ImmunoTargets and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Recombinant Antithrombin Alleviated Pulmonary Injury and Inflammation in LPS-Induced ARDS by Inhibiting IL17a/NF-κB Signaling

Chen Yang[1,*], Cong Fu[2,*], Mengxue Wang[2], Junbo Zheng[3], Yang Gao[4], Huiting Zhu[2], Haoxuan Li[2], Dongxu Li[2], Lichen Guo[2], Bing Yu[5], Qingqing Dai[2]

[1]Department of Anesthesia, the Obstetrics & Gynecology Hospital of Fudan University, Shanghai, People's Republic of China; [2]Department of Critical Care Medicine, The Obstetrics & Gynecology Hospital of Fudan University, Shanghai, People's Republic of China; [3]Department of Critical Care Medicine, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, People's Republic of China; [4]Department of Critical Care Medicine, The Sixth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, People's Republic of China; [5]Department of Cell Biology, Navy Medical University, Shanghai, People's Republic of China

*These authors contributed equally to this work

Correspondence: Qingqing Dai, Department of Critical Care Medicine, The Obstetrics & Gynecology Hospital of Fudan University, No. 128, Shenyang Road, Shanghai, People's Republic of China, Tel +086-15124523220, Email daiqingqing888@126.com

Background: Recombinant antithrombin (rAT) has been shown to protect lungs from ARDS and modulate immune responses, but its anti-inflammatory mechanisms remain unclear. This study aimed to explore the immunomodulatory effects and mechanisms of rAT in LPS-induced ARDS mice.

Methods: ARDS mouse model was established by intraperitoneally administration of 20 mg/kg LPS. After 3 hours of LPS administration, rAT or PBS was injected intravenously. Lung injury, alveolar permeability, serum inflammatory cytokines, immune cell infiltration in lung tissue, and the proportion of Th17 were assessed 36 hours after rAT administration. The functional roles of the differential expressed genes (DEGs), obtained from LPS-induced ARDS mice treated with or without rAT, were analyzed by GO, KEGG and GSEA enrichment analysis. The activation of NF-κB and NLRP3 inflammasome was evaluated by Western blot and immunofluorescence staining.

Results: We found that rAT alleviated lung injury, reduced pulmonary permeability, decreased serum inflammatory cytokines, and suppressed immune cell infiltration and NLRP3 inflammasome activation. Moreover, rAT decreased the proportion of Th17 cells in lung tissues and peripheral blood, downregulated IL17a expression, and inhibited NF-κB signaling pathway in lung tissues. Additionally, the administration of IL-17A diminished the efficacy of rAT in mitigating lung injury, suppressing the immune response, and inhibiting the activation of the NF-κB signaling pathway in LPS-induced ARDS mice.

Conclusion: The findings of this study suggest that rAT alleviates lung injury and suppresses inflammatory responses by inhibiting the IL17a/NF-κB signaling axis, suggesting that rAT may serve as a potential therapeutic agent for mitigating pulmonary inflammation and improving the prognosis of ARDS induced by sepsis. Furthermore, this study provides important research data and theoretical basis for the clinical translation and application of rAT.

Keywords: ARDS, antithrombin, lipopolysaccharide, Th17 cells, IL17a, NF-κB

Introduction

Sepsis is typically a systemic inflammatory response syndrome mainly caused by infections.[1] The results of global statistics indicate that sepsis is one of the main reasons for increased admission rates and mortality in intensive care units (ICU), with an incidence of sepsis among hospitalized patients as high as 9%, and an even higher incidence among ICU patients. The mortality rate for sepsis patients ranges from 30% to 60%.[2] Lung injury is one of the most common complications in sepsis patients, often manifested as ARDS, which characterized by increased permeability of the alveolar-capillary membrane, infiltration of inflammatory cells, release of cytokines, and direct

Received: 4 November 2024
Accepted: 6 March 2025
Published: 7 April 2025

damage to lung tissues, ultimately leading to pulmonary edema, impaired gas exchange, and respiratory failure.[3,4] Currently, despite significant advancements in critical care medicine for the treatment of ARDS, various therapeutic approaches such as infection control, ventilatory management, and extracorporeal membrane oxygenation (ECMO) are employed to treat ARDS, the therapeutic outcomes remain suboptimal. The lack of effective pharmacological interventions for ARDS may be one of the major challenges faced in the treatment process.

Recombinant antithrombin (rAT), a vitamin K-independent glycoprotein that inactivates several enzymes in the coagulation system and plays a crucial role as a physiological serine protease inhibitor during coagulation,[5,6] has both anti-inflammatory and anticoagulant activities and has been suggested as an adjunctive therapy for patients with sepsis, particularly those with sepsis-induced coagulopathy.[7] However, a Phase III randomized controlled trial showed no beneficial effect of rAT in patients with sepsis-induced coagulopathy.[8] Subsequent analysis studies found that rAT significantly reduced 28-day and in-hospital mortality in sepsis patients with high fibrin degradation products (FDPs) and D-dimer levels.[9] Another randomized controlled trial also showed that rAT had therapeutic benefits in the subgroup of patients who were given high-dose rAT without heparin.[10] Although rAT has demonstrated some therapeutic effects in the treatment of sepsis, there are no widely reported large-scale clinical trials specifically focusing on the use of rAT for the treatment of ARDS. However, in recent years, several experimental studies have shown that rAT can prevent organ failure and improve survival in sepsis-induced ARDS models by reducing cell damage and inflammatory responses.[11–14] Iba T et al reported that rAT effectively attenuated lung endothelial damage and maintained lung vascular integrity in a rat sepsis model by protecting the glycocalyx, which is essential for vascular homeostasis.[12] Okamoto H et al reported that rAT attenuates ARDS by promoting DNA repair and ciliogenesis in endothelial cells and reducing inflammation in a mouse sepsis model induced by LPS.[13] In addition, rAT also possesses strong anti-inflammatory properties that are independent of its anticoagulation characteristics.[5] Komura et al reported that rAT reduced the production of TNF-α, an essential proinflammatory cytokine, in LPS-induced human peripheral monocytes.[15] These reported studies demonstrated that rAT can effectively reduce lung damage, regulate immune responses, and lower mortality rates caused by sepsis. These findings also suggest that rAT holds significant potential for translational therapy in the treatment of ARDS patients. However, the mechanisms underlying the anti-inflammatory effects and alleviation of lung injury by rAT in LPS-induced ARDS are still unclear.

IL-17A is a pro-inflammatory cytokine produced by Th17 cells, playing a dual role in regulating the immune response by promoting T cell survival and proliferation, which is crucial for pathogen clearance.[16] However, excessive T cell activation may lead to exacerbated pulmonary inflammation in ARDS.[17] The levels of IL-17A are significantly elevated in ARDS patients and are closely associated with alveolar inflammation and poor prognosis.[18] This suggests that IL-17A could be a potential target for ARDS treatment. NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a key transcription factor involved in regulating numerous inflammatory responses. NF-κB plays a central role in lung injury and inflammation in ARDS by promoting the expression of pro-inflammatory factors such as tumor necrosis factor (TNF-α) and interleukins (IL-1, IL-6, etc.), thereby advancing the inflammatory process.[19] Conversely, the activation of NF-κB can lead to dysfunction in alveolar epithelial cells and endothelial cells, worsening ARDS symptoms.[19] Studies have indicated that IL-17A promotes the production and secretion of inflammatory factors by activating the NF-κB signaling pathway.[20] IL-17A can bind to the IL-17RA/IL-17RC heterodimer, which subsequently activates downstream signaling pathways, including the NF-κB pathway, triggering an immune response.[21] Furthermore, research has shown that IL-17A can activate the NF-κB signaling pathway through Act1-mediated TRAF6 ubiquitination.[22] Therefore, IL-17A plays a significant role in the inflammatory response by activating the NF-κB signaling pathway. Interventions targeting the IL-17a/NF-κB pathway may become a new strategy for treating ARDS. Although rAT has shown anti-inflammatory effects in ARDS, it is still uncertain whether it exerts its anti-inflammatory action by inhibiting the IL-17a/NF-κB axis.

In this study, we explored the protective effects of rAT on lung tissue injury and the anti-inflammatory effects in the LPS-induced ARDS mouse model, as well as its potential mechanisms. This study provides an experimental basis for the application of rAT in the treatment of sepsis-induced lung injury.

Materials and Methods

Mouse Models

Eight-week-old male C57BL/6 mice were obtained from Lingchang Biotechnology Co., Ltd. (Shanghai, China) and were maintained under standard pathogen-free conditions with ad libitum access to water and food. The PS: Power and Sample Size Calculations (Version 3.0) was used to estimate the sample size for each group,[23] and the parameters were as follows: $\alpha = 0.05$, $\delta = 0.3$, $\sigma = 0.15$, power = 0.9, and m = 1. Consequently, the minimum number of mice per group was determined to be 6. In brief, 30 mice were randomly divided into three groups after a 16-hour starvation period: (1) LPS group: 20 mg/kg LPS (Millipore Sigma, Burlington, MA) was administered intraperitoneally. Then, 400 ul of PBS was given intravenously 3 hours after LPS administration. (2) LPS + rAT group: 400 ul of rAT (750 IU/kg) (Kyowa Kirin Co., Ltd., Tokyo, Japan) was administered intravenously 3 hours after LPS administration. (3) Control group: An equal volume of PBS was given at the corresponding time points when LPS and rAT were injected. All the mice were euthanized after being anesthetized with tribromoethanol 36 hours after rAT administration.

Wet/Dry (W/D) Lung Weight Ratio

The lungs were excised, blotted dry, weighed to obtain the wet weight, and then placed in an oven at 60°C for 48 h to obtain the dry weight. The W/D ratio was used to evaluate the degree of pulmonary edema.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

After the mice were anesthetized, the trachea was exposed, and the lungs were flushed three times with sterile saline, with 1 mL used each time. BALF aliquots were collected for the determination of cell counts, protein concentrations, and immunoglobulin M (IgM) concentrations. Centrifugation was performed immediately after BALF collection, and the protein concentration in the supernatant was determined via the bicinchoninic acid assay. The cell pellet was resuspended, and the total cell count was determined by an automated cell counter (Countstar, Shanghai, China). The IgM (900 kDa) level in the BALF was assessed via enzyme-linked immunosorbent assay (ELISA) (E-EL-M3036; Elabscience Biotechnology Co., Ltd., Wuhan, China) to determine the degree of transalveolar–capillary membrane protein transit.

