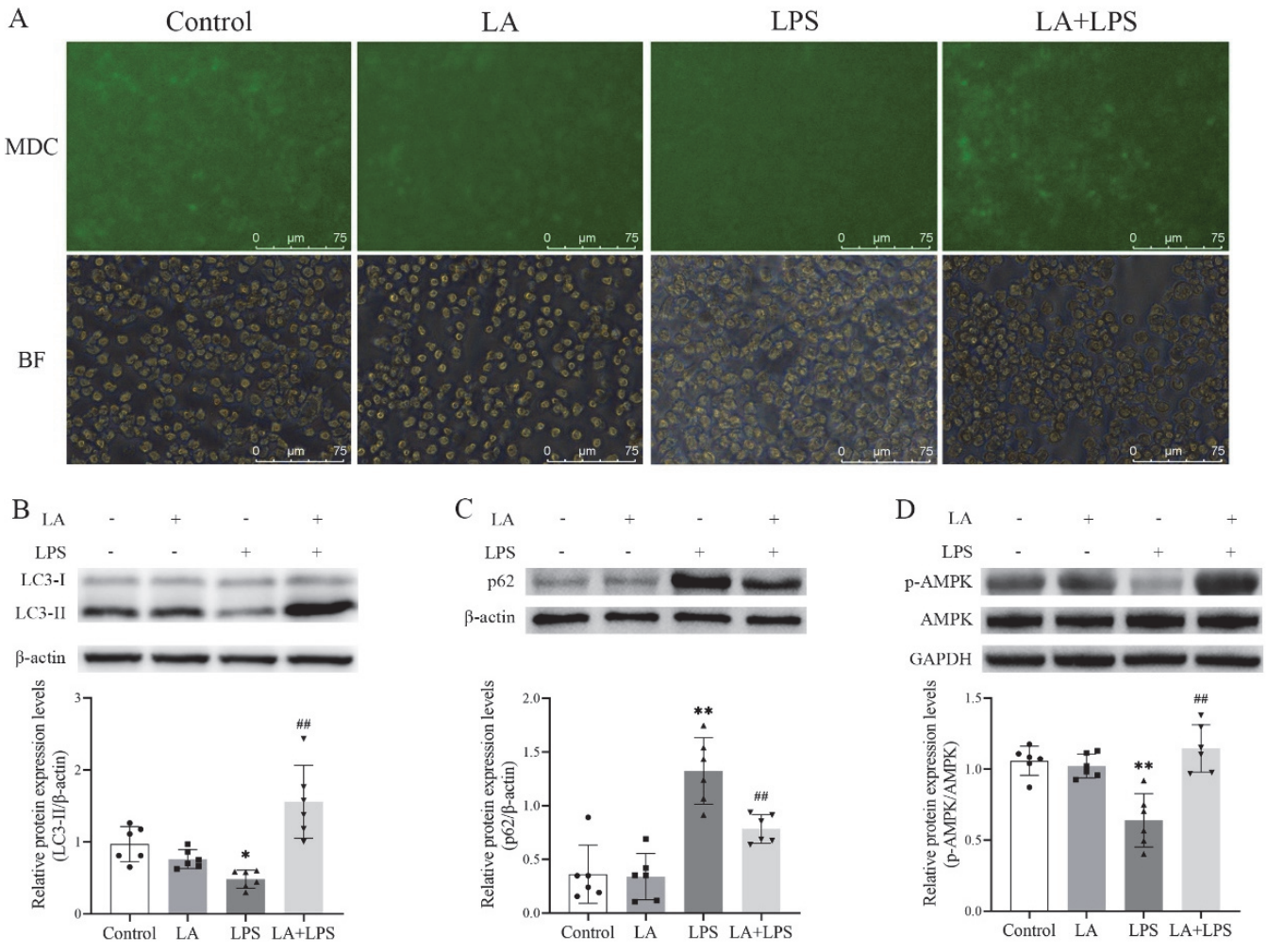

Fig. 4. LA promotes autophagy and AMPK activation. (**A**) The detection of autophagy was performed using MDC staining and the BF mean bright field. (**B–D**) RAW264.7 cells were treated with LA (10 μM) in the presence of LPS (1 μg/ml) for 24 h and protein levels of LC3-I, LC3-II, p62, AMPK, and p-AMPK were measured by immunoblot; * $p < 0.05$, ** $p < 0.01$ versus Control; ## $p < 0.01$ versus LPS.

