Article

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Curcumin Alleviates Airway Inflammation in Cough-Variant Asthmatic Rats by Modulating M1/M2 Macrophage Polarization

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Background: With the increase of environmental pollution and atypical pathogen infections, the incidence of cough variant asthma (CVA) has been increasing annually, making it a pressing issue of the medical community. This study aims to observe the ameliorative effect of curcumin on a rat model of cough variant asthma.

Methods: A rat model of cough variant asthma was induced by sensitization with ovalbumin combined with aluminum hydroxide ($Al(OH)_3$), followed by repeated excitations. The drug was administered on the day of the initial nebulized attack, and gavage was administered for 14 d. Pathological changes in the lung tissues were observed, along with the assessment of cough susceptibility and airway resistance. The number of inflammatory cell eosinophils and leukocytes were determined in alveolar lavage fluid. Additionally, serum inflammatory factors and lung tissues Matrix Metalloproteinase-9 (MMP-9) protein were assessed. The level of M1/M2 macrophages was also detected.

Results: Following the administration of curcumin, there was reduced inflammatory infiltration, less disordered arrangement of the lung tissue, and decreased abnormal proliferation of lung tissues in cough variant asthma rats compared to the model group. Curcumin treatment led toa notable reduction in cough frequency, a significant decrease in pro-inflammatory factor concentration levels in serum and inflammatory cell counts in the alveolar lavage fluid, and a marked increase in anti-inflammatory factor levels ($p < 0.05$). Additionally, curcumin administration led to a significant increase in M2-type macrophage levels, while simultaneously decreasing the levels of M1-type macrophages ($p < 0.05$).

Conclusions: The administration of curcumin effectively ameliorates ovalbumin-induced airway inflammation in cough-variant asthma rats. This effect is attributed to modulating macrophage polarization towards the anti-inflammatory M2 phenotype, thereby reducing airway inflammation, airway hyperresponsiveness, and lung tissue injury.

Keywords: curcumin; cough variant asthma; macrophage; airway inflammation; rat

Introduction

The primary clinical manifestation of cough variant asthma (CVA) is a chronic cough devoid of the normal signs of asthma. However, its pathophysiologic changes are similar to those of typical asthma, manifesting a persistent chronic airway inflammatory response with hyperresponsiveness [1]. A survey on etiology conducted across five regions in China revealed that CVA contributed to 32.6% of chronic cough cases, representing one of the primary causes of chronic cough in the country. Furthermore, it may predispose certain patients to develop typical asthma [2]. CVA is typified by a gradual start, prolonged duration, and recurrent episodes. Colds, dust, pollutants, and cold air can all easily trigger or exacerbate CVA. Currently, Western medical treatment for CVA primarily involves drugs such as inhaled glucocorticoids, bronchodilators, and leukotriene receptor antagonists [3]. However, most patients found the long-term use of glucocorticoid drugs burdensome and the therapeutic effect is often unsatisfactory. Additionally, some patients still progress to typical asthma. In recent years, the incidence of CVA has been gradually increasing, due to rising environmental pollution and atypical pathogen infections. This trend has emerged as a pressing concern within the current medical community.

The immune-inflammatory response is central to the airway inflammation in asthma, with macrophage-mediated airway inflammatory response playing a crucial role in CVA [4]. Macrophages, serving as vital antigen-presenting cells and phagocytes, constitute a key element of innate, intrinsic, and adaptive immunity. They hold a pivotal role in orchestrating inflammatory responses and maintaining tissue homeostasis. Upon exposure to external stimuli and local inflammation, lung-resident macrophages positioned

along the respiratory tract become activated. They engage in regulating respiratory homeostasis by recruiting monocyte-derived macrophages [5]. Macrophages exhibit functional diversity and phenotypic variability. Under various physiopathological conditions, mature macrophages can undergo polarization, primarily categorized into two phenotypes: M1-type (classically activated) and M2-type (alternatively activated) [6]. M1 macrophages typically exert pro-inflammatory effects, whereas M2 macrophages are associated with anti-inflammatory responses. Overall, macrophages possess both pro-inflammatory and anti-inflammatory functions [7]. It has been documented that increased M1 macrophage polarization and activation are evident in asthma and may contribute significantly to allergic asthma. However, they also facilitate M2 macrophage polarization by upregulating Interleukin-4 (IL-4) expression, which, in turn, regulates M2 macrophages and attenuates airway remodeling in asthma [8,9]. In asthmatic lungs, alveolar macrophage (AM) undergoes an alternative activation giving rise to alternatively activated (M2) macrophages that play an important role in modulating allergic airway inflammation [10].