Permeability Assay

The mice were injected with FITC–dextran (25 mg/kg) via the tail vein 34 h after LPS administration. After 2 h, the BALF and serum were collected after the mice were anesthetized with avertin. After the collection of BALF and serum, the whole-lung tissues were harvested and immersed in 4% paraformaldehyde for 12 h. After the serum was diluted 10-fold, 100 μL of diluted serum and BALF were added to a 96-well plate. The intensity of the fluorescence in the BALF and serum was measured with Tanon ABL X6 in the FITC channel. The ratio of BALF fluorescence to serum fluorescence was subsequently used to indicate the permeability of endothelial and epithelial cells according to previously reported methods.[24] The paraformaldehyde-fixed lung tissues were embedded in optimal cutting temperature (OCT) compound for frozen sectioning. Then, 10-μm-thick sections were cut and stained with Hoechst 33542. The fluorescence of the sections was measured with a microscope (Nikon).

Western Blot

Western blotting was performed as described previously.[25] In brief, total proteins were extracted from lung tissues with RIPA buffer (89,901; Thermo Fisher) and quantified with a BCA kit (23,225; Thermo Fisher). Then, 40 μg of total protein was loaded per lane and separated via SDS–PAGE. After the proteins were transferred to PVDF membranes, the membranes were blocked with 5% skim milk for 30 min and incubated with a primary antibody at 4°C overnight. The membranes were washed three times with TBST and subsequently incubated with an HRP-conjugated secondary antibody at 37°C for 1 h. The immune complexes were visualized by using Pierce™ ECL Western Blotting Substrate (32,132, Engreen). The primary antibodies used were as follows: IL17a (66148-1-Ig), RORγt (29910-1-AP), P65 (66,535-1-Ig), NLRP3 (68,102-1-Ig), Caspase 1 (22,915-1-AP), GAPDH (60004-1-Ig) and α-Tubulin (66,031-1-lg) from Proteintech. Cleaved Caspase 1 (CASP1) (AF4022), p-P65 (AF2006), IκBα (AF5002), IKK (AF6014), IL-1β

(AF5103) and Cleaved IL-1β (AF4006) from Affinity. STAT3 (#9139), p-STAT3 (#9145), p-IκBα (Ser32) (#2859) and p-IKKα/β (Ser176/180) (#2697) were obtained from Cell Signaling Technology.

Real-Time PCR

Total RNA was purified with RNAiso Plus reagent (9108, Takara) and reverse transcribed into cDNA with MMLV Reverse Transcriptase (M530A, Promega) according to the manufacturer's protocols. Real-time PCR analysis was performed in a 20 μL mixture containing 10 μL of 2 × ChamQ SYBR qPCR Master Mix (Q321–02; Vazyme), 0.4 μL of each forward and reverse primer (10 mM), 0.5 μL of cDNA and 8.7 μL of ddH2O. The target genes were amplified via a two-step PCR procedure: annealing at 95°C and amplification at 60°C. All the samples were examined in triplicate. The fold changes in the expression of each target gene were calculated via the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry (IHC) and Immunofluorescence (IF)

Immunohistochemistry and immunofluorescence staining were performed as previously described.[26] Briefly, fixed lung tissues were embedded in paraffin and cut into 3 μm sections. The rehydrated sections were immersed in Tris-EDTA (pH 9.0) antigen retrieval buffer and maintained at 121°C for 2 minutes. Then, the antigen-retrieving sections were blocked with 3% H_2O_2 for 10 minutes at room temperature. After being washed with PBS-T 3 times, the sections were blocked with 1% BSA for 30 min at room temperature. The sections were incubated with primary antibodies at 4°C overnight. For IHC staining, the sections were incubated with HRP-conjugated secondary antibodies at 37°C for 30 min and visualized with DAB substrate (34,002; Thermo Fisher). The nuclei were counterstained with hematoxylin. For IF staining, the sections were incubated with fluorescein-conjugated secondary antibodies at 37°C for 30 min, after which the nuclei were counterstained with Hoechst 33542.

ELISA

The levels of inflammatory factors, including IL-6, TNF-α, Cxcl15 (known as IL-8) and hypersensitive C reactive protein (hs-CRP), in the serum were detected via ELISA according to the manufacturer's instructions. A mouse IL-6 ELISA kit (KE10007) and a mouse TNF-alpha ELISA kit (KE10002) were purchased from Proteintech. A mouse CXCL15 ELISA Kit (E-EL-M0269) and a mouse hs-CRP ELISA Kit (E-EL-M0677) were purchased from Elabscience (Wuhan, China).

Flow Cytometry Assay

The proportions of Th17 in PBMCs were detected via flow cytometry according a reported protocol.[27] In briefly, lymphocytes, isolated from periphery blood with ACK lysis buffer, were first stimulated with phorbol ester, phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin at 37°C for 6 hours in cell culture incubator. The lymphocytes were then stained with PE FITC anti-mouse CD4 Antibody (100405, BioLegend) and PE anti-mouse IL-17A Antibody (506903, BioLegend). The the stained cells were resuspended in Cell Staining Buffer and analyzed with a Beckman Coulter CytoFLEX flow cytometer. To detect the proportions of Th17 in the lung tissues, lung tissues were dissociated into single cells with a MACS Lung Dissociation Kit (Miltenyi Biotec, United States), and then the $CD4^+$ cells were enriched by magnetic sorting with a MojoSort™ Mouse CD4 Nanobeads (480069, BioLegend). The sorted cells were detected using the same method as lymphocytes isolated from periphery blood.

Statistical Analysis

The data are presented as the mean ± standard deviation (SD). The statistical methods are indicated in the figure legends, and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

rAT Attenuated Pulmonary Injury and Exudation in LPS-Induced ARDS Model Mice

In a mouse sepsis model established by intraperitoneal injection of 20 mg/kg LPS, the survival rate of the LPS +rAT group (100%; 10 of 10) was greater than that of the LPS group (50%; 5 of 10) at 36 hours after LPS

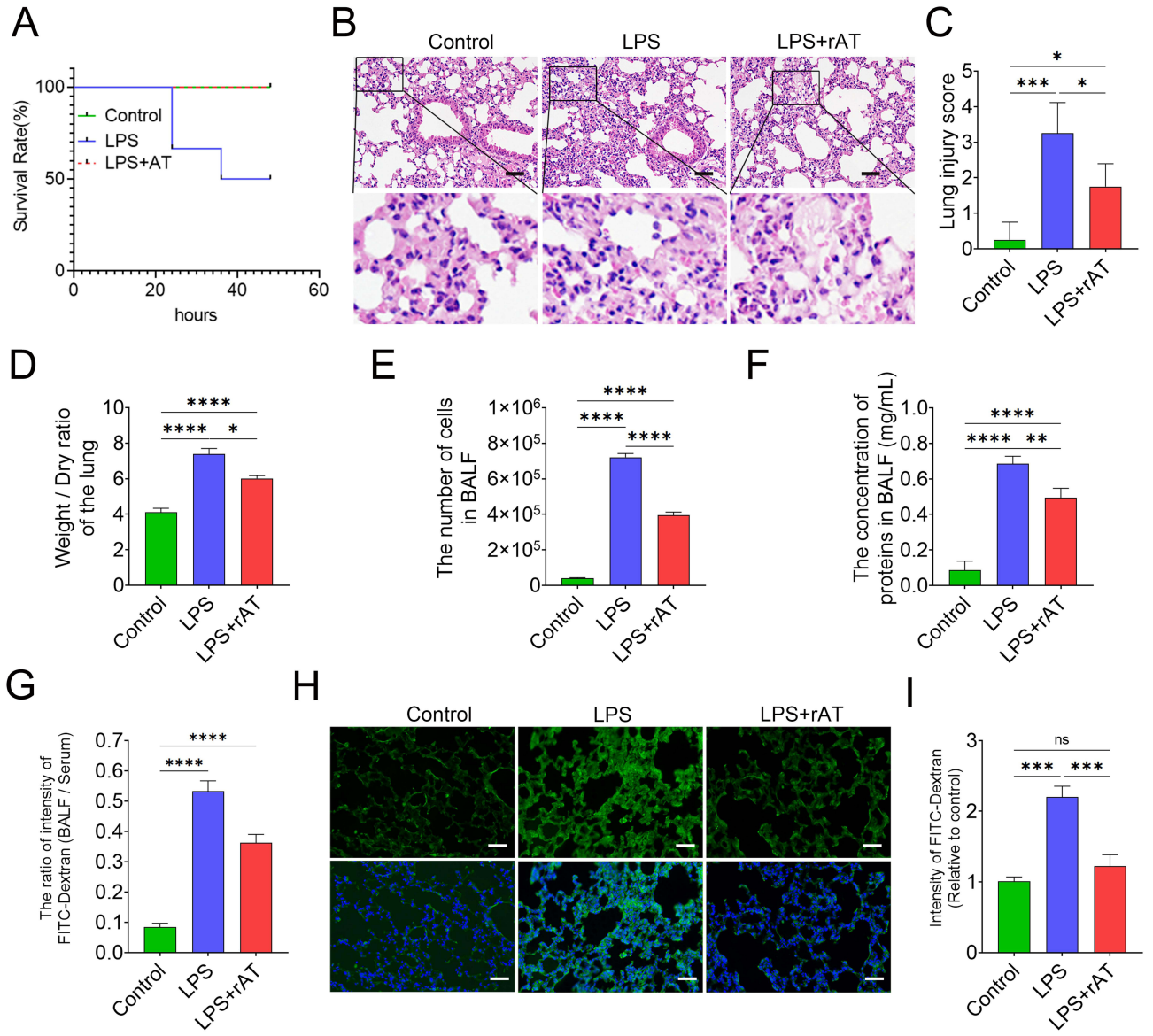

Figure 1 rAT attenuated pulmonary injury and exudation in LPS-induced ARDS model mice. (**A**) Kaplan–Meier survival curves of control group treated with PBS (n=10), LPS group treated with LPS without rAT (n=10), and LPS + rAT group treated with LPS and rAT (n=10). Log rank test revealed differences between the groups of LPS and LPS + rAT ($p < 0.05$). (**B**) Representative results of H&E staining of lung sections from the indicated groups. (**C**) Lung injury index scores of the indicated groups indicated that the treatment of rAT alleviated the lung injury in LPS-induced ARDS model mice. (**D**) The wet/dry weight ratio of the lung tissue showed that the treatment with rAT alleviated pulmonary exudation in LPS-induced ARDS model mice. (**E**) The treatment with rAT decreased the number of cells in the BALF of LPS-induced ARDS model mice. (**F**) The treatment with rAT decreased the concentrations of proteins in the BALF of LPS-induced ARDS model mice. (**G**) The ratio of the fluorescence intensity of FITC-dextran in the BALF and serum showed that the treatment with rAT reduced the exudation of FITC-dextran into the alveoli. (**H**) The fluorescence intensity of FITC-dextran in lung tissue was analyzed in frozen sections. (**I**) Histogram of the statistical analysis of the FITC-dextran fluorescence intensity in frozen sections. All the data are presented as the means ± SDs. One-way ANOVA, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, ns, not significant; scale bar, 50 μm.

