Tropical Journal of Pharmaceutical Research September 2022; 21 (9): 1879-1884
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i9.10

Original Research Article

Abietic acid attenuates LPS-induced acute lung injury by restoring Th17/Treg balance

Xiang Li[1], Bo Liao[1], Rongming Liu[1], Yanli Ma[2]*

[1]Department of Critical Care Medicine, The Affiliated Jiangning Hospital of Nanjing Medical University, Nanjing City, Jiangsu Province 211100; [2]Department of Public Health Management, Ankang Central Hospital, Ankang City, Shaanxi Province 725000, China

For correspondence:* ***Email:* *yanli_m77@163.com;* ***Tel:*** *+86-0915-3216866*

Sent for review: 1 June 2022 *Revised accepted: 16 August 2022*

Abstract

Purpose: *To investigate the mechanism of action and effects of abietic acid (AA) in a mouse acute lung injury (ALI) model.*
Methods: *A mouse ALI model was established by lipopolysaccharide (LPS) induction. Lung tissues were examined for histological alterations and scored based on the degree of injury. Myeloperoxidase (MPO), IL-6, IL-1β, and TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA) while the numbers of Th17 and Treg cells were assessed by immunofluorescence. Protein expression levels of p-STAT3, p-STAT5, RORrt, and FOXP3 were analyzed by immunoblot assay. Expression of peroxisome proliferator-activated receptor γ (PPARγ) was assessed by immunoblot.*
Results: *AA ameliorated LPS-induced lung injury in mice. Furthermore, AA ameliorated LPS-induced pneumonia in mice ($p < 0.05$) and restored Th17/Treg balance and Th17/Treg transcription factor expression that was altered by LPS induction. AA also activated PPARγ expression to restore Th17/Treg balance ($p < 0.05$).*
Conclusion: *The results indicate that AA attenuates LPS-induced ALI in mice by restoring Th17/Treg balance. Thus, AA is a potential drug for the management of ALI; however, AA must first be evaluated in clinical studies.*

Keywords: *Acute lung injury, Abietic acid, Lipopolysaccharide, Peroxisome proliferators-activated receptor γ (PPARγ)*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Acute lung injury (ALI) is a leading cause of death in patients with respiratory failure [1]. ALI may occur as a result of pneumonia, inhalation of harmful substances, or indirect injuries such as ischemia-reperfusion. ALI induces damaging hypoxic and ischemic stress and the release of lipopolysaccharide (LPS), a bacterial endotoxin. LPS stimulates the release of severe inflammatory signals, leading to lung injury by inflammatory leukocyte infiltration [2]. Therefore, it is important to identify drugs that suppress the inflammatory response in ALI.

Immune homeostasis *in vivo* depends on a balance among T-helper lymphocyte subsets, including Th1, Th2, Th17, and Treg cells. Th17

cells secrete IL-17, which induces the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, thereby exacerbating the inflammatory response [3]. In contrast, Tregs secrete anti-inflammatory mediators such as IL-10 and TGF-β to mediate immune homeostasis. Imbalance between Th17 cells and Treg cells contributes to the development of various diseases [4]. Mediation of the balance of Th17 cells and Treg cells can improve sepsis-induced ALI. Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor family and one of the PPAR isotypes [5]. PPARγ activation plays anti-inflammatory and anti-apoptotic roles in several inflammatory diseases, including ALI. There is growing evidence that nuclear receptors, especially PPARγ, play crucial roles in regulating the Th17/Treg balance.

Abietic acid (AA) is an abietic diterpene compound, mainly derived from *Pimenta racemosa* var grissea [6], that has been reported to have anti-allergy, anti-inflammation, anti-obesity, anti-convulsion, and anti-tumor effects. Rosinic acid inhibits IL-1β-induced inflammation in human osteoarthritis chondrocytes by activating PPAR-γ and has a protective effect on allergic asthma in mice induced by OVA [7]. In this study, the effect of AA on the treatment of ALI was investigated.

