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Original Article

Aspirin-triggered lipoxin A4 attenuates lipopolysaccharide-induced acute lung injury by inhibiting activation of mitogen-activated protein kinases and NF-κB in mice

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Abstract: Despite advances in patient care, effective approaches to acute lung injury (ALI), are currently unceratin. Aspirin-triggered lipoxin A4 (ATL), an endogenous lipid mediator, has been found to have potent anti-inflammatory and pro-resolving effects. In this study, we found ATL exerted protective effects on LPS-induced ALI in mice through inhibiting activations of MAPKs and NF-κB. Our findings revealed that pretreatment by ATL alleviated lung histopathologic changes and injury scores, and reduced leukocytes and protein concentration in the bronchoalveolar lavage fluid (BALF). We further found down-regulation of pulmonary myeloperoxidase (MPO) activity, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein (MCP)-1, and up-regulation of interleukin-10 levels after treatment with ATL in a dose-dependent manner. Moreover, ATL blocked phosphorylations of p38 MAPK, ERK1/2 and JNK, activation of NF-κB, and DNA-binding activity of activator protein-1 (AP-1) and NF-κB in the lung tissues of LPS-challenged mice. Our study suggested that the anti-inflammatory effects of ATL on LPS-induced ALI in mice were at least partly correlated with inhibited activations of mitogen-activated protein kinases and NF-κB.

Keywords: Aspirin-triggered lipoxin A4, lipopolysaccharide, acute lung injury, mitogen-activated protein kinases, NF-κB

Introduction

Acute lung injury (ALI) and its more severe form acute respiratory distress syndrome (ARDS) are life-threatening syndromes that are characterized by acute onset, diffuse pulmonary infiltrates, lung inflammation, protein-rich edema and severe arterial hypoxemia [1]. Despite advances in patient care, ALI/ARDS has a high mortality, of about 43% [2]. As of now, there is no precise treatment to cure ALI/ARDS. Therefore, it is necessary to exploreeffective treatments and further investigate the mechanisms involved.

Lipoxins (LXs), endogenous lipid mediators derived from arachidonic acid, are known to have anti-inflammatory and pro-resolution effects [3]. It was found that aspirin acetylates cyclooxygenase-2 to form 15-epimers of LXs, named aspirin-triggered LXs (ATLs), which have more potent ability and longer biologic half-life than native LXs [4]. This may also partly explain the beneficial impacts of aspirin in many inflammatory diseases. Aspirin-triggered LXA_4 (ATL) is one of the ATLs and its administration *in vivo* or *in vitro* also has potent anti-inflammatory and pro-resolving effects [3]. It is found that ATL facilitates the resolution of ALI induced by carrageenan plus MPO, whereas the explicit molecular mechanism remains elusive [5].

In the present study, we investigated the effects of ATL on LPS-induced ALI and the inflammatory response in mice, as well as the signaling transduction pathways involved. Our data demonstrated that ATL improved lung histopathologic changes, reduced leukocytic infiltration, and pro-inflammatory cytokines in LPS-induced ALI at least partly by inhibiting MAPK/AP-1 and NF-κB signaling pathways.

Materials and methods

Animals and model of ALI

The animal experiments were approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Male BALB/C mice (25-28 g) from Hubei Provincial Laboratory Animal Research Center were used in the experiments and maintained under specific pathogen-free conditions. Mice were anesthetized by intraperitoneal injection of 4 ml/kg body weight of ketamine (20 mg/ml) and xylazine (2 mg/ml) mixture. ALI was induced by instilling *Escherichia coli* LPS (055:B5, Sigma-Aldrich L2880, USA) at 3 mg/kg body weight into the lung via a 22-gauge catheter according to the methods described previously [6, 7], immediately followed by 3 insufflations of 0.8 mL of air and by rotating for 1 minute in order to equally distribute LPS in the lungs. Mice instilled with endotoxin-free saline (1.5 ml/kg body weight) were set as the control group. When the mice recovered from anesthesia, they were returned to their cages with free access to food and water.