administration (Figure 1A). Compared with the LPS group, the lung tissue structure in the LPS+rAT group was significantly improved (Figure 1B and C). Moreover, the wet/dry weight ratio of the lung (Figure 1D), the number of cells (Figure 1E) and the concentration of proteins (Figure 1F) in the BALF were significantly lower in the LPS +rAT group than those in the LPS group, indicating that rAT reduced exudation in LPS-induced injury lung tissues. To further evaluate the effect of rAT on exudation, a permeability assay with FITC-dextran, a polymer of glucose that cannot freely penetrate the tight junctions that develop between the normal vascular endothelium and alveolar epithelium,[28] was performed. The ratio of the intensity of FITC-dextran in BALF to that in serum (Figure 1G) and the fluorescence intensity of frozen sections of lung tissues (Figure 1H and I) were both

https://doi.org/10.2147/ITT.S502925

obviously decreased in the LPS+rAT group, indicating that the penetration of FITC-dextran from blood into BALF was decreased and suggesting that rAT may alleviate damage to vascular endothelial and alveolar epithelium.

rAT Reduces Pulmonary Inflammation in LPS-Induced ARDS Model Mice

A severe inflammatory response is a factor that can further damage lung tissue. Therefore, changes in the levels of inflammatory factors, such as IL-6, TNF-α, IL-8, and hs-CRP, in the serum obtained from each group were further detected via ELISA, and the results revealed that rAT treatment reduced the levels of IL-6 (Figure 2A), TNF-α (Figure 2B), IL-8 (Figure 2C), and hs-CRP (Figure 2D) in the serum of mice with LPS-induced lung injury. Furthermore, the results of immunohistochemical staining revealed that rAT treatment decreased the number of $F4/80^{+}$ macrophages in the lung tissues of the mice with LPS-induced injury (Figure 2E). However, the number of M2-like macrophages, which express Mrc1 (encoding the protein CD206), was increased[29] (Figure 2F), and the infiltration of neutrophils was reduced (Figure 2G). These results indicate that rAT can effectively reduce the pulmonary inflammatory response in LPS-induced ARDS model mice.

rAT Attenuates NLRP3 Inflammasome Activation in LPS-Induced Lung Injury

Due to the activation of inflammasomes plays a significant role in the pathogenesis of ARDS,[30] we explored the effect of rAT on NLRP3 inflammasome activation in a mouse model of LPS-induced ARDS by detecting the protein expression of NLRP3, caspase-1 (CASP1), cleaved CASP1, IL-1β and cleaved IL-1β in lung tissues. Immunofluorescence staining revealed obvious upregulation of NLRP3 and cleaved CASP1 expression in lung tissues from mice with LPS-induced injury, whereas rAT treatment significantly downregulated the expression of NLRP3 and cleaved CASP1 (Figure 3A). The results of Western blot analysis also revealed that the protein expression levels of NLRP3, CASP1, cleaved CASP1, IL-1β, and cleaved IL-1β were significantly increased in LPS-induced injury lung tissues and that treatment with rAT downregulated the expression of these proteins (Figure 3B–G). Therefore, rAT attenuated NLRP3 inflammasome activation in the lung tissues of LPS-induced ARDS model mice.

rAT Inhibits the Activation of IL17a Signaling Pathway

To investigate the potential mechanism by which rAT alleviates LPS-induced lung injury and reduces inflammation, we analyzed DEGs in LPS-induced lung injury tissues from mice treated with and without rAT from the GSE160929 dataset (Supplementary Table 1). The results of GO enrichment analysis of the 1058 DEGs revealed that the top 20 GO terms were involved mainly in immune regulation, such as macrophage chemotaxis, leukocyte chemotaxis, monocyte chemotaxis, lymphocyte chemotaxis, neutrophil chemotaxis, and the inflammatory response (Figure 4A). The results of KEGG enrichment analysis also showed that the DEGs were involved in cytokine–cytokine receptor interactions, chemokine signaling pathways, the IL-17 signaling pathway and the TNF signaling pathway (Figure 4B), which are also the main regulators of immune regulation. Additionally, the results of GSEA revealed that the IL-17A signaling pathway was downregulated in rAT treated LPS-induced ARDS mice, when compared with LPS-induced ARDS mice (Figure 4C). Many studies have shown that the IL-17 signaling pathway plays an important role in the immune regulation of LPS-induced lung injury, and IL-17a is the most important activator of IL-17 signaling pathway activation. Therefore, we analyzed the expression levels of IL17a in the GSE160929 dataset. The results showed that the expression of IL17a in rAT treated LPS-induced ARDS mice was significantly lower than that in LPS-induced ARDS mice (Figure 4D). Furthermore, real-time PCR (Figure 4E), Western blotting (Figure 4F) confirmed that rAT treatment significantly decreased IL17a expression in lung tissues from mice with LPS-induced injury.

rAT Down-Regulates the Proportion of Th17 Cells in LPS-Induced ARDS Mice

IL17a, an important proinflammatory factor, is mainly secreted by Th17 cells and plays an important role in the process of pulmonary infection.[16] Therefore, we analyzed the proportion Th17 cell in the mice with LPS-induced ARDS treated with or without rAT via flow cytometry. The results revealed that the proportion of $CD4^{+}$ $IL17A^{+}$ Th17 cells in both periphery blood and in lung tissue were significantly greater in the LPS-induced ARDS mice than in the control mice, whereas rAT treatment obviously reduced the percentage of Th17 cells in the LPS-induced ARDS model mice

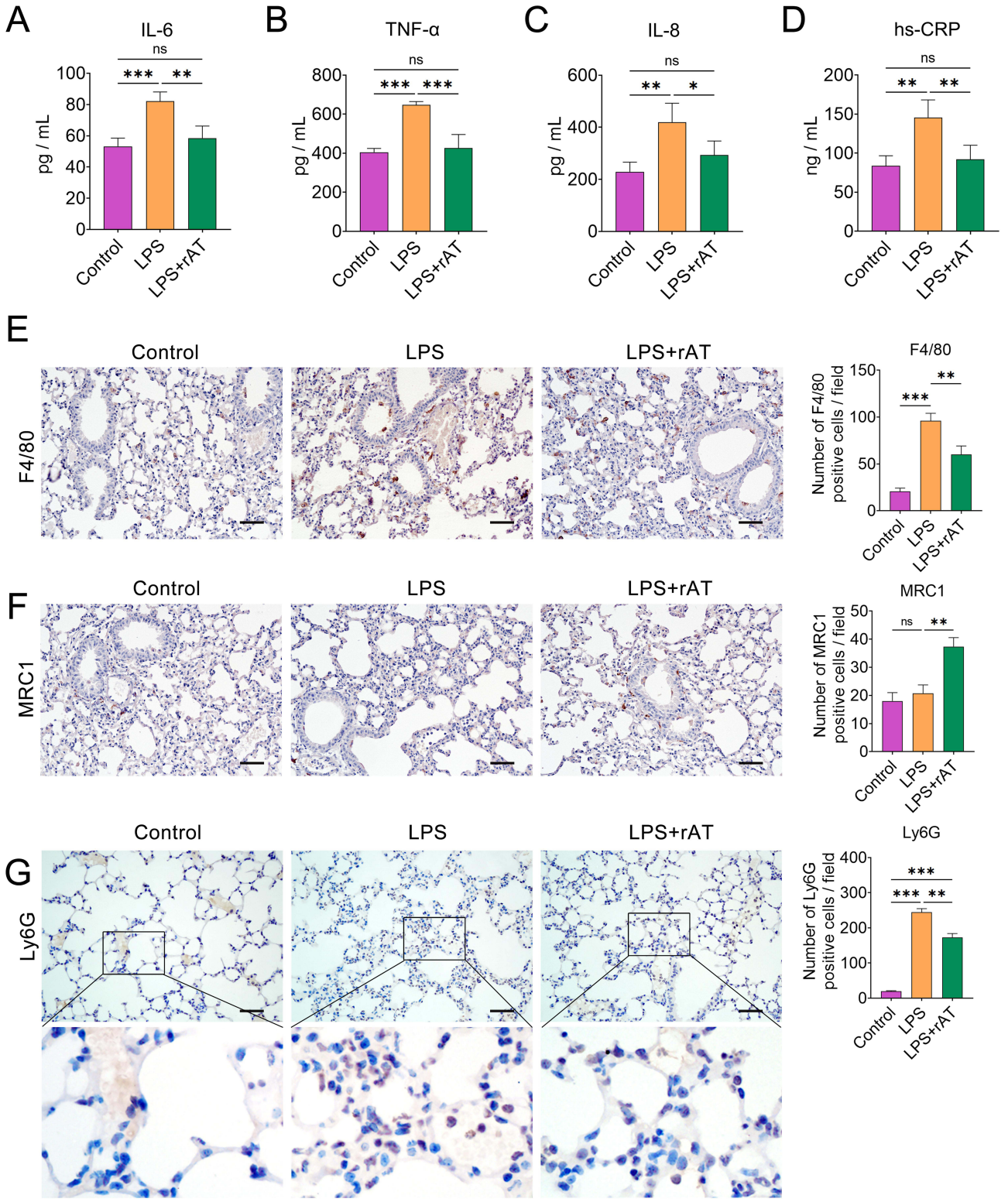

Figure 2 rAT reduced pulmonary inflammation in LPS-induced ARDS model mice. (**A–D**) The levels of inflammatory factors, including IL-6 (**A**), TNF-α (**B**), IL-8 (**C**), and hs-CRP (**D**), were decreased in the serum of the rAT-treated group, as detected by ELISA. (**E**) Immunohistochemical staining for F4/80 revealed that rAT treatment reduced the proportion of macrophages in the lung tissue of LPS-induced ARDS model mice. (**F**) Immunohistochemical staining for MRCI, a marker of M2 macrophages, showed that rAT treatment increased the proportion of M2 macrophages in the lung tissue of LPS-induced ARDS model mice. (**G**) Immunohistochemical staining for Ly6G, a marker of neutrophils, showed that rAT treatment reduced the proportion of neutrophils in the lung tissue of LPS-induced ARDS model mice. All the data are presented as the means ± SDs of three independent experiments. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant; scale bar, 50 μm.