EXPERIMENTAL

Animals and treatments

Male C57BL/6 mice (~8 weeks old) were obtained from SLAC (Shanghai, China) and kept in a sterile environment. All experimental procedures in this study were approved by the Ethics Committee of Ankang Central Hospital (approval no. 2021-021) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [8]. The mice were anesthetized by injection of 4 % chloral hydrate (ClHy, 0.1 ml/10 g) and instilled with sterile PBS (control group) or 3 mg/kg LPS (Sigma, *Escherichia coli* 0111:B4) dissolved in 50 µL PBS. AA (10, 20, or 40 mg/kg) was administered by oral gavage 1 h after LPS treatment. Bronchoalveolar lavage fluid (BALF) and lung tissue samples were collected, and pathological changes such as cytokine production, total cells, and neutrophils were assessed.

Histological examination

Lung tissues were fixed with 5 % PFA, embedded in paraffin, and sectioned. The sections were counterstained with hematoxylin and eosin (H&E).

Lung injury scores

Lung injuries were scored based on hemorrhage, alveolar wall thickness, inflammatory infiltration, alveolar congestion, and hyaline membrane formation. A score of 0 represents no damage, 1 represents mild damage, 2 represents moderate damage, 3 represents severe damage, and 4 represents very severe histological alterations.

Evaluation of wet-to-dry (W/D) lung weight ratio

Mice were sacrificed by neck breakage, and their lungs were removed and weighed immediately. The lungs were dried in an oven until they reached a constant weight. The wet/dry weight ratios of the lungs were calculated.

Enzyme-linked immunosorbent assay (ELISA)

IL-1β, TNF-α, and IL-6 levels in BALF samples were assessed using ELISA kits (Beyotime, Beijing, China) according to the manufacturer's instructions. Briefly, samples were aspirated into wells and incubated with biotin-conjugated primary antibodies. Then, avidin-conjugated horseradish peroxidase (HRP) was added to each well. Determination was performed by the addition of substrate, and absorbance was measured at 350 nm using a microplate reader.

Immunofluorescence

Lung tissues were fixed with 5 % PFA, embedded in OCT, and sectioned. Sections were incubated with primary antibodies to CD4, IL-10, and IL-17A at 4°C overnight. After rinsing with PBS, sections were incubated with the respective secondary antibodies and counterstained with DAPI. Images were captured using a fluorescence microscope.

Western blotting

Lysates were collected from tissue samples using RIPA buffer, and protein concentrations were determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked in 5 % BSA and incubated with primary antibodies to p-STAT3 (1:1000, Thermo Scientific, Waltham, MA, USA), STAT3 (1:1000, Abcam, Cambridge, UK), p-STAT5 (1:1000, Abcam), STAT5 (1:1000, Abcam), RORγt

(1:1000, Abcam), FOXP3 (1:1000, Abcam), PPARγ (1:1000, Abcam), and GAPDH (1:10000, Abcam). The membranes were rinsed with TBST for 15 min and then incubated with HRP-conjugated secondary antibodies (1:1000) for 2 h. Detection was performed using an ECL detection kit.

Statistical analysis

Statistical analysis was performed using GraphPad 7.0. Data are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

AA relieved LPS-induced lung injury in mice

After induction of ALI by LPS, lung tissues were examined histologically. Mice in the LPS group displayed tissue injury characterized by irregular alveolar integrity and size, partial collapse of alveoli, enlarged spaces between alveoli, and inflammatory cell infiltration. However, these injuries were reversed after the administration of AA (Figures 1 A and B). In addition, the wet/dry ratio of lung tissue increased in the LPS group, but this increase was reversed upon AA treatment (Figure 1 C). These results suggested that AA may ameliorate lung injury.