Experimental design

Forty mice were randomly divided into 4 groups (10 per group), including a control group, LPS group, 1 μg ATL plus LPS group (ATL 1 μg-LPS) and 5 μg ATL plus LPS group (ATL 5 μg-LPS). Mice were injected intravenously with ATL (1 μg or 5 μg) in 100 μL 0.9% endotoxin-free saline or vehicle (appropriately diluted ethanol with saline) 30 min prior to LPS instillation. ATL (5 (S), 6 (R), 15 (R)-Lipoxin A4) was purchased from Cayman Chemical. At 24 h after LPS instillation, mice were sacrificed by anesthetic and exsanguinated through the inferior vena cava.

Histological analysis

The upper lobe of right lung was fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Slices at 5 μm were stained with hematoxylin and eosin. Then a histologist blinded to the study analyzed histological changes of all the slices and scored as previously description [8]. Histologic changes in ALI included neutrophilic infiltration, hemorrhage, alveolar congestion, and thickness of alveolar wall/hyaline membrane formation. Each histological change was scored on a scale as follow: 0 = minimal, 1 = mild, 2 = moderate, 3 = severe, 4 = maximal. A lung injury score was calculated as the sum of each score. The lower a lung injury score is, the less damage the lung has.

Oxygenation index analysis and wet/dry weight ratio

Mice were endotracheally incubated with a 20-gauge catheter after anesthesia and ventilated with air (7 ml/kg tidal volume, 120 breaks/min) for 15 min, with the carotid artery cannulated for collecting blood to monitor arterial blood gases (ABL80, Radiometer). After anesthetic overdose and exsanguination (by severing of the inferior vena cava and the abdominal aorta), all the excised lung lobes per mouse were weighed (wet weight), placed in an oven and weighed daily until its weight unchanged (dry weight). The wet-to-dry ratio was then calculated.

BALF cell counts, total proteins and pulmonary myeloperoxidase (MPO) activity

After ligating the hilum of right lung, left lung was lavaged according to the methods described previously [9]. Briefly, the left lung was gently lavaged via a 20-gauge cannula with 0.5 ml PBS (kept at 4°C) and withdrawn. This procedure was repeated three times and the total volume of fluid recovered averaged 1.2-1.35 ml/mouse. The bronchoalveolar lavage fluid (BALF) was centrifuged at 1000 r/min for 10 min at 4°C (3K 15 refrigerated centrifuge, Sigma). The cell-free supernatants were stored in aliquot at -80°C until assayed. Total cells in the BALF were resuspended in PBS and counted. Differential cell counts were done on cytocentrifuge preparations (Cytospin 4 Cytocentrifuge; Thermo Shandon) and stained with Wright-Giemsa stain. After BAL, the right lungs were collected and stored correctly for further analysis. Total protein concentration in BALF was measured by using a Pierce BCA Protein Assay Kit (Thermo scientific). Pulmonary MPO activities of the left lung were assayed by using a MPO kit (Nanjing Jiancheng Bioengineering Institute).

Enzyme-linked immunosorbent assay for cytokines in the BALF

The levels of BALF TNF-α, IL-6 and MCP-1 were quantified by commercial enzyme-linked immunosorbent assay (ELISA) kits (NeoBioscience