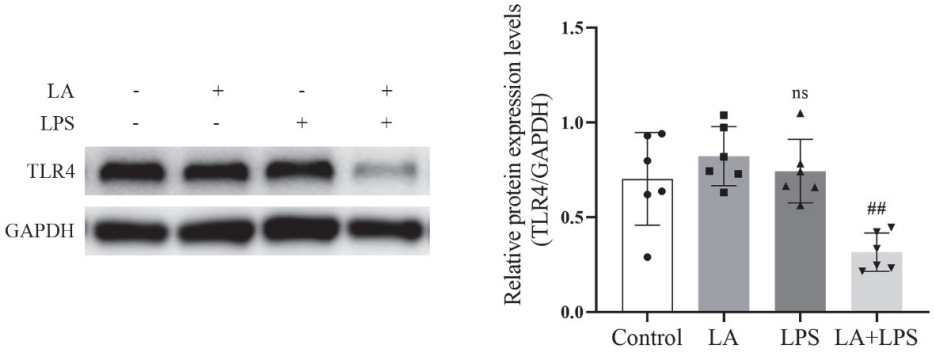

Fig. 5. LA reduces TLR4. RAW264.7 cells were treated with LA (10 μM) in the presence of LPS (1 μg/ml) for 24 h and protein levels of TLR4 were measured by immunoblot; ns = not significant versus Control; ## $p < 0.01$ versus LPS.