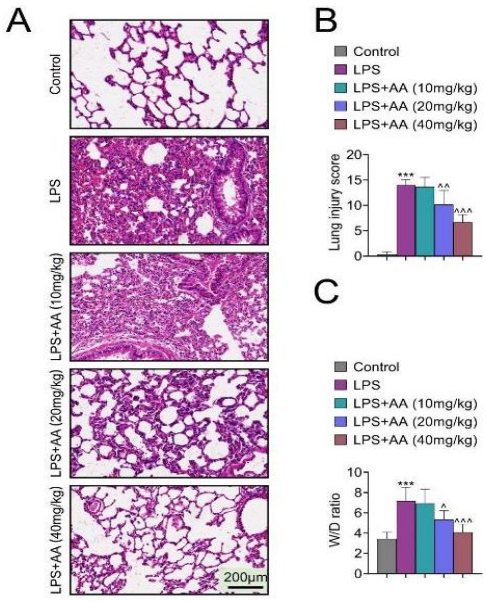

Figure 1: AA relieved lung injury in mice induced with LPS**.** A, Histological analysis of lung tissues in control, LPS, and LPS+AA mice. B, Injury scores of lung tissues in control, LPS, and LPS+AA mice. C, W/D ratios of lungs in control, LPS, and LPS+AA mice. ***$P < 0.001$ vs control; ^$p < 0.05$, ^^$p < 0.01$, and ^^^$p < 0.001$ vs LPS

AA inhibited inflammatory cytokine production in mice

Total cells and neutrophils in the BALF samples from each group were evaluated. Total cell and neutrophil numbers in the BALF samples from LPS-induced mice increased significantly compared with those in control samples, and AA treatment reversed these increases (Figure 2 A). MPO activity reflects neutrophil infiltration into damaged tissue. The MPO level increased in LPS-induced mice, and this increase was reversed by AA treatment (Figure 2 B). Levels of the inflammatory cytokines IL-6, IL-1β, and TNF-α also increased in LPS-induced mice, and this increase was reversed by AA treatment (Figure 2 C).

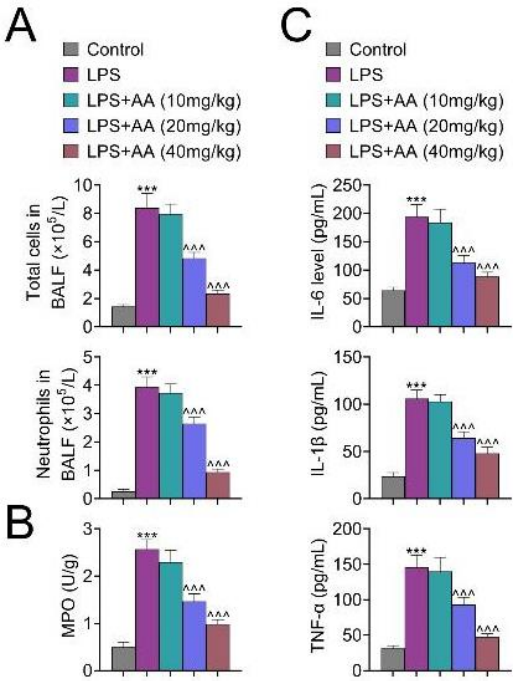

Figure 2: AA inhibited inflammatory cytokine production in LPS-induced mice. A, Total cells and neutrophils in BALFs from control, LPS, and LPS+AA mice. B, MPO levels in control, LPS, and LPS+AA mice. C, Levels of IL-1b, TNF-a, and IL-6 in control, LPS, LPS+AA mice. ***$P < 0.001$ vs control; ^$p < 0.05$, ^^$p < 0.01$, and ^^^$p < 0.001$ vs LPS

AA restored TH17/Treg balance in mice

Differentiated Th17 and Treg cells in the lungs of control, LPS, and LPS+AA mice were evaluated by immunofluorescence. The number of IL-17A and IL-10 T cells increased in the lungs of LPS-induced mice (Figure 3). AA treatment decreased the number of IL-17A cells and increased the numbers of CD4 and IL-10 cells (Figure 3). Thus, AA restored the balance of TH17/Treg cells in LPS-induced mice.