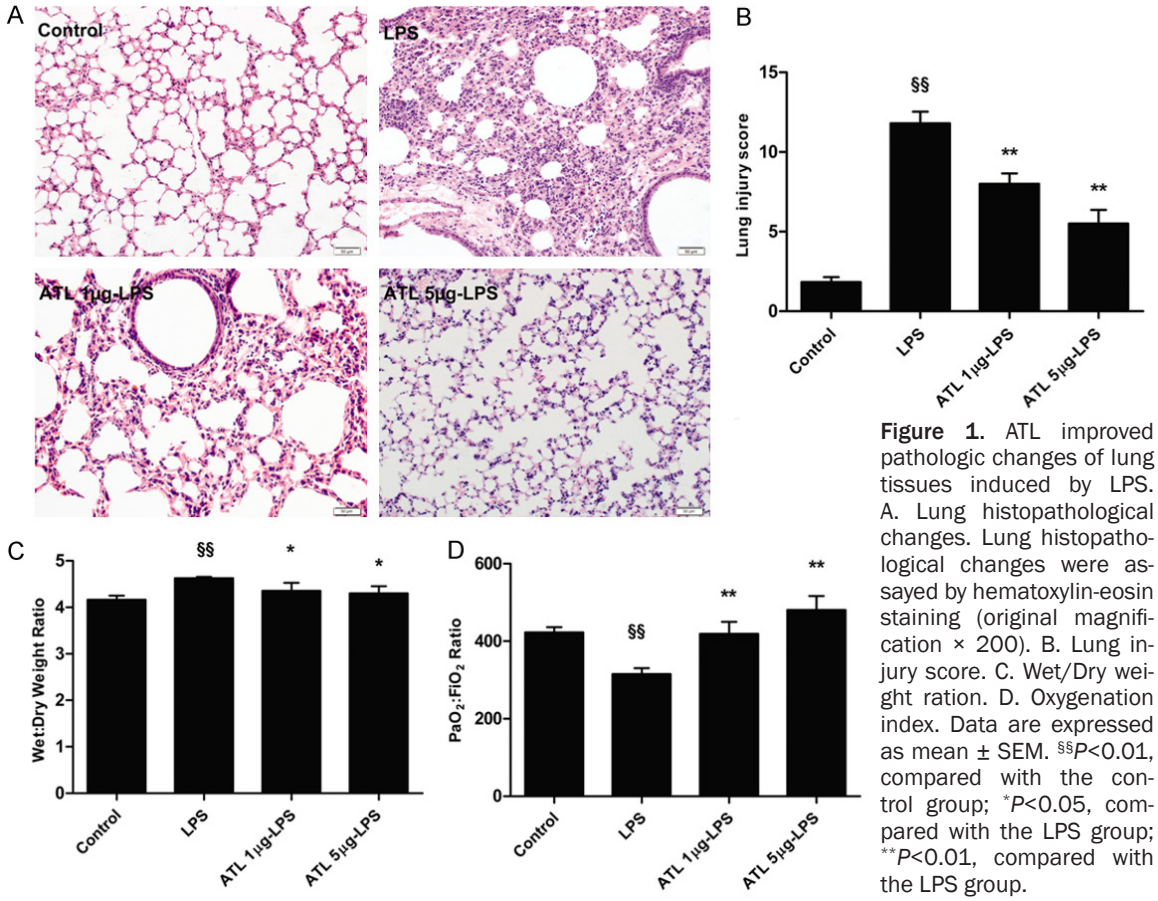

Figure 1. ATL improved pathologic changes of lung tissues induced by LPS. A. Lung histopathological changes. Lung histopathological changes were assayed by hematoxylin-eosin staining (original magnification × 200). B. Lung injury score. C. Wet/Dry weight ration. D. Oxygenation index. Data are expressed as mean ± SEM. §§P<0.01, compared with the control group; *P<0.05, compared with the LPS group; **P<0.01, compared with the LPS group.

Technology). IL-10 expression in BALF was also measured by an IL-10 ELISA kit (eBioscience).

Western blotting analysis

The total protein extraction from lung tissue samples were obtained using T-PER® Tissue Protein Extraction Reagent kit (Thermo scientific). Nuclear and cytoplasmic protein fraction was isolated from lung tissues according to the protocols of NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific). The procedure of western blot was used previously [10]. An aliquot containing 40 μg total protein, as determined by Pierce BCA Protein Assay Kit (Thermo Scientific), was loaded on a 10% sodium dodecyl sulfate polyacrylamide gel to electrophorese, and then transferred onto a polyvinylidene difluoride membrane (Millipore). The transformed membrane was blocked for 1 h and incubated with primary antibodies at 4°C overnight. Primary antibodies used were as followed: rabbit anti-p38 (1:500, Cell Signaling), ERK1/2 (1:1000, Cell signaling), JNK (1:500, Cell Signaling), NF-κB p65 (1:500, Santa Cruz), IκB-α (1:500, Santa Cruz,), β-actin (1:3000, Bioworld Technology) and anti-phosphorylated p38 (1:500, Santa Cruz), ERK1/2 (1:1000, Cell Signaling), JNK (1:500, Cell signaling). The membrane was washed three times with Tris-buffered saline containing 0.05-0.1% Tween 20 (TBST) for 10 min and incubated with goat anti-rabbit IgG-horseradish peroxidase (1:4000, Bioworld Technology) at room temperature for 1 h. Immunoreactive bands were detected by enhanced chemilumine-scent substrate system (Beyotime). Images of blot were captured by using a gel documentation system (ChemiDoc XRS+, Bio-Rad). The intensity of bands was quantitated with Image J software and normalized against proper controls.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were collected as described above. EMSA was performed using the LightShift chemiluminescent EMSA kit (Pierce). Oligonucleotides probe sequences of AP-1