https://doi.org/10.2147/ITT.S502925

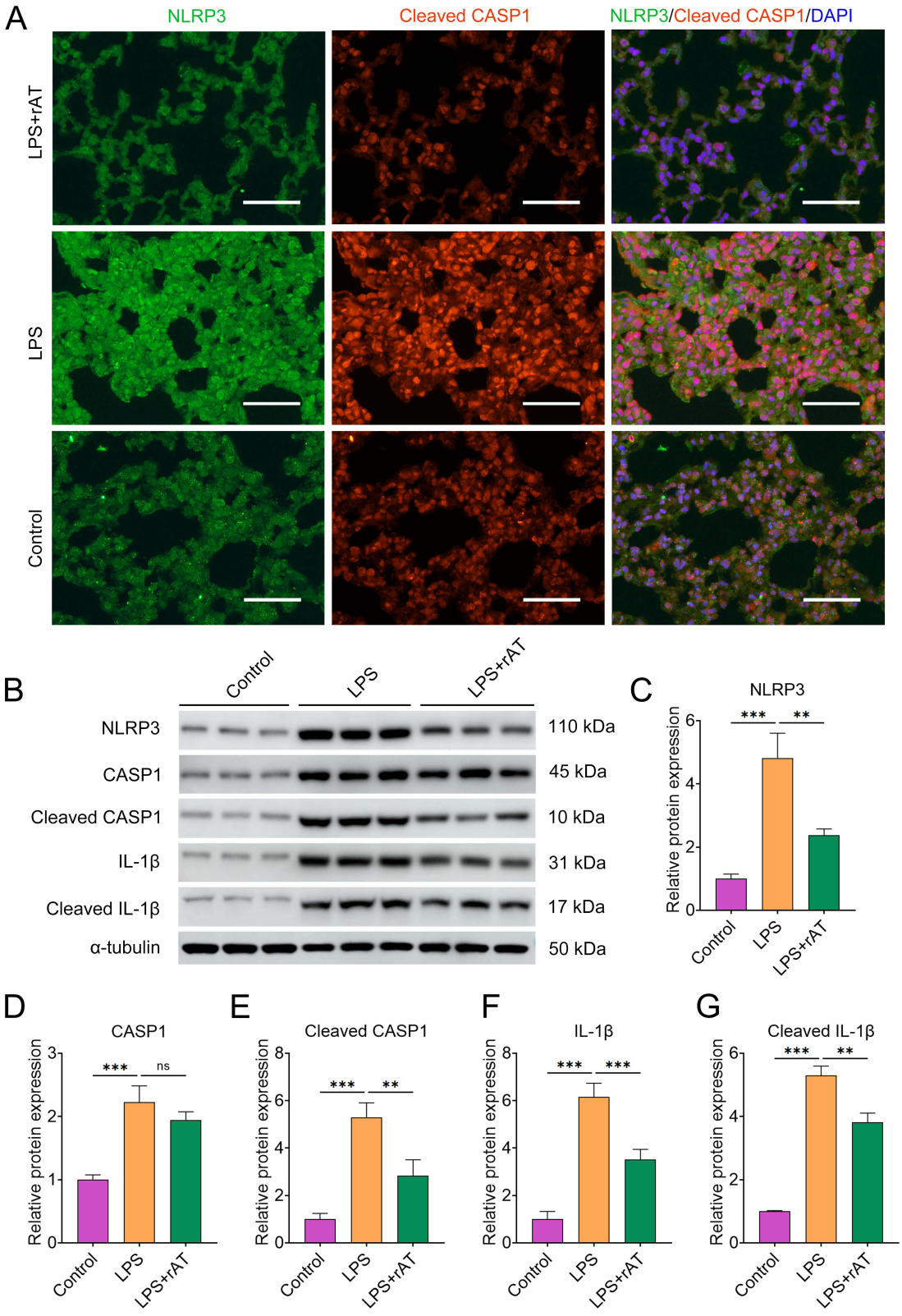

Figure 3 rAT attenuated NLRP3 inflammasome activation in the lung tissues of LPS-induced ARDS model mice. (**A**) Immunofluorescence staining of NLRP3 (green) and cleaved caspase-1 (CASP1) (red) in lung tissues from mice with LPS-induced injury. (**B**) The protein levels of NLRP3, CASP1, cleaved CASP1, IL-1β, and cleaved IL-1β in lung tissues from model mice with LPS-induced injury were detected via Western blotting. (**C–G**) The protein expression levels of NLRP3 (**C**), CASP1 (**D**), cleaved CASP1 (**E**), IL-1β (**F**), and cleaved IL-1β (**G**) were significantly reduced following rAT treatment in LPS-induced ARDS model mice, as assessed by the gray intensity analysis of the blots shown in (**B**). The data are expressed as the means ± SDs (n=3 in each group). One-way ANOVA, **p < 0.01, ***p < 0.001, and ns not significant. Scale bar: 50 μm.

https://doi.org/10.2147/ITT.S502925

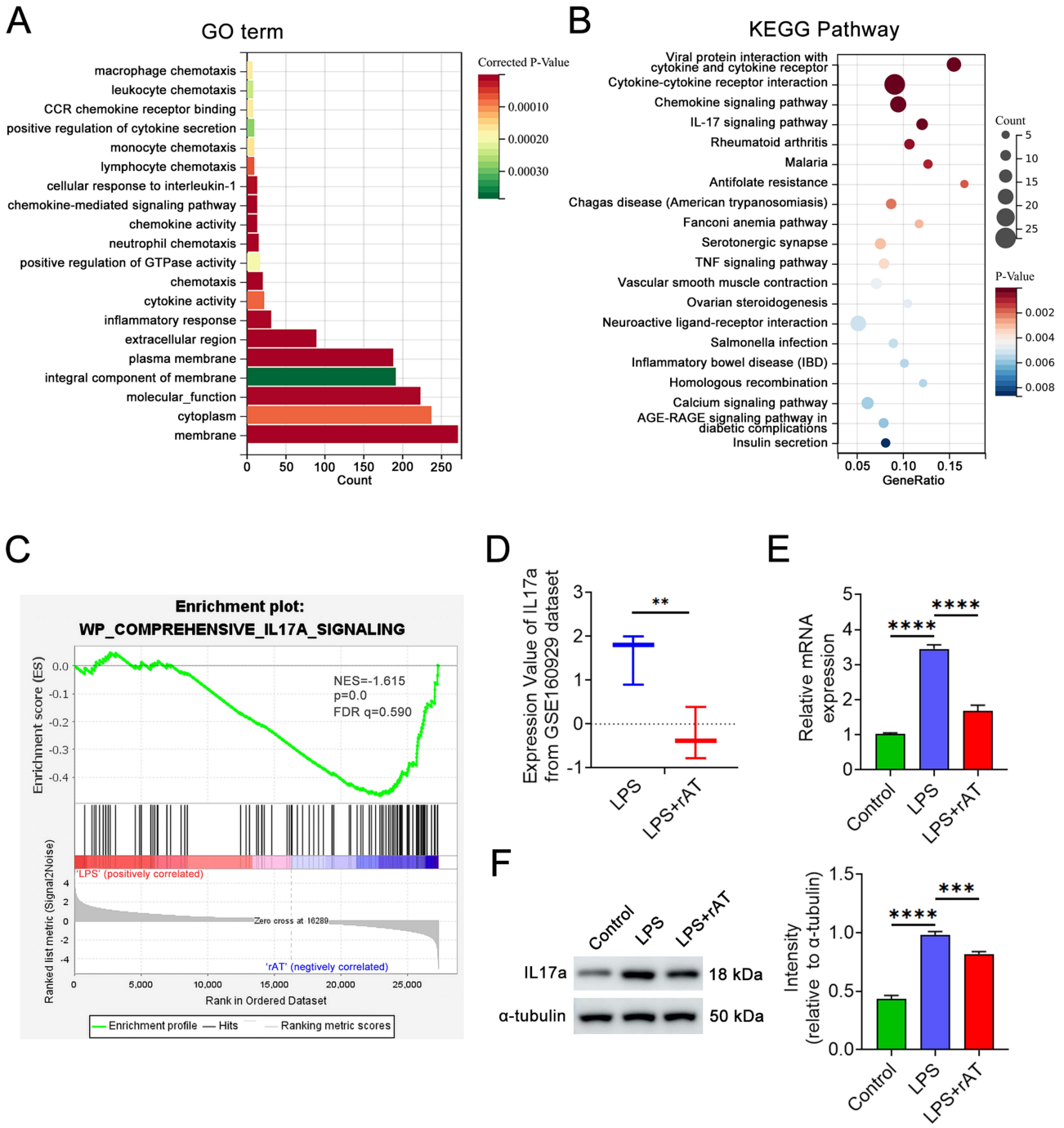

Figure 4 The activity of IL17a signaling pathway were inhibited by rAT. (**A**) The top 20 GO terms from the results of GO enrichment analysis of the DEGs obtained from model mice treated with or without rAT in the GSE33690 dataset. (**B**) The top 20 signaling pathways from the results of KEGG enrichment analysis of the DEGs obtained from model mice treated with or without rAT in the GSE33690 dataset. (**C**) The results of GSEA showed that IL17a signaling pathway were inhibited after the treatment of rAT in LPS-induced ARDS mice. (**D**) The expression of IL17a in LPS-induced lung injury tissues was significantly reduced following treatment with rAT, as evidenced by data from the GSE33690 dataset. The data are expressed as the means ± SDs (n=3 in each group); Student's *t* test, **p < 0.01. (**E**) The mRNA expression of IL17a in LPS-induced lung injury tissues was significantly downregulated after treatment with rAT, as measured by real-time PCR. One-way ANOVA, ****p < 0.0001. (**F**) The protein expression of IL17a in LPS-induced lung injury tissues was significantly reduced following treatment with rAT, as confirmed by Western blot analysis. The data are expressed as the means ± SDs (n=3 in each group). One-way ANOVA, ***p < 0.001 and ****p < 0.0001.