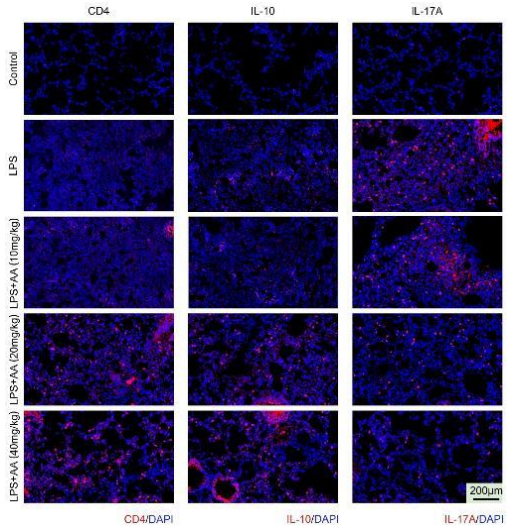

Figure 3: CD4, IL-10, and IL-17A cells in the lungs of control, LPS, and LPS+AA mice

AA mediated the Th17/Treg balance via the STAT pathway

To unravel the mechanism of action of AA, the effects of AA on molecules in the STAT pathway were investigated. AA treatment increased p-STAT5, RORrt, and FOXP3 levels and decreased the level of p-STAT3 in LPS-induced mice (Figure 4). These data confirmed that AA restores the Th17/Treg balance by modulating the STAT pathway.

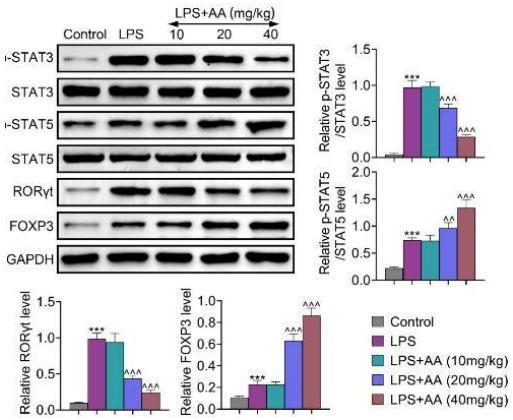

Figure 4: AA modulated the Th17/Treg balance via the STAT pathway. p-STAT3, p-STAT5, RORrt, and FOXP3 protein levels in control, LPS, and LPS+AA mice are shown. ***P < 0.001 vs. control; ^p < 0.05, ^^p < 0.01, and ^^^p < 0.001 vs. LPS

AA modulated Th17/Treg balance by activating PPARγ

To further understand the molecular mechanism of AA, the effect of AA on PPARg was examined. The level of PPARg decreased in LPS-induced mice, and this decrease was reversed with AA treatment (Figure 5 A). To verify the effect of AA on PPARg, the PPARg inhibitor GW9662 was used. As shown above, levels of p-STAT5 and FOXP3 increased and levels of p-STAT3 and RORrt decreased in LPS+AA mice, and these alterations were reversed with administration of GW9662 (Figure 5 B). Thus, AA modulated the Th17/Treg balance by activating PPARγ.

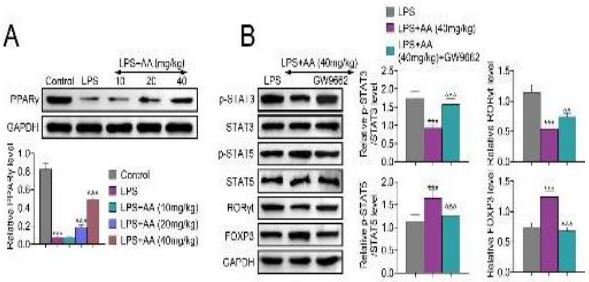

Figure 5: AA modulated the Th17/Treg balance by activating PPARγ. A, PPARg protein levels in control, LPS, and LPS+AA mice. B: p-STAT3, STAT3, p-STAT5, STAT5, RORrt, and FOXP3 protein levels in control, LPS, LPS+AA, and LPS+AA+ GW9662 mice. ***P < 0.001 vs. control; ^ p < 0.05, ^^, p < 0.01, and ^^^ p < 0.001 vs. LPS