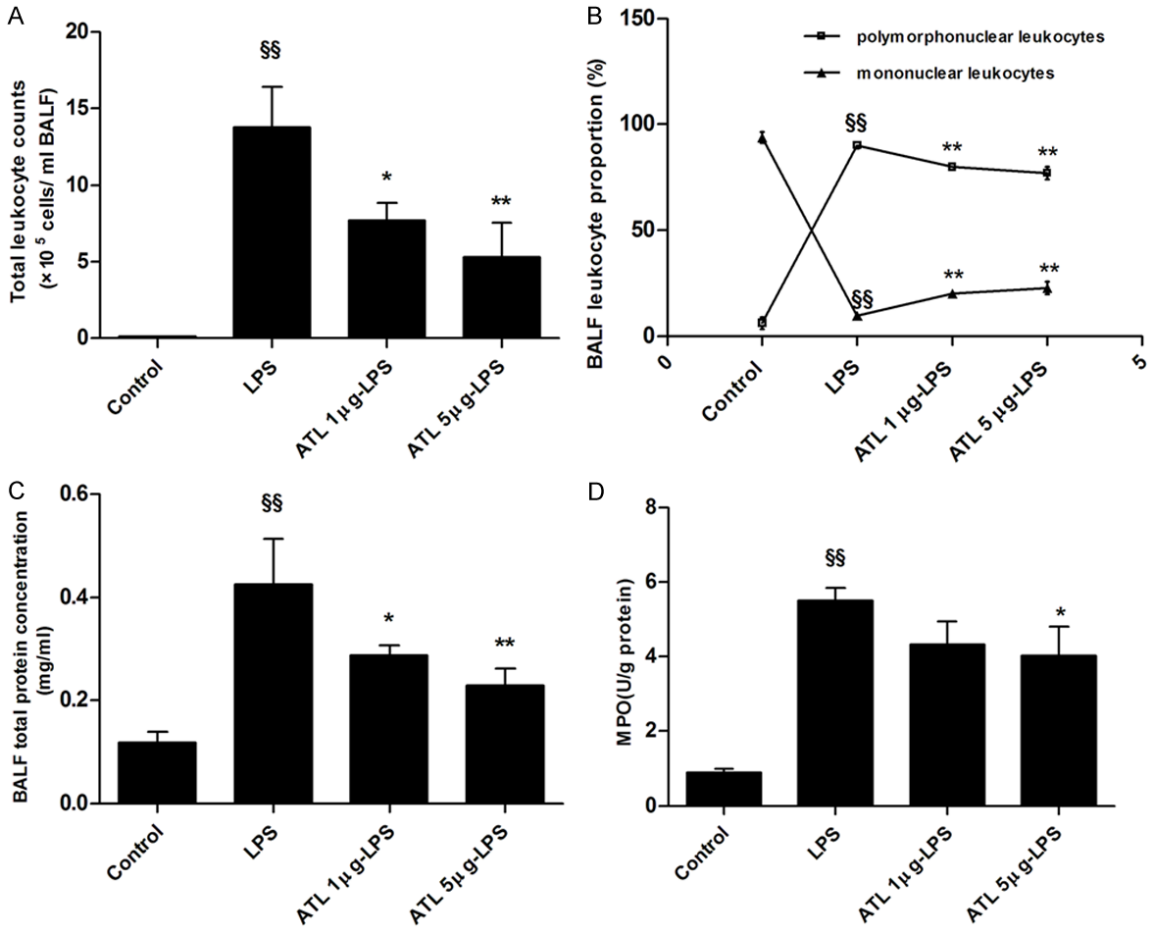

Figure 2. ATL attenuated LPS-regulated changes of leukocytes and MPO activity in BALF. A. Total cell counts in BALF. B. Polymorphonulear and mononuclear leukocytes in BALF. C. Total protein concentration in BALF. D. Pulmonary MPO activity. §§*P*<0.01, compared with the control group; *P<0.05, compared with the LPS group; **P<0.01, compared with the LPS group.