(Figure 5A-D). Furthermore, the results of IHC also revealed that the percentage of $IL17A^+$ cells were significantly increased in LPS-induced ARDS lung tissue, while, the treatment of rAT obviously decreased the percentage of $IL17A^+$ cells in LPS-induced ARDS lung tissue (Figure 5E). These findings suggest that rAT treatment reduced the percentage of Th17 cells in the lung tissue and PBMCs in the LPS-induced ARDS mice, and also indicate that rAT treatment prevents

https://doi.org/10.2147/ITT.S502925

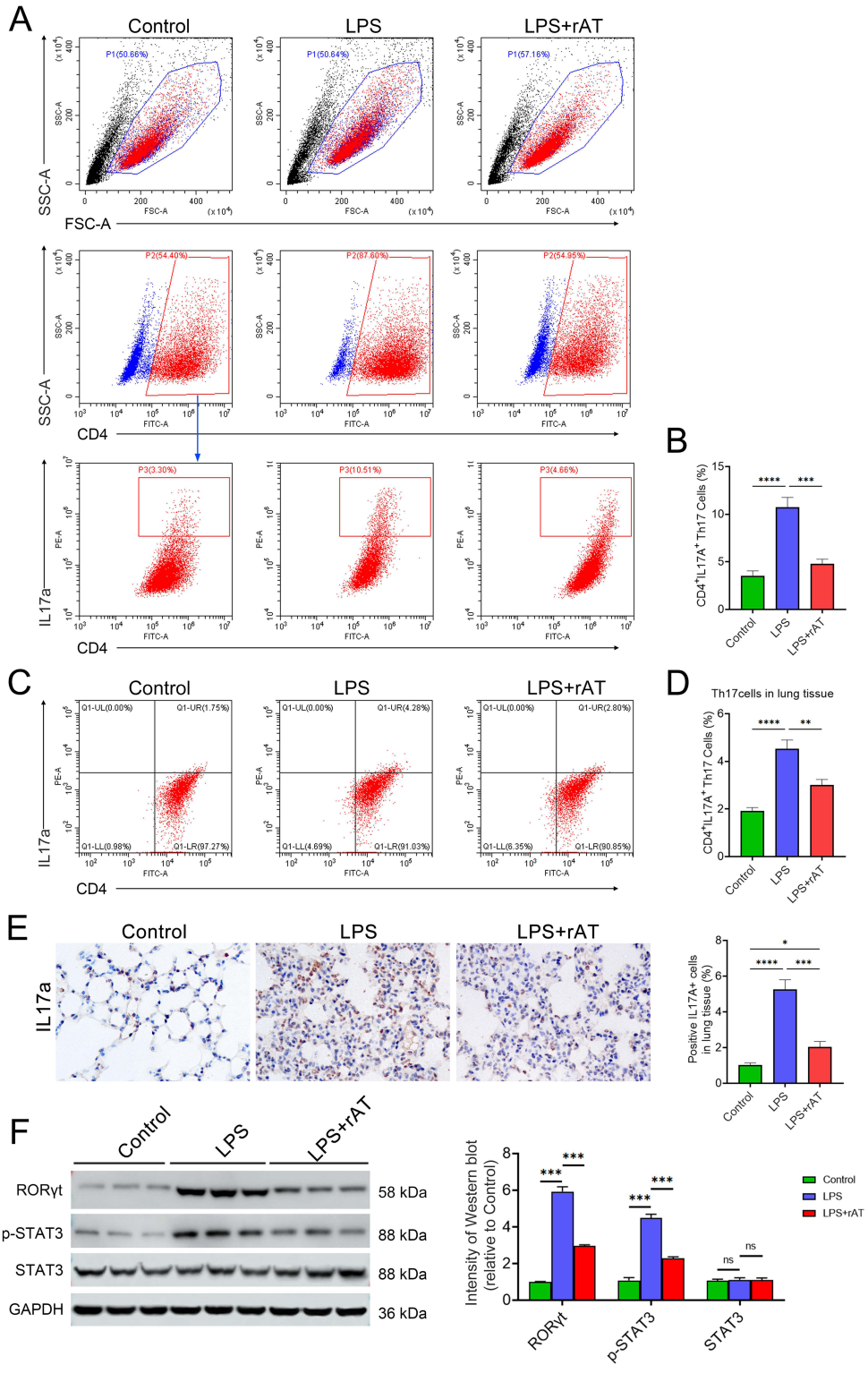

Figure 5 rAT reduces the proportion of Th17 cells in LPS-induced ARDS model mice. (**A**) Scatter plot of the flow cytometry results for the proportion of Th17 cells in periphery blood. (**B**) Statistical histogram of the proportion of Th17 cells in periphery blood (**A**) showed that treatment with rAT significantly decreased the proportion of Th17 cells. (**C**) Scatter plot of flow cytometry results for the proportion of Th17 cells in lung tissue. (**D**) Statistical histogram of the proportion of Th17 cells in lung tissue (**C**) showed that treatment with rAT significantly decreased the proportion of Th17 cells. (**E**) Representative immunohistochemical staining results for IL17a showed that rAT treatment decreased the proportion of IL17a-positive cells in the lung tissue of LPS-induced ARDS model mice. (**F**) The protein levels of RORγt, STAT3, and p-STAT3 in the lung tissues of each group were evaluated by Western blotting. Gray intensity analysis demonstrated that the expression of RORγt and p-STAT3 was significantly downregulated following rAT treatment in LPS-induced ARDS model mice. The data are shown as the means ± SDs (n=3 in each group). One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns not significant.

 https://doi.org/10.2147/ITT.S502925

the excessive activation of Th17 cells, thereby reducing the heightened immune response that further exacerbates lung tissue damage.

The development of Th17 cells is dependent on signal transducer and activator of transcription 3 (STAT3) and the retinoid-related orphan receptor RORγt, which are Th17-specific transcription factors.[31] Thus, the protein expression of RORγt, p-STAT3, and STAT3 in lung tissues was detected. Western blot analysis revealed that the protein levels of RORγt and p-STAT3 were significantly increased in lung tissues from LPS-induced ARDS model mice (Figure 5F). Treatment with rAT downregulated the expression of RORγt and the phosphorylation of STAT3 (Figure 5F). Taken together, these results suggest that rAT down-regulated Th17/IL-17A axis in the mice with LPS-induced injury.

rAT Downregulates NF-κB (P65) Signaling in LPS-Induced Injured Lung Tissue

The NF-κB signaling pathway not only plays a critical role in the pathogenesis of ARDS,[32] but also modulates the development of Th17 cells.[33] Under normal circumstances, the dimer formed by NF-κB p50 and p65 binds to IκB and exists in the cytoplasm as an inactive form.[34] However, activation of NF-κB signaling pathway requires phosphorylation of IκB and subsequent ubiquitination modifications which lead to the release of the bounded NF-κB dimers and translocation to the nucleus. This process depends on the activation of the IKK (IκB kinase) complex through phosphorylation.[34,35] To assess this pathway, we measured the protein levels of p-IκBα, IκBα, p-IKKα/β, IKKα/β, p-P65, and P65 in lung tissues. The results revealed that the levels of p-IκBα (Figure 6A and B), p-IKKα/β (Figure 6C and D), and p-P65 (Figure 6E and F) were significantly elevated in LPS-induced injured lung tissues. Conversely, rAT treatment significantly diminished the phosphorylation of IκBα (Figure 6A and B), IKKα/β (Figure 6C and D), and P65 (Figure 6E and F). These results suggested that rAT could inhibit the NF-κB signaling pathway in the lung tissues of LPS-induced ARDS model mice.

IL17a Blocks the Inhibitory Effect of rAT on LPS Induced Inflammation and Activation of NF-κB Signaling Pathway

To further elucidate rAT inhibits inflammation in LPS-induced mice via the IL17a/NF-κB axis, mice were pretreated with IL17a two hours before rAT injection. After 24 hours of rAT treatment, serum inflammatory factors such as IL6, IL8, and TNFa were significantly higher in the LPS+IL17a+rAT group compared to the LPS+rAT group (Figure 7A), indicating that IL17a effectively blocked the ability of rAT to reduce inflammatory factors in serum of LPS-induced ARDS mice. Simultaneously, the effect of rAT in reducing lung tissue permeability was also diminished by IL17a (Figure 7B-D). Real-time PCR results showed that IL17a blocked the reduction of rAT in chemokines (Cxcl1 and Cxcl5) and inflammatory factors (Ccl2, Ccl3, Muc5ac, Muc5b, S100a7, and S100a8) in LPS-induced ARDS lung tissue (Figure 7E), which are regulated by the IL17a/NF-κB signaling pathway. The results of Western blot showed that IL17a weakened the effect of rAT in diminishing the phosphorylation of IκBα, IKKα/β, and P65 (Figure 7F), suggesting that IL17a blocked the inhibitory effect of rAT on NF-κB signaling pathway activation. These results indicate that rAT inhibits inflammation and reduces lung exudation by downregulating the activity of the IL17a/NF-κB axis.

Discussion

In this study, we showed that rAT not only attenuated pulmonary injury but also inhibited the inflammatory response, such as by reducing the infiltration of inflammatory cells in lung tissues, inhibiting the activation of the NLRP3 inflammasome in lung tissues, and decreasing the secretion of inflammatory cytokines in the blood, in an LPS-induced ARDS mouse model. Although previous studies have reported that rAT has anti-inflammatory effects,[12,13,36] the underlying anti-inflammatory mechanism and pathway have not been fully elucidated. Here, we found that rAT reduced the expression of IL17a and down-regulated the proportion of Th17 cell in periphery blood and injured lung tissues via inhibiting the activity of NF-κB signaling pathway. Furthermore, we also confirmed that the supplementation of IL-17a diminished the effects of rAT in alleviating injury, suppressing immune response, and inhibiting the activation of the NF-κB signaling pathway in LPS-induced ARDS mice. Therefore, our results suggest that rAT alleviated pulmonary injury and suppressed inflammation in LPS-induced ARDS via inhibiting IL17a/NF-κB signaling.