Derived from the rhizomes of turmeric plants, curcumin (CUR) is a polyphenolic molecule with a broad range of pharmacological characteristics, such as anti-inflammatory, antioxidant, and anticancer activities [11]. Prior investigations have demonstrated that CUR can effectively mitigate inflammatory responses by modulating M1/M2 macrophage polarization and Toll-like receptors (TLRs) signaling pathways in a murine model of colitis [12]. Based on this rationale and recognizing the crucial role of macrophage polarization in asthma, the present study focus on CVA model rats as the research object. The aim is to explore the potential ameliorative effect of CUR on CVA by observing the effect of CUR on macrophage phenotype in their peripheral blood and to provide experimental bases for the clinical application of CUR.

Materials and Methods

Reagents and Instruments

Reagents: Tumor Necrosis Factor-alpha (TNF-α) Enzyme-Linked Immunosorbent Assay (ELISA) kit (ml002859, Mlbio, Shanghai, China); IL-10 ELISA kit (ml002813, Mlbio, Shanghai, China); IL-5 ELISA kit (ml002975, Mlbio, Shanghai, China); OVA (9006-59-1, Macklin, Shanghai, China); aluminum hydroxide ($Al(OH)_3$) (21645-51-2, Macklin, Shanghai, China); NaCl (7647-14-5, Macklin, Shanghai, China); montelukast tablets (H20181210, ANBISON LAB, Shanghai, China); curcumin, capsaicin (MB2147-S, MB6732-1, MeiLunBio, Liaoning, China); anti-Matrix Metalloproteinase-9 (MMP-9) antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (ab76003, ab59164, 1:500, Abcam, Shanghai, China); corresponding secondary antibody (A0208, 1:200, Beyotime, Shanghai, China).

Instruments: YB3002 analytical balance (Li-Neng Electronic Instrument Co., Ltd., Shanghai, China); DZF-6050 vacuum drying oven (Cispel Industrial Science and Technology Co., Ltd., Shanghai, China); NANO 2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); MR-96A enzyme labeling instrument (Myriad Bio-medical Electronics Co., Ltd., Shenzhen, China); VE-180 Western electrophoresis instrument (Tianyi Biomedical Electronics Co., Ltd., Shanghai, China); Nikon Microscope E100 (Shanghai Tonghao Photoelectric Technology Co., Ltd., Shanghai, China).

Animal Modeling and Grouping

Male Sprague Dawley (SD) rats, aged 6~8 weeks with a body mass (160 $\pm$ 10) g, were purchased from Jinan Panyue Experimental Animal Breeding Co., Ltd., Laboratory Animal License No. SCXK (Lu) 20180003. The rats were kept in specific pathogen-free (SPF) grade animal environment, with access to food and water. Thirty-two rats were randomly assigned to four groups based on body weight: the control (CON) group, the model (MOD) group, the Montelukast sodium (MLS) group, and the Curcumin (CUR) group, each containing eight rats. The rat CVA model was replicated by using OVA and $Al(OH)_3$-inspired sensitization and OVA-inspired sensitization [13]. Except for the CON group, all rats (the MOD, MLS and CUR group) were sensitized by intraperitoneal injection of 1 mL of a mixture of 10% OVA and $Al(OH)_3$ [100 mg of OVA, 100 mg of $Al(OH)_3$, and an appropriate amount of physiological saline] on the 1st and 8th days. Starting from day 15, the rats were placed in a closed plexiglass enclosure and were stimulated by ultrasonic nebulization of 0.9% NaCl containing 1% OVA for 2 min once a day for 10 d. The drug was administered on the day of the nebulization attack by gavage 1 h before the nebulization, continuously for 14 d, the MLS group was given montelukast sodium [montelukast sodium (0.9 mg/kg) mixed in 2 mL of saline] by gavage, the CUR group was given curcumin [curcumin (100 mg/kg) mixed in 2 mL of saline] by gavage, and equal amount of saline was given to the CON and MOD groups. After data collection, rats were euthanized by intraperitoneal injection of 3% pentobarbital (150 mg/kg), followed by cervical dislocation.