(5'-CGCTTGATGAGTCAGCCG GAA-3') and NF-κB (5'-AGTTGAGGGGACTTTCCCA-GGC-3') binding site were synthesized and end-labeled with biotin. The DNA-nuclear protein complexes were electrophoresed in 6% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA (TBE) buffer at 100 V and then electroblotted onto Hybond nylon membranes (Roche) at 380 mA on ice for 50 min. The membranes were cross-linked on a transilluminator at 312 nm for 15 min, and the biotinylated protein-DNA bands were detected with HRP-conjugated streptavidin using the chemiluminescent nucleic acid detection system (Pierce).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistics were conducted using the SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variances (ANOVA) followed by least significant difference (LSD) post hoc test for multiple comparisons. P<0.05 was considered statistically significant.

Results

ATL improved pathological changes of lung tissues induced by LPS

As shown in **Figure 1A**, no pathologic changes were observed in the lung tissue of the control group. However, significant leukocytic infiltration, severe pulmonary interstitial edema, alveolar atelectasis, and hemorrhage appeared in the lung tissues of LPS group mice. ATL markedly improved these pathologic changes in lung tissues induced by LPS. Lung injury scores

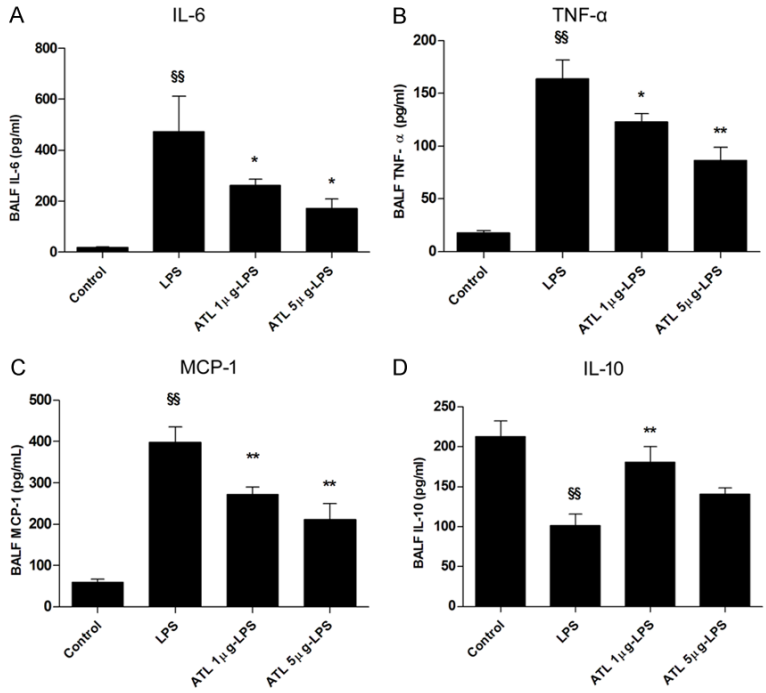

Figure 3. ATL influenced the expression of inflammatory factors. A. The IL-6 levels in BALF. B. The TNF-α levels in BALF. C. The MCP-1 levels in BALF. D. The IL-10 levels in BALF. Results are reported as mean ± SEM. §§*P*<0.01, compared with the control group; *P<0.05, compared with the LPS group; **P<0.01, compared with the LPS group.

were in accordance with lung histology, and were significantly lower in control and ATL groups than that in the LPS group (P<0.01) (**Figure 1B**). LPS induced a lower PaO_2/FiO_2 ratio than that in the control group (P<0.01), whereas ATL significantly elevated the PaO_2/FiO_2 ratio (all P<0.01) (**Figure 1C**). ATL also decreased LPS-induced lung W/D ratio (**Figure 1D**).

ATL attenuated LPS-regulated changes of leukocytes and MPO activity in BALF

As shown in **Figure 2A**, while total leukocytes in BALF in the LPS group were significantly increased compared with the control group (P<0.01). Addition of ATL led to significant and dose-dependent decrements in total cell numbers in contrast to the LPS group (P<0.05). The polymorphonuclear and mononuclear leukocytes were further determined. The results demonstrated that ATL significantly reduced the proportion of polymorphonuclear leukocytes in BALF, whereas markedly elevated the proportion of mononuclear leukocytes (**Figure 2B**). Moreover, total protein concentration in BALF of the LPS group was significantly elevated compared with the control group (P<0.01; **Figure 2C**); pretreatment with ATL caused a dose-dependent reduction in total proteins in BALF (P<0.05). Additionally, ATL suppressed LPS-induced pulmonary myeloperoxidase (MPO) activity (P<0.05; **Figure 2D**).