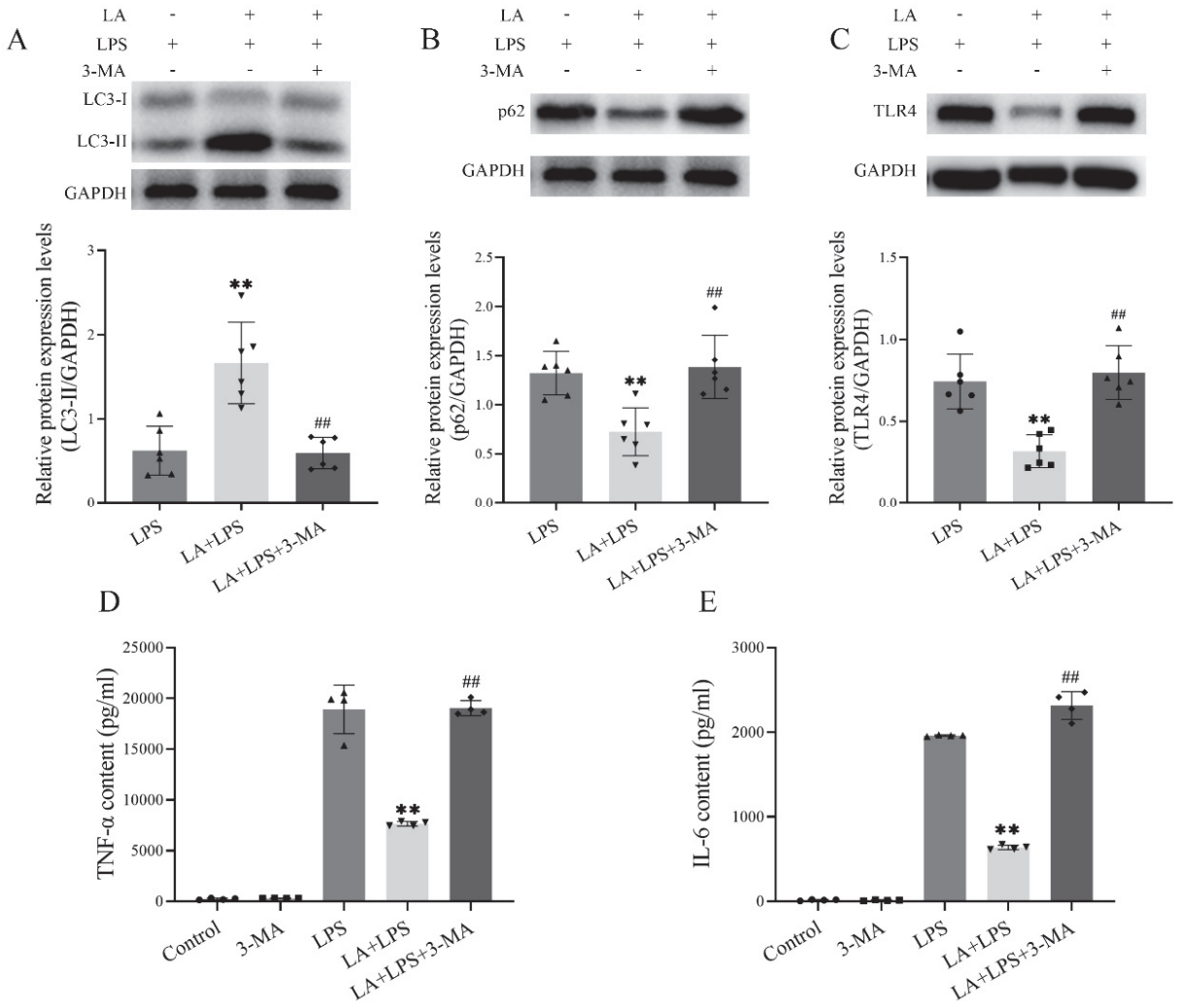

Fig. 6. 3-MA inhibits autophagy, increases levels of TLR4 and pro-inflammatory cytokines. RAW264.7 cells were pretreated with 3-MA (5 mM) for 2 h, then treated with LA (10 μM) and LPS (1 μg/ml) for 24 h. (**A–C**) Protein levels of LC3-I, LC3-II, p62, and TLR4 were measured by immunoblot. (**D** and **E**) TNF-α and IL-6 in supernatant were determined by ELISA; ** $p < 0.01$ versus LPS; ## $p < 0.01$ versus LA+LPS.

Discussion

Emerging findings indicate that LA could potentially exert inhibitory effects on the inflammatory response (Burns et al., 2018; Innes and Calder, 2018; Marangoni et al., 2020). A prior investigation has exhibited the regulatory impact of LA on the signaling pathway linked to inflammation in macrophages when exposed to LPS (Qian et al., 2015). In a prior investigation, it was observed that LA exhibited the ability to mitigate septic liver injury induced by LPS through the inhibition of inflammation and oxidative stress (Zhang et al., 2024). Consistent with these findings, our present study reveals that treatment with LA effectively suppresses the LPS-induced production of pro-inflammatory cytokines *in vivo* and *in vitro*, evidenced by the decreased levels of pro-inflammatory cytokines in plasma, BALF, PLF, and culture medium supernatant of RAW264.7 macrophages. Furthermore, LA mitigated LPS-induced lung injuries. Therefore, LA holds promising potential for intervention in inflammatory injuries.

Network pharmacology prediction, based on topological analysis (Luo et al., 2020; Wang et al., 2021), integrates drug information data and disease-related genes to analyze and predict the potential targets and signaling pathways of drugs for treating diseases (Luo et al., 2020). Specifically, in our study, the top 30 pathways from KEGG pathway analysis have extensively been investigated for their association with inflammation regulation, including C-type lectin receptor signaling pathway (Brown et al., 2018), cAMP signaling pathway (Chen et al., 2022a; Yan et al., 2015), PI3K-Akt signaling pathway (Yu et al., 2024), and Toll-like receptor signaling pathway (Gong et al., 2020). Additionally, GO enrichment analysis revealed significant enrichment of LA-related targets in inflammatory response in the biological process aspect. Furthermore, putative core targets identified through constructing PPI network were closely related to inflammation such as IL-6 (Jones and Jenkins, 2018), MAPK3 (Jager et al., 2011), and TLR4 (Li et al., 2020). According to the results obtained, our hypothesis suggests that LA could potentially function as a modulator of inflammation in sepsis, and this has subsequently been confirmed through experimental validation.

To investigate the underlying mechanism of LA's regulatory role in inflammation, we conducted a comprehensive analysis of network pharmacology predictions. The activation of

autophagy in large yellow croaker hepatocytes by LA has been demonstrated (Yang et al., 2020), which has captured our attention and prompted us to select autophagy for further investigation based on the results of network pharmacology analysis. Additionally, KEGG pathway analysis identified several pathways that have been investigated as potential regulators of autophagy, including the C-type signaling pathway (Ding et al., 2015; Li et al., 2022a; Vilser et al., 2010), PI3K-Akt signaling pathway (Yang et al., 2018; Zhang et al., 2019a), FoxO signaling pathway (Lee et al., 2019), and AMPK signaling pathway (Hu et al., 2021; Inoki et al., 2012; Mihaylova and Shaw, 2011). Autophagy itself was also mentioned in the KEGG pathway analysis. Therefore, our hypothesis was that LA may inhibit inflammation by functioning as a regulator of autophagy. Cellular homeostasis is effectively maintained through the degradation of intracellular macromolecules within lysosomes, which is a significant function performed by autophagy (Mizushima and Komatsu, 2011). There is an increasing amount of evidence suggesting that the encouragement of autophagy is emerging as a fresh strategy for managing inflammation (Matsuzawa-Ishimoto et al., 2018; Qiu et al., 2019). In this study, we evaluated the levels of LC3-II and SQSTM1/p62 to assess autophagy activity (Mizushima and Yoshimori, 2007). Our findings demonstrated that the application of LA enhanced autophagy. However, co-administration of the autophagy inhibitor 3-MA counteracted LA's inhibitory effects on cytokines generation. Therefore, activation of autophagy may underlie the bioactivity of LA.