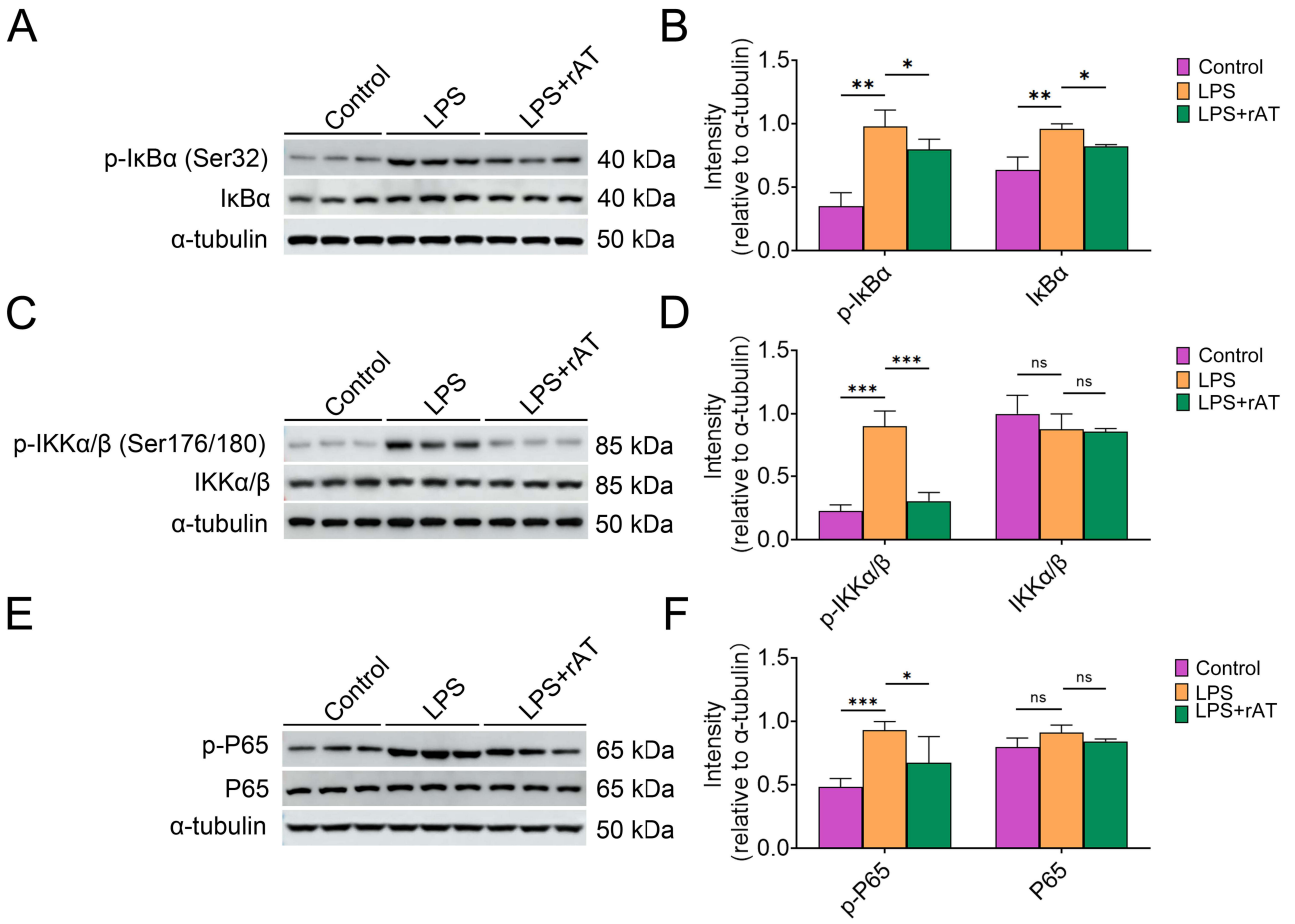

Figure 6 rAT downregulated NF-κB signaling pathway activity in the lung tissues of LPS-induced ARDS model mice. (**A**) The protein expression levels of p-IκBα and IκBα in lung tissues were detected via Western blotting. (**B**) The gray intensity analysis of the blots in (**A**) showed that the elevated p-IκBα in LPS-induced injured lung tissues were downregulated following the treatment with rAT. (**C**) The protein expression levels of p-IKKα/β and IKKα/β in the lung tissues were detected via Western blotting. (**D**) The gray intensity analysis of the blots in (**C**) showed that the elevated p-IKKα/β in LPS-induced injured lung tissues were downregulated following the treatment with rAT. (**E**) The protein levels of P65 and p-P65 in the lung tissues were detected via Western blotting. (**F**) The gray intensity analysis of the blots in (**E**) showed that the elevated p-P65 in LPS-induced injured lung tissues were downregulated following the treatment with rAT. The data are expressed as the means ± SDs (n=3 in each group). One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, and ns not significant.

Many kinds of immune cells, including neutrophils, macrophages and dendritic cells, are involved in the development of ARDS. Recently, $CD4^+$ T cells have been shown to participate in the pathogenesis of ARDS. TH17 cells, which differentiate from naïve $CD4^+$ precursor cells under stimulation with antigen-presenting cells and certain cytokines, such as IL-6, IL-23, and TGFβ, play critical roles in antimicrobial defense and autoimmune diseases through secreting a variety of pro-inflammatory cytokines, including IL-17A, IL-17F, IL-21, and IL-22.[37,38] However, excessive activation of Th17 cells often leads to a "cytokine storm", which exacerbates tissue damage.[39] Inhibiting the over-activation of Th17 cells may be one of the strategies to control the "cytokine storm" during the development of ARDS. In this study, our results showed that rAT significantly reduced the proportion of Th17 cells in the peripheral blood and injured lung tissues of LPS-induced mice. Concurrently, it decreased the levels of inflammatory cytokines in the serum and reduced the infiltration of inflammatory cells (such as macrophages and neutrophils) in the lung tissues. These results suggests that rAT effectively alleviates the inflammatory response and lung tissue damage in LPS-induced ARDS mice by inhibiting the over-activation of Th17 cells.

IL17A, mainly produced by Th17 cells, plays protective roles in host defense against bacterial and fungal pathogens and promotes the clearance of viruses by neutrophil inflammation.[40,41] Liu et, al showed that Th17 cells are increased in chronic obstructive pulmonary disease patients, and there is a negative correlation between IL-17A levels and lung function.[42] Several animal studies have shown that cigarette smoke promotes pathogenic Th17 differentiation and

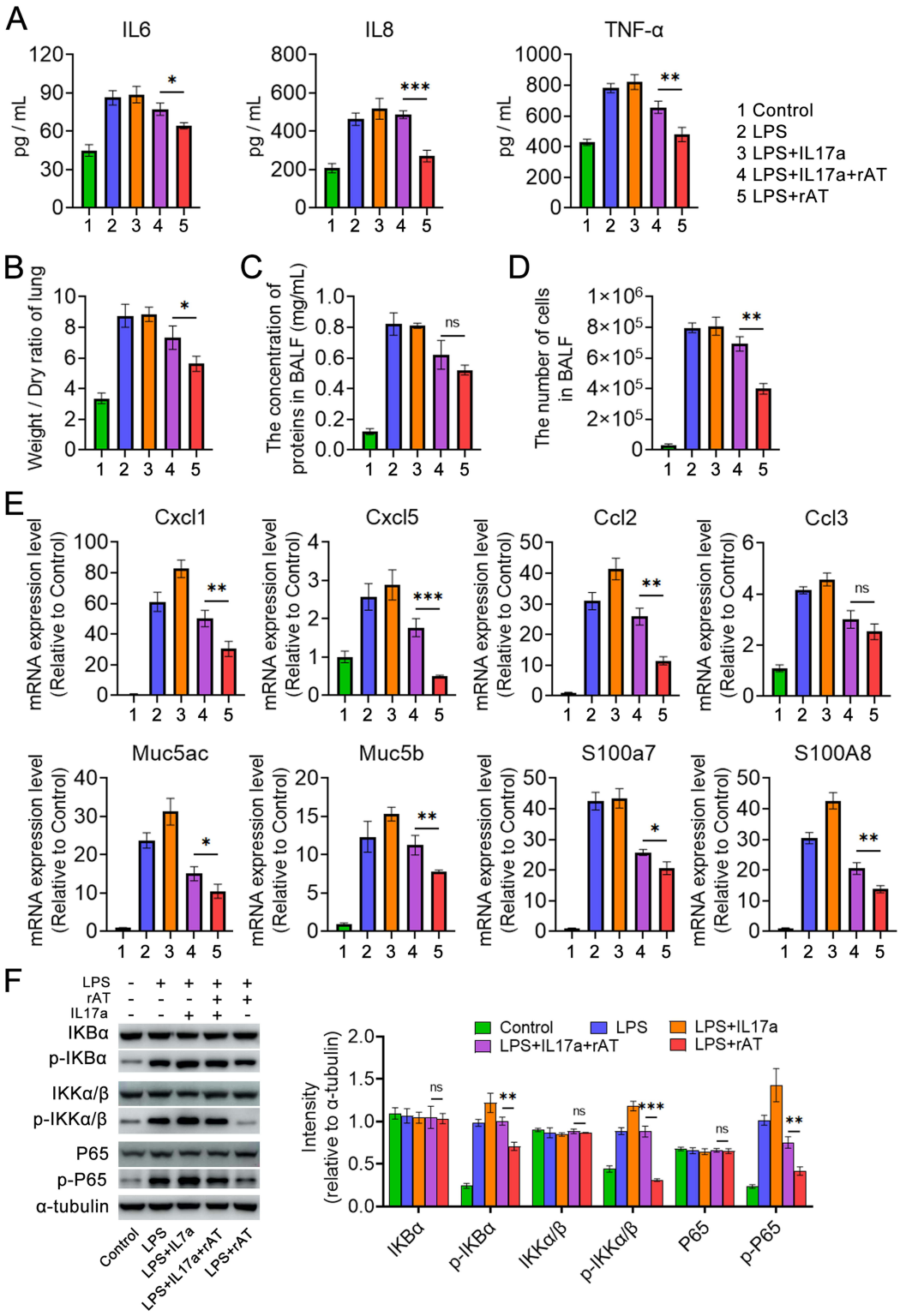

Figure 7 The efficacy of rAT in mitigating lung injury, suppressing the immune response, and inhibiting the activation of the NF-κB signaling pathway in LPS-induced ARDS mice were diminished by the administration of IL-17a. (**A**) ELISA results demonstrated that the administration of IL17a inhibited the ability of rAT to reduce inflammatory factors, including IL-6, TNF-α, and IL-8, in the serum of LPS-induced ARDS mice. (**B**) The analysis of the wet/dry weight ratio of the lung tissue revealed that the administration of IL17a counteracted the ability of rAT to alleviate pulmonary exudation in LPS-induced ARDS mice. (**C**) The administration of IL17a did not significantly affect the ability of rAT to reduce the number of cells in the BALF of LPS-induced ARDS mice. (**D**) The administration of IL17a attenuated the ability of rAT to reduce the concentrations of proteins in the BALF of LPS-induced ARDS mice. (**E**) Real-time PCR results showed that the administration of IL17a blocked the ability of rAT to downregulate the expression of target genes in the IL17a/NF-κB signaling pathway. (**F**) The protein levels of the NF-κB signaling pathway were assessed by Western blotting, and gray intensity analysis of the blots showed that the administration of IL17a in LPS-induced ARDS mice counteracted the ability of rAT to suppress the phosphorylation of IκBα, IKKα/β, and P65. The data are expressed as the means ± SDs (n=3 in each group). One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, and ns not significant.

https://doi.org/10.2147/ITT.S502925

induces emphysema and that blocking IL-17A with a neutralizing antibody significantly reduces neutrophil recruitment and the pathological score of airway inflammation in mice exposed to tobacco smoke.[43] However, IL-17A exerts different effects on different cells. IL-17A affects endothelial cells by leading to inflammation, procoagulant activity and cell senescence.[20,44] When acting on epithelial cells and fibroblasts, lL-17A leads to the production of cytokines and enzymes. When acting on monocytes and dendritic cells, lL-17A promotes inflammation by increasing the production of proinflammatory cytokines. In this study, we found that rAT not only decreases the number of Th17 cells but also inhibits the expression levels of IL17A in lung tissues from an LPS-induced ARDS mouse. The decreased expression of IL17A after rAT treatment further reduced the secretion of inflammatory cytokines, such as IL6, IL8, TNFα and hs-CRP, and the infiltration of inflammatory cells, including macrophages and neutrophils, into injured lung tissues. Therefore, the attenuation of the inflammatory response by rAT may occur through the inhibition of the IL17A signaling pathway, thereby reducing damage to lung tissue. These results also indicate that the IL17A signaling pathway may be an effective therapeutic target in the treatment of ARDS.