ATL influenced the expression of inflammatory factors

Levels of IL-6, TNF-α, MCP-1 and IL-10 in BALF from mice were further explored after ATL treatment. As shown in **Figure 3A**, the levels of IL-6 in BALF of the LPS-treated mice were dramatically increased compared with the control ones (P<0.01). Pretreatment with ATL significantly suppressed LPS-induced up-regulation of IL-6 (P< 0.05). Similar results were obtained in regulation of TNF-α and MCP-1 by ATL (**Figure 3B** and **3C**). However, the IL-10 level in LPS-treated mice was significantly lower than that in control group BALF (P<0.01), which was highly elevated by low-dose ATL administration (P<0.01; **Figure 3D**).

ATL inhibited LPS-activated MAPK and NF-κB pathway

As shown in **Figure 4A**, the phosphorylations of p38, ERK1/2 and JNK were increased in the lung tissues of LPS-treated mice, and were further inhibited by pretreatment with high-dose ATL. These results were further confirmed by calculating ratios of phosphorylated- to total-protein abundance (**Figure 4B**). While the cytoplasmic expression of IκB-α and NF-κB p65 in the lung tissues was suppressed by LPS, pretreatment with 5 μg ATL rescued these events (**Figure 4C**). Moreover, the EMSA results showed that DNA-binding activities of AP-1 and NF-κB were increased after treatment with LPS, whereas 5 μg ATL administration dramatically abolished the activation of AP-1 and NF-κB (**Figure 4D**).