Measurement of Airway Sensitivity

Each group of rats underwent a capsaicin cough provocation test to assess airway sensitivity. Following the final administration, the rats were placed in a transparent nebulizer box and exposed to a 10^{-4} mol/L capsaicin solution aerosol for 60 seconds. Subsequently, the rats remained in the box for an additional 60 seconds after cessation of stimulation, during which the number of coughs was recorded for the subsequent 2 minutes.

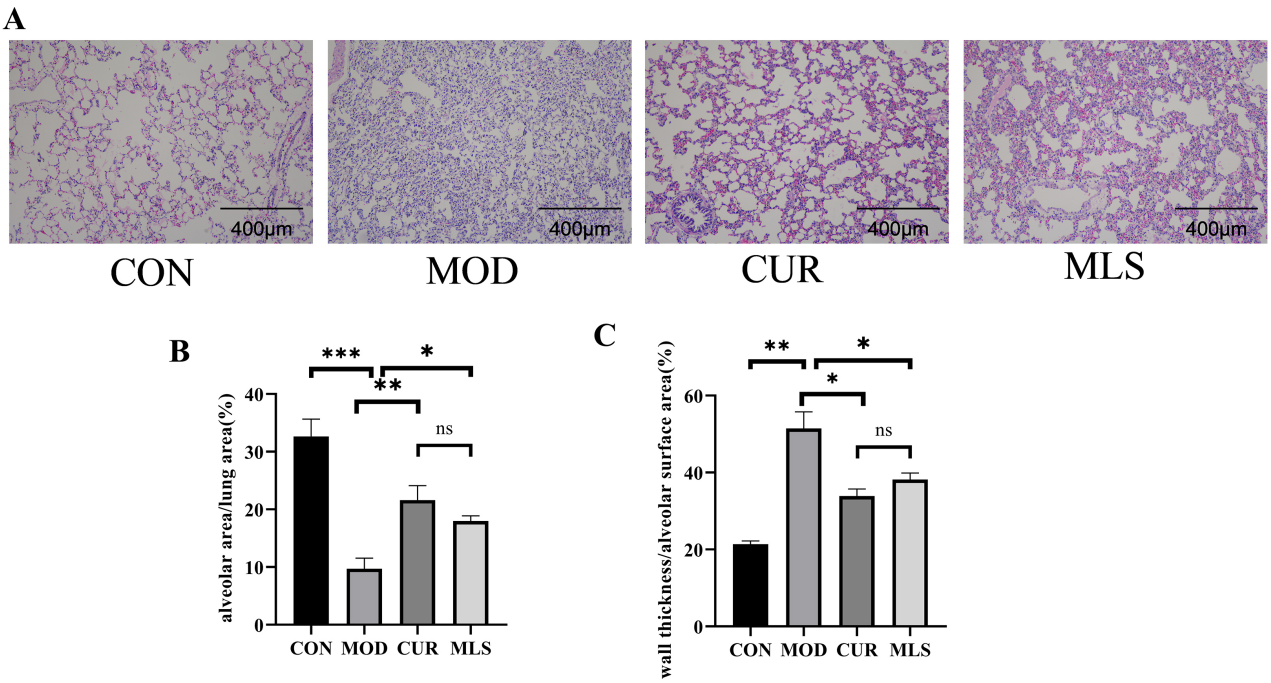

Fig. 1. The impact of curcumin on morphological characteristics of lung tissue in cough variant asthma (CVA) rats. (A) Histopathological changes in the lungs of rats in each group were observed using Hematoxylin and Eosin (H&E) staining (Scale bar = 400 μm). (B) Alveolar area. (C) Alveolar wall. ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. CON, control; MOD, model; CUR, Curcumin; MLS, Montelukast sodium. n = 8.

Determination of Special Airway Resistance (sRaw) in Rats

Two days following the final stimulation, conscious rats were placed in the dual-channel animal noninvasive airway detection system (DSI, Minneapolis, MN, USA) to measure specific airway resistance after being sedated with isoflurane inhalation. After the rats' breathing was stabilized, the pre-prepared acetyl methacholine solution (Ach) was inhaled in sequence for 30 s, and the changes in airway resistance were recorded within 3 min after the rats inhaled acetyl methacholine in the gradient of the various concentrations.

Hematoxylin and Eosin Staining (H&E Staining)

Following standard procedures, paraffin slices of fixed lung tissue were produced, sealed, dehydrated, and stained with H&E before the histomorphology was examined.