The balance between Th17 cells and regulatory T (Treg) cells, another subtype of $CD4^+$ T cells that maintain immune tolerance by secreting anti-inflammatory factors, such as IL-10 and TGF-β, inhibiting excessive immune responses and preventing the development of autoimmune diseases,[45] is crucial for maintaining immune homeostasis.[46] Many studies have demonstrated that the delicate balance between Th17 cells and Treg cells is often disrupted in various inflammatory diseases, including autoimmune diseases, allergies, and infections.[47–49]Compared with those in healthy individuals, the frequency of Th17cell, the level of IL17a, and the Th17/Treg ratio in the peripheral blood are increased in ARDS patients.[50] Moreover, a higher Th17/Treg ratio is associated with more adverse outcomes in ARDS patients. Hence, modulating the balance between Th17 and Treg cells may be an effective method of intervention in ARDS. For example, in an LPS-induced ARDS mouse model, LPS directly stimulated Th17 cell differentiation through modulating the phosphorylation of RelB and NF-κB1, whereas the differentiation of Th17 cells stimulated with LPS was inhibited by the TLR4-specific inhibitor CLI-095.[51] Another study revealed that secreted phosphoprotein 1 (SPP1) exacerbates lung inflammation in ARDS by modulating the Th17/Treg balance through reducing the ubiquitination and degradation of HIF-1α, which leads to an increased Th17/Treg ratio.[52] However, the effect of rAT on Treg cells in not been explored in this study. Therefore, whether rAT inhibits the immune response in LPS-induced mice by balancing the ratio of Th17/Treg requires further investigation.

NF-κB, which is generally considered a proinflammatory transcription factor, plays a key role in the inflammatory response and NLRP3 inflammasome activation in a variety of inflammatory diseases.[53,54] In addition, NF-κB is required for the differentiation and functional maturation of proinflammatory Th17 cells by inducing the expression of the transcription factor RORγt, which is specific to Th17 cells.[55–58] Bacterial or viral infection activates NF-κB through Toll-like receptors to produce proinflammatory cytokines, including TNF-α, IL-1β, and IL-6.[55] NF-κB also acts as a priming signal to promote the transcription of NLRP3 and pro-IL-1β.[59] Activation of the NLRP3 inflammasome triggers airway inflammation by activating immune cells through the secretion of IL-1β and IL-18.[60] In this study, we found that rAT significantly downregulated the phosphorylation of IKK, IκB and NF-κB p65 as well as the protein levels of NLRP3, cleaved caspase-1, IL-1β and cleaved IL-1β, leading to a reduction in proinflammatory cytokines in lung tissues. Thus, rAT alleviated inflammatory responses in an LPS-induced ARDS mouse model partially through the inhibition of the NF-κB pathway.

Conclusion

In summary, our study revealed that rAT attenuated injury to and permeability of lung tissue, decreased serum inflammatory cytokines, and reduced immune cell infiltration and activation of the NLRP3 inflammasome in lung tissue in an LPS-induced ARDS mouse model. rAT reduced the proportion of proinflammatory Th17 cells in both lung tissue and PBMCs. In addition, rAT reduced the expression of IL17A in lung tissue and inhibited the activation of the NF-κB signaling pathway. Our findings demonstrate that rAT alleviates the inflammatory response by downregulating the proportion of Th17 cells through inhibition of the IL17a/NF-κB axis, suggesting that rAT may serve as a promising therapeutic agent for managing lung inflammation and improving the prognosis of sepsis-induced ARDS. However, it is important to note that this study was conducted in a murine ARDS model, and the translational relevance to human

ARDS requires further validation through clinical trials. Additionally, the precise mechanisms by which rAT modulates Th17 cell differentiation and IL17a production remain incompletely elucidated and warrant more in-depth investigation. Future research should focus on clarifying the molecular pathways underlying immunomodulatory effects of rAT, assessing its efficacy in human ARDS, and exploring its potential synergistic effects with other therapeutic agents, such as corticosteroids, SGLT2 inhibitors, or other existing treatments. These efforts will be critical for optimizing therapeutic application of rAT and enhancing outcomes for ARDS patients.

Abbreviations

ARDS, Acute respiratory distress syndrome; BALF, Bronchoalveolar lavage fluid; CASP1, Cleaved Caspase 1; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; hs-CRP, Hypersensitive C-reactive protein; IF, Immunofluorescence; IHC, Immunohistochemistry; IL-10, Interleukin 10; IL-1β, Interleukin 1 beta; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, Lipopolysaccharide; NF-κB, Nuclear transcription factor-κB; NLRP3, NOD-like receptor pyrin domain containing 3; rAT, Recombinant antithrombin; RORγt, Retinoid-related orphan receptor gamma t; TGF-β, Transforming growth factor-β; Th17, T helper 17 cells; Tregs, Regulatory T cells; TNF-α, Tumor necrosis factor-alpha.

Declaration of Ethical Approval

All animal experiments were approved by the Institutional Ethics Committee of the Obstetrics & Gynecology Hospital of Fudan University (Approval Number: 2023-FCKYY-019). All animal procedures were performed following the Guidelines for Care and Use of Laboratory Animals of Fudan University.

Author Contributions

All authors have made significant contributions to the work presented, including its conception, study design, execution, data acquisition, analysis, and interpretation. Each author participated in drafting, revising, or critically reviewing the article. They have all endorsed the final version of the manuscript for publication, agreed on the choice of journal for submission, and accept accountability for all aspects of the work.

Funding

This study was supported by the National Key Research and Development Program of China (2021YFC2501800), the National Natural Science Foundation of China (82002077), and the 6th Shanghai Three-year Action Plan to Strengthen the Construction of Public Health System (GWVI-2.1-3).

Disclosure

The authors declare that they have no competing interests in this work.

References

1. Cajander S, Kox M, Scicluna BP. et al. Profiling the dysregulated immune response in sepsis: overcoming challenges to achieve the goal of precision medicine. *Lancet Respir Med*. 2024;12(4):305–322. doi:10.1016/s2213-2600(23)00330-2
2. Markwart R, Saito H, Harder T, et al. Epidemiology and burden of sepsis acquired in hospitals and intensive care units: a systematic review and meta-analysis. *Intensive Care Med*. 2020;46(8):1536–1551. doi:10.1007/s00134-020-06106-2
3. Fan E, Brodie D, Slutsky AS. Acute respiratory distress syndrome: advances in diagnosis and treatment. *JAMA*. 2018;319(7):698–710. doi:10.1001/jama.2017.21907
4. Wick KD, Aggarwal NR, Curley MAQ, et al. Opportunities for improved clinical trial designs in acute respiratory distress syndrome. *Lancet Respir Med*. 2022;10(9):916–924. doi:10.1016/s2213-2600(22)00294-6
5. Wiedermann CJ. Clinical review: molecular mechanisms underlying the role of antithrombin in sepsis. *Crit Care*. 2006;10(1):209. doi:10.1186/cc4822
6. Rosenberg RD, Damus PS. The purification and mechanism of action of human antithrombin-heparin cofactor. *J Biol Chem*. 1973;248(18):6490–6505. doi:10.1016/S0021-9258(19)43472-8
7. Yamakawa K, Ogura H, Fujimi S, et al. Recombinant human soluble thrombomodulin in sepsis-induced disseminated intravascular coagulation: a multicenter propensity score analysis. *Intensive Care Med*. 2013;39(4):644–652. doi:10.1007/s00134-013-2822-2
8. Vincent JL, Francois B, Zabolotskikh I, et al. Effect of a recombinant human soluble thrombomodulin on mortality in patients with sepsis-associated coagulopathy: the SCARLET randomized clinical trial. *JAMA*. 2019;321(20):1993–2002. doi:10.1001/jama.2019.5358