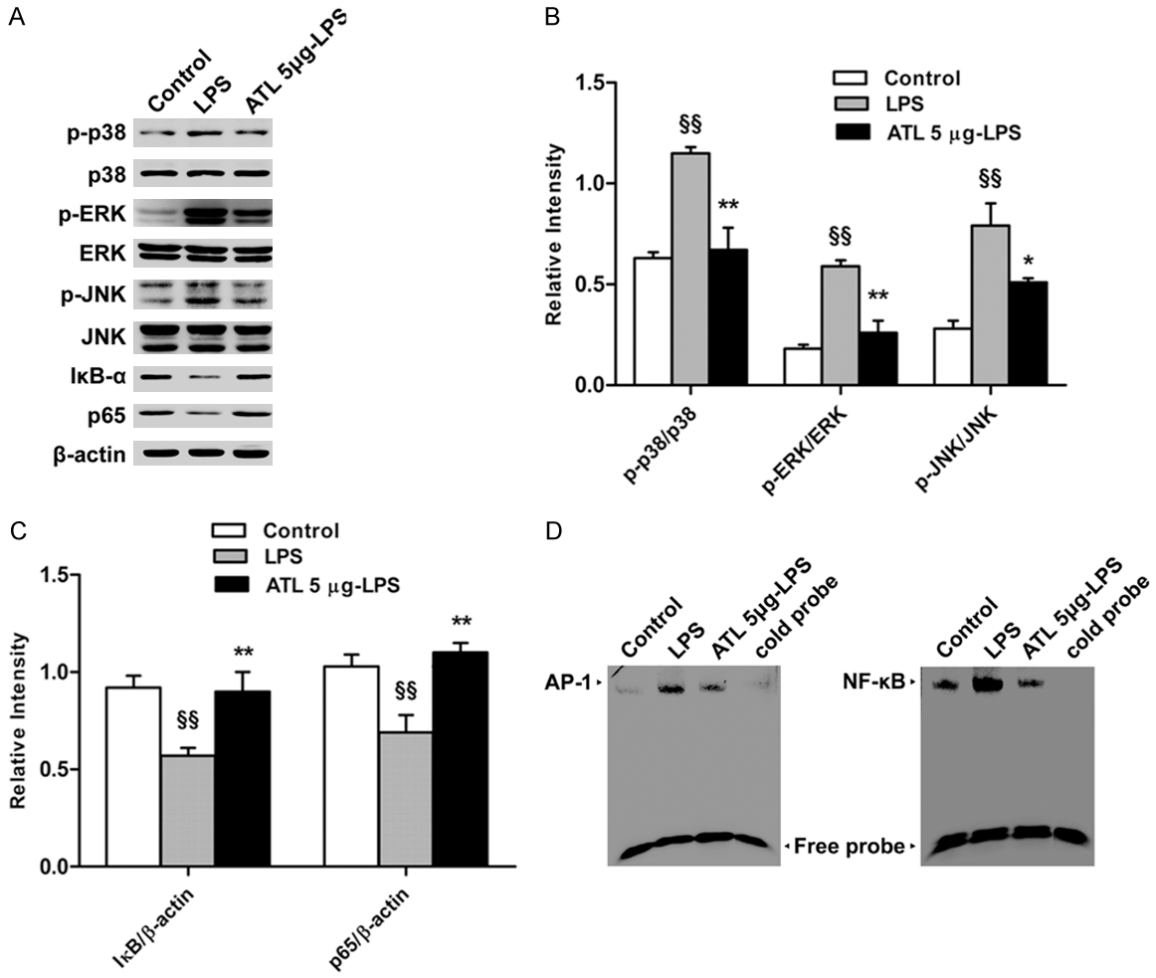

Figure 4. ATL inhibited LPS-activated MAPK and NF-κB pathway. A. The total protein extraction from the lung tissues was prepared, and the activation of MAPK and NF-κB pathway were detected by Western blotting. B. 3 independent experiments were performed, and the ratios of p-p38/p38, p-ERK/ERK and p-JNK/JNK were calculated. C. The expression of IκB and p65 in cytoplasm protein extraction from the lung tissues were valued. D. The DNA-binding activities of NF-κB and AP-1 were examined by EMSA. Data are shown as mean ± SEM. §§P<0.01, compared with the control group; *P<0.05, compared with the LPS group; **P<0.01, compared with the LPS group.

Discussion

In this study, we found that ATL attenuated LPS-induced ALI, and this may be mediated by MAPK/AP-1 and NF-κB signaling pathways. These results were demonstrated by the improvement in the lung histopathology and oxygenation, reduction of Wet/Dry ratio, leukocytic infiltration and pro-inflammatory factors, elevation of anti-inflammatory cytokine IL-10, inhibition of MAPK and NF-κB activations, and blockage of DNA binding activations of AP-1 and NF-κB.

As we know by now, no single animal ALI model can totally reproduce all characteristics of ALI in human. There are some reasons to choose LPS as a stimulant to induce ALI in this study. Firstly, LPS, a key component of the outer membrane of Gram-negative bacteria, can activate innate immune defenses and play important roles in the pathogenesis of inflammatory activation [11]. Thus, this model can be used to investigate the molecular mechanism of acute inflammation. Secondly, this model is characterized by lung epithelial and endothelial injury, leukocytic infiltration in the interstitium and alveolus, cytokine production, edema, intravascular coagulation, and congestion. [12]. The model reproduces the characteristics of ALI in humans. Thirdly, this model is well-reproduced, easy to make and not lethal. Therefore,

we used LPS to induce ALI in mice and mimic the clinical ALI induced by Gram-negative bacteria.

Acute inflammation plays key roles in the host defense of pathogens, virus, and other noxious stimuli. Uncontrolled inflammation may either cause chronic inflammation or cause global disruption of the host response to lead to more severe injury, even death [13, 14]. However, acute inflammation can resolve promptly sometimes through the actions of endogenous anti-inflammatory and pro-resolving mediators [14]. Lipoxins (LXs) and ATLs are considered to act as "braking signals" in the inflammation [3]. They have dual anti-inflammatory and pro-resolving effects in many experimental models of disorders, such as models of glomerulonephritis, ischemic renal injury, and airway inflammation [3]. Lack of lipoxins causes deterioration or impaired reparation after organ injury. Previous studies have shown that the significant lower levels of lipoxin A4 associated with exacerbated chronic obstructive pulmonary disease (COPD), severe asthma and exercise-induced bronchoconstriction in children asthma [15, 16]. Therefore, lipoxins are importantly endogenous mediator in keeping homeostasis. In the current study, we investigate whether ATL can turn off LPS-induced severe inflammation in ALI in mice and its mechanism involved.