Inflammatory Cell Count in Alveolar Lavage Fluid

The neck skin of each group of rats was incised to expose the trachea, and a small incision was made. Subsequently, 2 mL of saline was injected using a lavage needle, and lavage was performed three times. The lavage fluid was recovered and combined. The fluid was centrifuged at 1500 rpm/min for 10 minutes, and then the precipitate was resuspended with PBS (10100147C, Thermo Fisher, Waltham, MA, USA). The cells in the suspension were stained with Wright's stain, and examined under the microscope (N-SIM, Nikon, Tokyo, Japan) for leukocyte counting.

Enzyme-Linked Immunosorbent Assay (ELISA)

An appropriate amount of rat blood was taken, and the serum levels of inflammatory factors in each group of rats were detected by the ELISA method using an enzyme labeling instrument in strict accordance with the instruction manual of the corresponding reagent kits.

Western Blotting (WB)

Lung tissue samples from rats were collected, and protein samples were extracted. The protein concentration was quantified using the BCA protein detection kit (P0012S, Beyotime, Shanghai, China). Subsequently, the proteins were separated by sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis, transferred onto a membrane, and blocked. The membrane was then washed with Tris-Buffered Saline and Tween 20 (TBST) and incubated with primary antibodies overnight at 4 °C. Afterward, the corresponding secondary antibodies were added and incubated at room temperature for 30 minutes. The membrane was washed again with TBST and further incubated at room temperature for 30 minutes. The relative expression level of MMP-9 protein in rat lung tissue was determined using GAPDH as an internal reference. Finally, Enhanced Chemiluminescence (ECL, P0018AS, Be-

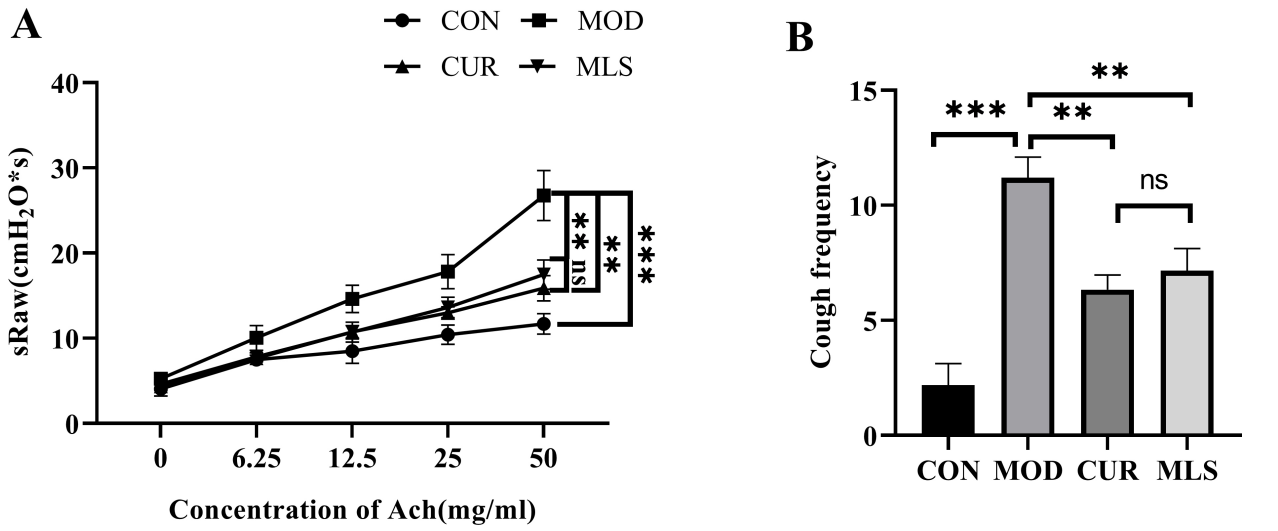

Fig. 2. Effect of curcumin on cough counts and specific airway resistance in CVA rats. (A) Special Airway Resistance (sRaw). (B) Cough count assay. ns $p > 0.05$; **$p < 0.01$; ***$p < 0.001$. n = 8. Ach, acetyl methacholine solution.

yotime, Shanghai, China) chemiluminescence was utilized to develop and analyze the images. Quantification of the intensity of the blot bands by ImageJ software (V1.8.0.112, NIH, Madison, WI, USA).