The binding of TLR4 to LPS triggers a robust production of various pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, through the activation of signaling cascades such as the TRAF6-TAK1-NF-κB/MAPK axis (Dajon et al., 2017; Monlish et al., 2016). This signaling pathway is responsible for TLR4-mediated hyperinflammation. The regulation of TLR4 has emerged as a pivotal mechanism in maintaining homeostasis. For example, a previous study has investigated the downregulation of TLR4 protein levels through Sirt1 overexpression to mitigate inflammation in periodontal ligament fibroblasts induced by LPS (Li et al., 2018). Similarly, punicalin treatment was found to reduce TLR4 protein levels and alleviate LPS-induced neuroinflammation while preventing the development of Alzheimer's disease (Chen et al., 2024). Additionally, endocytosis, ubiquitination, and degradation of TLR4 via Hsp27 phosphorylation were investigated as mechanisms to inhibit inflammation in Thp1 cells exposed to LPS (Li et al., 2019). The miR-499b-5p/TLR4 axis deactivated by LncRNA UCA1 was also shown to reduce TLR4 protein levels and alleviate LPS-mediated neonatal pneumonia (Zhao et al., 2021). Previous studies have explored the role of autophagy-mediated degradation process on TLR4 function during autophagy induction (Puneet et al., 2011; Wang et al., 2022; Zhang et al., 2019b). Interestingly, both pathway enrichment analysis (Toll-like receptor signaling pathway) and PPI network analysis mentioned the involvement of TLR4 in this study. Furthermore, some recent literature suggests that TLR4 acts as a link between nutrient metabolism and inflammation in obesity (Velloso et al., 2015). Therefore, we hypothesized that understanding the involvement of LA-induced autophagy in TLR4 modulation could be significant for studying inflammation. In our cellular experiments, we observed a reduction in TLR4 levels concurrent with autophagy induction following LA treatment. However, this effect was reversed upon administration of 3-MA, along with changes observed in pro-inflammatory cytokines production. Consequently, the interplay between LA and TLR4 may facilitate the reduction of TLR4 by promoting autophagy while exposing LPS.

AMPK plays a pivotal role as an upstream regulator of autophagy, and our previous study has demonstrated that LPS-induced dephosphorylation of AMPK leads to suppressed autophagy in pulmonary inflammation. In this study, the LPS-induced dephosphorylation of AMPK in RAW264.7 cells *in vitro* was also observed. Conversely, treatment with LA stimulates the phosphorylation of AMPK and enhances autophagy induction. These findings are consistent with previous studies indicating that LA modulates the function of AMPK (Taha et al., 2020; Yang et al., 2020). These results align with prior research suggesting that the function of AMPK is influenced by LA. It is intriguing that the expression of TLR4 in BV-2 cells exposed to morphine is downregulated when AMPK is activated (Pan et al., 2016). In addition, the activation of TAK1 by AMPK has been found to play a crucial role in regulating TLR4-mediated inflammatory signaling. Interestingly, when AMPK-α is knocked down in THP-1 cells exposed to LPS, there is an increased production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Kim et al., 2012). Hence, the activation of AMPK may also be responsible for the anti-inflammatory effects of LA, in addition to its promotion of autophagy. Consequently, further investigation into the interplay between TLR4 and AMPK modulated by LA in LPS-induced inflammation holds significant potential for future exploration.

Conclusion

This research has provided novel insights into the mechanism through which LA demonstrates its anti-inflammatory properties. We have elucidated that LA exerts its inhibitory effects on inflammation through downregulation of TLR4 by promoting autophagy, thereby enhancing our understanding of the beneficial impacts of LA supplementation in disorders associated with inflammation.

Data availability statement

Supporting data for the conclusions drawn in this research can be obtained from the corresponding author upon reasonable request.

Author contributions

GL and *YJ* formulated and devised the experimental procedures. YQ and *KL* conducted the experiments. *YQ, KL*, and *QZ* analyzed the data. *JL, YX, XW, TZ*, and *LZ* contributed to the materials, data analysis, and provided constructive discussion. *YQ* and *KL* wrote and revised the manuscript. All authors have read this manuscript and agreed to publish it.

Ethics statement

The animal testing procedure carried out in this study adhered to the protocols specified in the Guide for the Ethical Treatment and Utilization of Laboratory Animals, and received ethical approval from the Ethics Committee at University-Town Hospital, Chongqing Medical University (approval no. LL-202238).

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Conflict of interest

The authors have no manuscript-related conflict of interest to declare.

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