Intratracheal LPS induces severe leukocyte infiltration in the interstitial and air spaces in the process of ALI in mice [7]. ATL pretreatment reduced the total cell numbers and polymorphonuclear leukocytes in dose-dependent manners in this study. Mononuclear leukocytes in ATL groups were slightly less than that in LPS group, whereas the proportion of mononuclear leukocytes in BALF in ATL groups were significantly higher than that in LPS group. We found that ATL accelerates neutrophil apoptosis and/or facilitate phagocytosis of the apoptotic ones by macrophages to promote the resolution of ALI induced by intratracheal carrageenan plus MPO as well as E. coli-induced pulmonary inflammation [5]. Therefore, in the current study, relatively more mononuclear leukocytes in ATL groups may survive to engulf the apoptosis neutrophils to promote the inflammation resolution. BALF total protein concentration is a very relevant measurement of alteration of the alveolar capillary barrier [17]. In this study, ATL dose-dependently decreased LPS-induced protein concentrations in BALF, which means ATL improves the alveolar capillary barrier, consist with a previous study [5]. Wet/Dry weight ratio is an estimate of the total water content of the lung. ATL significantly reduced the Wet/Dry weight ratio induced by LPS, which indicated that ATL attenuated lung edema.

MPO is a resident enzyme released from neutrophilic polymorphonuclear leukocytes [18], which can be considered as a symbol of neutrophil recruitment. Our findings showed that ATL inhibited LPS-induced MPO activity in a dose-dependent manner. This means that ATL inhibited pulmonary neutrophils recruitment, which was also in line with lung histopathology showing less neutrophil infiltration in the interstitium of lung tissue in ATL groups.

Pro-inflammatory cytokines (TNF-α, IL-6 and MCP-1) play vital roles in the development of ALI, and by recruiting leukocytes into the lung, propagate and amplify the inflammatory response initiated by LPS [19]. MCP-1, as a chemokine, plays critical roles in the recruitment and adherence of neutrophils and monocytes to infection and inflammation [20]. IL-10 is considered as "anti-inflammation mediator" in the inflammation, which downregulates some pro-inflammatory cytokine production, such as TNF-α, and has potential in promoting resolution of inflammation [21]. In this study, our findings showed that the pro-inflammatory factors (TNF-α, IL-6 and MCP-1) in BALF were significantly increased but anti-inflammatory cytokine IL-10 was reduced in mice exposure to LPS, which were all reversed by ATL pretreatment. The results further suggest that ATL attenuates LPS-induced lung inflammation through upregulation of anti-inflammatory cytokine IL-10, downregulation of leukocyte recruitment as well as pro-inflammatory cytokines. Consistent with our previous studies, LXs and its analogy reduced the production of pro-inflammatory cytokine TNF-α in cerebral ischemia reperfusion rat and LPS-induced BV-2 microglial cells [22], but increased anti-inflammatory cytokine IL-10.

MAPKs (JNK, ERK1/2 and p38 MAPK) are a group of conserved kinases that play important roles in many inflammatory diseases, such as cancer [23]. MAPKs are also known to be critical in regulating production of many pro-inflammatory mediators (IL-6 and TNF-α) and

enzymes (COX-2 and iNOS), and gene expression of many pro-inflammatory mediators (TNF-α, IL-6, MCP-1 and IFN-β) in LPS-induce ALI or sepsis [24]. Our data showed that pretreatment with 5 μg ATL inhibited phosphorylation of p38 MAPK, ERK1/2 and JNK after LPS exposure. Meanwhile, inflammation response and inflammatory cytokines all sharply decreased. It has been reported that LXA_4 inhibited activation of ERK1/2 and JNK (but no p38 MAPK) to attenuate brain damage in traumatic brain injury in mice [25]. Our previous study showed that ERK and p38 MAPK (but not JNK) activation were decreased in the presence of ATL in LPS-stimulated microglia [10]. The present results were not in total line with previous study. The discrepancies are mainly due to differences in experimental models. Our findings suggest that anti-inflammatory effects of ATL on decreasing the pro-inflammatory cytokines and inflammation response may be mediated by JNK and p38 MAPK.

In conclusion, the current study showed that pretreatment with 5 μg ATL significantly inhibited LPS-activated MAPK/AP-1 and NF-κB signaling pathways, the expression of downstream pro-inflammatory cytokines (TNF-α, IL-6 and MCP-1), pulmonary MPO activity and leukocytic infiltration, and alleviated the lung histology and function and enhanced the production of anti-inflammatory mediator IL-10. These findings suggest that ATL attenuates the LPS-induced ALI in mice at least partly by inhibiting MAPK/AP-1 and NF-κB signaling pathways. Therefore, ATL could be a useful therapeutic candidate for acute lung injury.

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Disclosure of conflict of interest

None.

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