DISCUSSION

ALI is characterized by acute hypoxic respiratory failure caused by injury of alveolar epithelial cells [9]. ALI is caused by inflammation; however, understanding the pathogenesis of ALI requires further study [10]. Currently, there are no effective treatments for the severe stage of ALI (oxygenation index < 200). In this study, it was found that AA, an abietic diterpene compound, may be an effective treatment for ALI.

H&E staining and lung injury scoring showed that AA ameliorated LPS-induced lung injury in mice. Furthermore, ELISA and FCM assays revealed that AA ameliorated LPS-induced pneumonia and restored LPS-induced Th17/Treg imbalance in mice. Immunoblot assays revealed that AA activated PPARγ and affected the expression of transcription factors that contribute to Th17 and Treg cell differentiation. It was previously shown that AA ameliorates psoriasis-like inflammation and modulates the gut microbiota in mice. In addition, AA suppressed non-small-cell lung cancer (NSCLC) cell growth by suppressing the IKKβ/NF-κB pathway. Similarly, AA ameliorated inflammation in our ALI mouse model. In this study, AA activated PPARγ and mediated the Th17/Treg balance, thus attenuating inflammation in the ALI model; however, the precise mechanism of AA requires further study.

AA modulated the Th17/Treg balance in the ALI mice model. Immune homeostasis depends on

the balance between lymphocyte subsets and Treg cells [11]. Th17 cells secrete IL-17 and induce production of pro-inflammatory cytokines, thereby exacerbating the inflammatory response [12]. Tregs secrete anti-inflammatory mediators to maintain immune homeostasis [13]. Imbalances in Th and Treg cells contribute to the development of various diseases [14]. Restoration of the balance of Th17 cells and Treg cells has been shown to improve ALI [15]. Mechanistically, we suggest that AA attenuates ALI by modulating the Th17/Treg balance.

PPARγ is a key member of the PPAR family [16], and PPARγ activation plays anti-inflammatory and anti-apoptotic roles in many inflammatory disease models. PPARγ also plays a crucial role in regulating the Th17/Treg balance [17]. In this study, LPS induced a Th17/Treg imbalance, and AA treatment restored the Th17/Treg balance by promoting the expression of transcription factors. In addition, AA activated PPARγ to regulate the Th17/Treg balance. Hence, PPARγ may serve as a target for the treatment of ALI and other inflammatory diseases.

CONCLUSION

The findings of this study show that AA attenuates LPS-induced ALI by activating PPAR-γ and restoring Th17/Treg balance. Thus, AA is a potential drug for the treatment of ALI.

DECLARATIONS

Acknowledgements

This study was supported by Nanjing Medical Health Science and Technology Development Foundation (Grant no. YKK20189).

Competing interests

There is no conflict of interest to disclose.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon reasonable request.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

Contribution of authors

Xiang Li, Bo Liao, Rongming Liu, and Yanli Ma designed and carried out the study, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the manuscript.