Detection of Macrophage Polarization

After the experiment, rats underwent a 12-hour fasting and dehydration period. Blood was then extracted from the tail vein, and lymphocyte isolation solution was added to obtain single-nucleated cell suspensions from peripheral blood. These cell suspensions were cultured in a medium for 24 hours. Following culture, individual cells were collected and labeled with M1-type macrophage markers: Anti-Rat CD80 (F3108002, MULTI SCIENCES, Zhejiang, China), anti-C-C-Motif Receptor 2 (CCR2) antibody (ab273050, Abcam, Shanghai, China); and M2-type macrophage markers: anti-CD163 antibody (ab182422, Abcam, Shanghai, China), CD206 Monoclonal Antibody (17-2061-82, Thermo Fisher, Waltham, MA, USA). The cells were then incubated for 30 minutes on ice, protected from light. Flow cytometry was performed to determine the ratio of M1 to M2-type macrophages.

Statistical Analysis

The SPSS 22.0 (IBM, Armonk, NY, USA) was utilized to conduct independent sample *t*-tests or one-way analysis of variance (ANOVA) for the sample data. For datasets showing significant differences, post hoc comparisons were performed using the sequential Bonferroni test. The results are presented as mean $\pm$ standard deviation and statistical significance was defined as $p < 0.05$.

Results

CUR's Effect on the Morphological Traits of the Lung Tissue in CVA Rats

In the MOD group, the bronchial tubes of lung tissue exhibited altered cell arrangement, aberrant proliferation, and marked inflammatory cell infiltration, contrasting sharply with the normal bronchial anatomy observed in the CON group. A comparative analysis between the CUR and MLS groups and the model group revealed a significant attenuation in both inflammatory cell infiltration and bronchial cell disarray. These findings underscore CUR's potential in effectively mitigating lung injury in CVA rats, as depicted in Fig. 1.

CUR's Effect on CVA Rats' Cough Frequency and sRaw in CVA Rats

Rats in the MOD group exhibited substantially greater airway resistance and cough frequency (Fig. 2, $p < 0.05$) than those in the CON group. Rats in the CUR and MLS groups had considerably fewer coughs and lower resistant airways (Fig. 2, $p < 0.05$) than rats in the MOD group. This suggests that CUR can effectively alleviate airway hyperresponsiveness and chronic airway inflammation in CVA rats.

CUR's Effect on MMP-9 Protein in Lung Tissue and Serum Inflammatory Factors of CVA Rats

Rats in the MOD group had considerably higher levels of TNF-α, MMP-9, and IL-5 than those in the CON group. Conversely, significantly lower levels of IL-10 (Fig. 3, $p < 0.05$) were observed. Rats in the CUR and MLS groups exhibited considerably lower levels of IL-5, TNF-α, and MMP-9 protein expression while the levels of IL-10 were significantly higher (Fig. 3, $p < 0.05$) compared to the