9. Kudo D, Goto T, Uchimido R, et al. Coagulation phenotypes in sepsis and effects of recombinant human thrombomodulin: an analysis of three multicentre observational studies. *Crit Care*. 2021;25(1):114. doi:10.1186/s13054-021-03541-5
10. Warren BL, Eid A, Singer P, et al. Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. *JAMA*. 2001;286(15):1869–1878. doi:10.1001/jama.286.15.1869
11. Murakami K, McGuire R, Cox RA, et al. Recombinant antithrombin attenuates pulmonary inflammation following smoke inhalation and pneumonia in sheep. *Crit Care Med*. 2003;31(2):577–583. doi:10.1097/01.Ccm.0000050444.52531.08
12. Iba T, Levy JH, Hirota T, et al. Protection of the endothelial glycocalyx by antithrombin in an endotoxin-induced rat model of sepsis. *Thromb Res*. 2018;171:1–6. doi:10.1016/j.thromres.2018.09.042
13. Okamoto H, Muraki I, Okada H, et al. Recombinant antithrombin attenuates acute respiratory distress syndrome in experimental endotoxemia. *Am J Pathol*. 2021;191(9):1526–1536. doi:10.1016/j.ajpath.2021.05.015
14. Wiedermann CJ. Antithrombin as therapeutic intervention against sepsis-induced coagulopathy and disseminated intravascular coagulation: lessons learned from COVID-19-Associated Coagulopathy. *Int J mol Sci*. 2022;23(20):12474. doi:10.3390/ijms232012474
15. Komura H, Uchiba M, Mizuochi Y, et al. Antithrombin inhibits lipopolysaccharide-induced tumor necrosis factor-alpha production by monocytes in vitro through inhibition of Egr-1 expression. *J Thromb Haemost*. 2008;6(3):499–507. doi:10.1111/j.1538-7836.2007.02869.x
16. Mills KHG. IL-17 and IL-17-producing cells in protection versus pathology. *Nat Rev Immunol*. 2023;23(1):38–54. doi:10.1038/s41577-022-00746-9
17. Dhawan M, Rabaan AA, Fawarah MMA, et al. Updated Insights into the T cell-mediated immune response against SARS-CoV-2: a step towards efficient and reliable vaccines. *Vaccines (Basel)*. 2023;11(1). doi:10.3390/vaccines11010101
18. Xie M, Cheng B, Ding Y, Wang C, Chen J. Correlations of IL-17 and NF-κB gene polymorphisms with susceptibility and prognosis in acute respiratory distress syndrome in a Chinese population. *Biosci Rep*. 2019;39(2). doi:10.1042/bsr20181987
19. Huang Q, Le Y, Li S, Bian Y. Signaling pathways and potential therapeutic targets in acute respiratory distress syndrome (ARDS). *Respir Res*. 2024;25(1):30. doi:10.1186/s12931-024-02678-5
20. Zhang L, Liu M, Liu W, et al. Th17/IL-17 induces endothelial cell senescence via activation of NF-κB/p53/Rb signaling pathway. *Lab Invest*. 2021;101(11):1418–1426. doi:10.1038/s41374-021-00629-y
21. Wright JF, Bennett F, Li B, et al. The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol*. 2008;181(4):2799–2805. doi:10.4049/jimmunol.181.4.2799
22. Liu C, Qian W, Qian Y, et al. Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. *Sci Signal*. 2009;2(92):ra63. doi:10.1126/scisignal.2000382
23. Dupont WD, Plummer WD Jr. Power and sample size calculations for studies involving linear regression. *Control Clin Trials*. 1998;19(6):589–601. doi:10.1016/s0197-2456(98)00037-3
24. Qian T, Qi B, Fei Y, et al. PLD2 deletion alleviates disruption of tight junctions in sepsis-induced ALI by regulating PA/STAT3 phosphorylation pathway. *Int Immunopharmacol*. 2023;114:109561. doi:10.1016/j.intimp.2022.109561
25. Li X, Chen W, Huang L, et al. Sinomenine hydrochloride suppresses the stemness of breast cancer stem cells by inhibiting Wnt signaling pathway through down-regulation of WNT10B. *Pharmacol Res*. 2022;179:106222. doi:10.1016/j.phrs.2022.106222
26. Yu B, Li H, Chen J, et al. Extensively expanded murine-induced hepatic stem cells maintain high-efficient hepatic differentiation potential for repopulation of injured livers. *Liver Int*. 2020;40(9):2293–2304. doi:10.1111/liv.14509
27. Wen S, He L, Zhong Z, et al. Stigmasterol restores the balance of Treg/Th17 cells by activating the butyrate-PPARγ axis in colitis. *Front Immunol*. 2021;12:741934. doi:10.3389/fimmu.2021.741934
28. Wang Q, Wu Q. Knockdown of receptor interacting protein 140 (RIP140) alleviated lipopolysaccharide-induced inflammation, apoptosis and permeability in pulmonary microvascular endothelial cells by regulating C-terminal binding protein 2 (CTBP2). *Bioengineered*. 2022;13 (2):3981–3992. doi:10.1080/21655979.2022.2031403
29. Wang J, Xu L, Xiang Z, et al. Microcystin-LR ameliorates pulmonary fibrosis via modulating CD206(+) M2-like macrophage polarization. *Cell Death Dis*. 2020;11(2):136. doi:10.1038/s41419-020-2329-z
30. Vora SM, Lieberman J, Wu H. Inflammasome activation at the crux of severe COVID-19. *Nat Rev Immunol*. 2021;21(11):694–703. doi:10.1038/s41577-021-00588-x
31. Chi X, Jin W, Zhao X, et al. RORγt expression in mature T(H)17 cells safeguards their lineage specification by inhibiting conversion to T(H)2 cells. *Sci Adv*. 2022;8(34):eabn7774. doi:10.1126/sciadv.abn7774
32. Han Y, Zhu Y, Almuntashiri S, et al. Extracellular vesicle-encapsulated CC16 as novel nanotherapeutics for treatment of acute lung injury. *Mol Ther*. 2023;31(5):1346–1364. doi:10.1016/j.ymthe.2023.01.009
33. Chen J, Shi X, Deng Y, et al. miRNA-148a-containing GMSC-derived EVs modulate Treg/Th17 balance via IKKB/NF-κB pathway and treat a rheumatoid arthritis model. *JCI Insight*. 2024;9(10). doi:10.1172/jci.insight.177841
34. Gerlo S, Kooijman R, Beck IM, Kolmus K, Spooren A, Haegeman G. Cyclic AMP: a selective modulator of NF-κB action. *Cell mol Life Sci*. 2011;68(23):3823–3841. doi:10.1007/s00018-011-0757-8
35. Yu H, Lin L, Zhang Z, Zhang H, Hu H. Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study. *Signal Transduct Target Ther*. 2020;5(1):209. doi:10.1038/s41392-020-00312-6
36. Levy JH, Sniecinski RM, Welsby IJ, Levi M. Antithrombin: anti-inflammatory properties and clinical applications. *Thromb Haemost*. 2016;115 (4):712–728. doi:10.1160/th15-08-0687
37. Waite JC, Skokos D. Th17 response and inflammatory autoimmune diseases. *Int J Inflam*. 2012;2012:819467. doi:10.1155/2012/819467
38. Eisenstein EM, Williams CB. The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. *Pediatr Res*. 2009;65(5 Pt 2):26r–31r. doi:10.1203/PDR.0b013e31819e76c7
39. Paroli M, Caccavale R, Fiorillo MT, et al. The double game played by Th17 cells in infection: host defense and immunopathology. *Pathogens*. 2022;11(12):1547. doi:10.3390/pathogens11121547
40. Chen K, Kolls JK. Interluekin-17A (IL17A). *Gene*. 2017;614:8–14. doi:10.1016/j.gene.2017.01.016
41. Li X, Bechara R, Zhao J, McGeachy MJ, Gaffen SL. IL-17 receptor-based signaling and implications for disease. *Nat Immunol*. 2019;20 (12):1594–1602. doi:10.1038/s41590-019-0514-y
42. Liu X, Nguyen TH, Sokulsky L, et al. IL-17A is a common and critical driver of impaired lung function and immunopathology induced by influenza virus, rhinovirus and respiratory syncytial virus. *Respirology*. 2021;26(11):1049–1059. doi:10.1111/resp.14141

43. Le Rouzic O, Pichavant M, Frealle E, Guillon A, Si-Tahar M, Gosset P. Th17 cytokines: novel potential therapeutic targets for COPD pathogenesis and exacerbations. *Eur Respir J*. 2017;50(4):1602434. doi:10.1183/13993003.02434-2016
44. Miossec P, Kolls JK. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov*. 2012;11(10):763–776. doi:10.1038/nrd3794
45. Qiu R, Zhou L, Ma Y, et al. Regulatory T cell plasticity and stability and autoimmune diseases. *Clin Rev Allergy Immunol*. 2020;58(1):52–70. doi:10.1007/s12016-018-8721-0
46. Wang J, Zhao X, Wan YY. Intricacies of TGF-β signaling in Treg and Th17 cell biology. *Cell mol Immunol*. 2023;20(9):1002–1022. doi:10.1038/s41423-023-01036-7
47. Liu X, Chen L, Peng W, et al. Th17/Treg balance: the bloom and wane in the pathophysiology of sepsis. *Front Immunol*. 2024;15:1356869. doi:10.3389/fimmu.2024.1356869
48. Thomas R, Qiao S, Yang X. Th17/Treg imbalance: implications in lung inflammatory diseases. *Int J mol Sci*. 2023;24(5):4865. doi:10.3390/ijms24054865
49. Fasching P, Stradner M, Graninger W, Dejaco C, Fessler J. Therapeutic potential of targeting the Th17/Treg axis in autoimmune disorders. *Molecules*. 2017;22(1):134. doi:10.3390/molecules22010134
50. Yu ZX, Ji MS, Yan J, et al. The ratio of Th17/Treg cells as a risk indicator in early acute respiratory distress syndrome. *Crit Care*. 2015;19(1):82. doi:10.1186/s13054-015-0811-2
51. Park JH, Jeong SY, Choi AJ, Kim SJ. Lipopolysaccharide directly stimulates Th17 differentiation in vitro modulating phosphorylation of RelB and NF-κB1. *Immunol Lett*. 2015;165(1):10–19. doi:10.1016/j.imlet.2015.03.003
52. Chen L, Yang J, Zhang M, Fu D, Luo H, Yang X. SPP1 exacerbates ARDS via elevating Th17/Treg and M1/M2 ratios through suppression of ubiquitination-dependent HIF-1α degradation. *Cytokine*. 2023;164:156107. doi:10.1016/j.cyto.2022.156107
53. Fann DY, Lim YA, Cheng YL, et al. Evidence that NF-κB and MAPK signaling promotes NLRP Inflammasome activation in neurons following ischemic stroke. *mol Neurobiol*. 2018;55(2):1082–1096. doi:10.1007/s12035-017-0394-9
54. He Y, Hara H, Núñez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci*. 2016;41(12):1012–1021. doi:10.1016/j.tibs.2016.09.002
55. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. *Signal Transduct Target Ther*. 2017;2(17023):17023. doi:10.1038/sigtrans.2017.23
56. Wang J, Yang J, Xia W, et al. Escherichia coli enhances Th17/Treg imbalance via TLR4/NF-κB signaling pathway in oral lichen planus. *Int Immunopharmacol*. 2023;119:110175. doi:10.1016/j.intimp.2023.110175
57. He Z, Wang F, Zhang J, et al. Regulation of Th17 Differentiation by IKKα-dependent and -independent phosphorylation of RORγt. *J Immunol*. 2017;199(3):955–964. doi:10.4049/jimmunol.1700457
58. Ruan Q, Chen YH. Nuclear factor-κB in immunity and inflammation: the Treg and Th17 connection. *Adv Exp Med Biol*. 2012;946:207–221. doi:10.1007/978-1-4614-0106-3_12
59. Zhao C, Zhao W. NLRP3 inflammasome-a key player in antiviral responses. *Front Immunol*. 2020;11:211. doi:10.3389/fimmu.2020.00211
60. Hikichi M, Mizumura K, Maruoka S, Gon Y. Pathogenesis of chronic obstructive pulmonary disease (COPD) induced by cigarette smoke. *J Thorac Dis*. 2019;11(Suppl 17):S2129–s2140. doi:10.21037/jtd.2019.10.43