REFERENCES

1. *Wittenstein J, Huhle R, Leiderman M, Mobius M, Braune A, Tauer S, Herzog P, Barana G, de Ferrari A, Corona A, et al. Effect of patient-ventilator asynchrony on lung and diaphragmatic injury in experimental acute respiratory distress syndrome in a porcine model. Br J Anaesth DOI: https://doi.org/10.1016/j.bja.2021.10.037.*
2. *Jia X, Huang J, Wu B, Yang M, Xu W. A Competitive Endogenous RNA Network Based on Differentially Expressed lncRNA in Lipopolysaccharide-Induced Acute Lung Injury in Mice. Front Genet 2021; 12: 745715.*
3. *Le Menn G, Jablonska A, Chen Z. The effects of post-translational modifications on Th17/Treg cell differentiation. Biochim Biophys Acta Mol Cell Res 2022; 1869(6): 119223.*
4. *Saini C, Sapra L, Bhardwaj A, Tarique M, Sharma A, Khanna N, Ramesh V, Puri P, Srivastava RK. IL-21 plays an important role in modulating "Th17-Treg" cell axis in leprosy Type 1 reactions. Cytokine 2022; 152: 155821.*
5. *Jin B, Dong W, Sun D, Cai B, Wu P. Yangjing capsule attenuates cyclophosphamide-induced deficiency of testicular microcirculation in mice. Trop J Pharm Res 2020; 19(3): 603-608.*
6. *Liu X, Chen W, Liu Q, Dai J. Abietic acid suppresses non-small-cell lung cancer cell growth via blocking IKKbeta/NF-kappaB signaling. Onco Targets Ther 2019; 12: 4825-4837.*
7. *Jung MJ, Jung HA, Kang SS, Hwang GS, Choi JS. A new abietic acid-type diterpene glucoside from the needles of Pinus densiflora. Arch Pharm Res 2009; 32(12): 1699-1704.*
8. *Institute of Laboratory Animal Resources (US). Committee on Care, Use of Laboratory Animals. Guide for the care and use of laboratory animals[M]. US*

Department of Health and Human Services, Public Health Service, National Institutes of Health, 1986.

9. *Chen X, Sun Z, Zhang H, Wang L. Correlation of Impaired NF-kB Activation in Sepsis-Induced Acute Lung Injury (ALI) in Diabetic Rats. J Healthc Eng 2021; 2021: 5657284.*
10. *Sandeep B, Xiao Z, Zhao F, Feng Q, Gao K. Role of Platelets in Acute Lung Injury after Extracorporeal Circulation in Cardiac Surgery Patients: A systemic review. Curr Probl Cardiol 2021: 101088.*
11. *Asila A, Liu J, Liu J, Li L, Liao J. Immunomodulatory effects of berberine on Staphylococcus aureus-induced septic arthritis through down-regulation of Th17 and Treg signaling pathways. Acta Biochim Pol 2022; 69(1): 215-226.*
12. *Zehra Okus F, Busra Azizoglu Z, Canatan H, Eken A. S1P analogues SEW2871, BAF312 and FTY720 affect human Th17 and Treg generation ex vivo. Int Immunopharmacol 2022; 107: 108665.*
13. *Yang J, Hao T, Liu Y, Huang J, Wu W, Wu J, Sun W. Th17/Treg balance and indoleamine 2,3 dioxygenase activity in periodontitis-associated atherosclerotic patients. J Int Med Res 2022; 50(2): 3000605221080877.*
14. *Song B, Li P, Yan S, Liu Y, Gao M, Lv H, Lv Z, Guo Y. Effects of Dietary Astragalus Polysaccharide Supplementation on the Th17/Treg Balance and the Gut Microbiota of Broiler Chickens Challenged With Necrotic Enteritis. Front Immunol 2022; 13: 781934.*
15. *Ishihara S, Sato T, Fujikado N, Miyazaki H, Yoshimoto T, Yamamoto H, Fukuda S, Katagiri K. Rap1 prevents colitogenic Th17 cell expansion and facilitates Treg cell differentiation and distal TCR signaling. Commun Biol 2022; 5(1): 206.*
16. *Ito Y, Ito T, Yamashiro K, Mineshiba F, Hirai K, Omori K, Yamamoto T, Takashiba S. Antimicrobial and antibiofilm effects of abietic acid on cariogenic Streptococcus mutans. Odontology 2020; 108(1): 57-65.*
17. *Gao Y, Zhaoyu L, Xiangming F, Chunyi L, Jiayu P, Lu S, Jitao C, Liangcai C, Jifang L. Abietic acid attenuates allergic airway inflammation in a mouse allergic asthma model. Int Immunopharmacol 2016; 38: 261-266.*