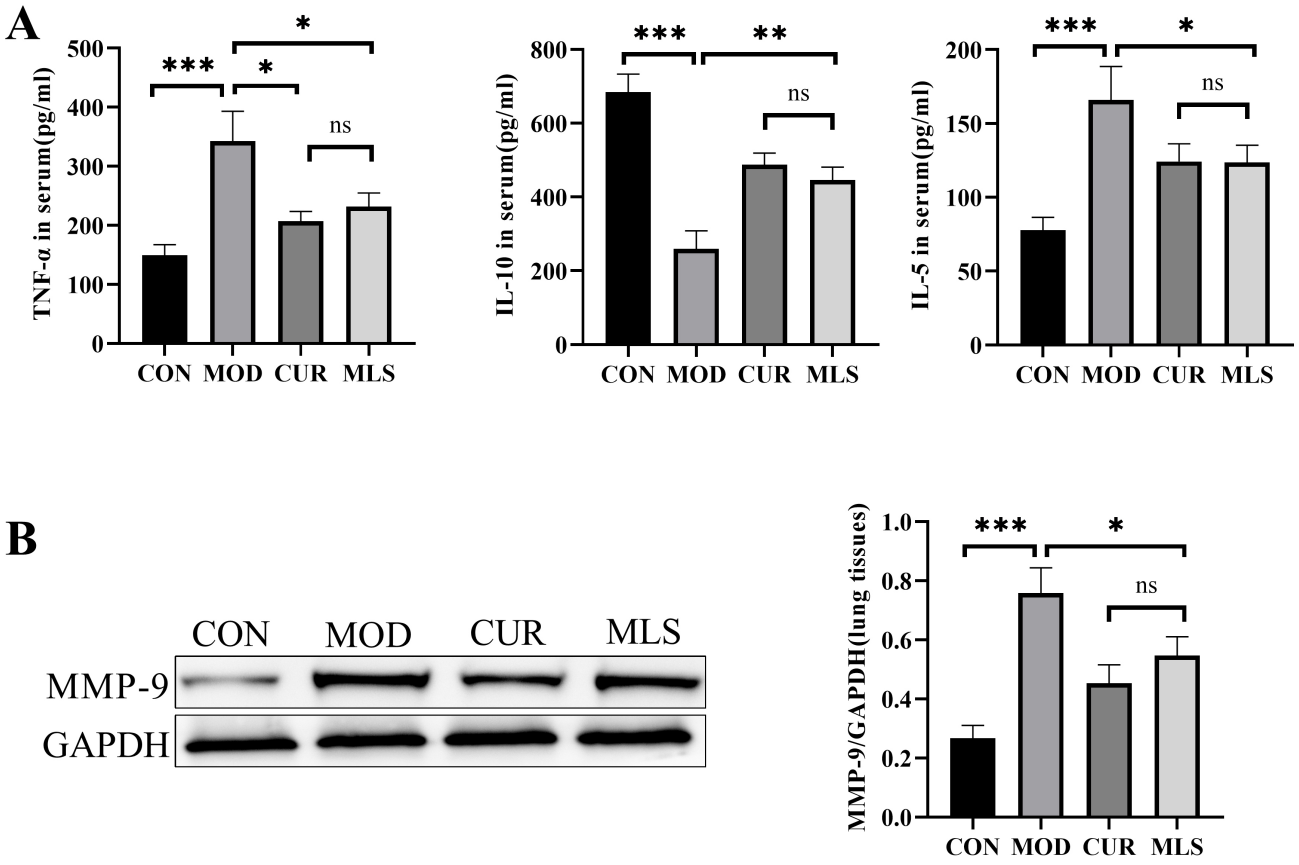

Fig. 3. Effect of curcumin on serum inflammatory factors and MMP-9 protein in lung tissue of CVA rats. (A) Determination of serum inflammatory factors concentrations by Enzyme-Linked Immunosorbent Assay (ELISA). (B) Detection of MMP-9 protein expression by Western blotting (WB) method. ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. CON, control; MOD, model; CUR, curcumin; MLS, montelukast sodium; TNF-α, Tumor Necrosis Factor-alpha; IL-10, Interleukin-10; MMP-9, Matrix Metalloproteinase-9; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. n = 8.

MOD group. This shows that CUR can both limit the production of pro-inflammatory factors and efficiently boost the release of anti-inflammatory factors.

Impact of CUR on the Quantity of Inflammatory Cells in CVA Rats' Alveolar Lavage Fluid

Rat alveolar lavage fluid from the MOD group contained considerably more leukocytes (Fig. 4, $p < 0.05$) than that of the CON group. The CUR and MLS groups of rats had significantly (Fig. 4, $p < 0.05$) fewer eosinophils and leukocytes than the MOD group. This suggests that CUR can effectively reduce the inflammatory response in CVA rats.

CUR's Effect on Macrophage Polarization in CVA Rats

Flow cytometry was employed to evaluate the effect of CUR on macrophage polarization by detecting the M1-type polarization marker CD80/CCR2 and the M2-type polarization marker CD163/CD206. The results revealed a significant increase in the CD80/CCR2 (Fig. 5A, $p < 0.05$), but not CD163/CD206 cell populations in the MOD group compared to the control group (Fig. 5B, $p > 0.05$). Conversely, after CUR and MLS administration, a significant decrease in the CD80/CCR2 cell population and a significant increase in the CD163/CD206 cell population were observed compared to the MOD group (Fig. 5, $p < 0.05$). These observations suggest that CUR effectively adjusts the M1/M2 macrophage balance in CVA rats, fostering anti-inflammatory M2 polarization and curtailing the shift towards pro-inflammatory M1 polarization.

Discussion

CVA is an atypical type of asthma with diastolic reactivity and airway hyperresponsive cough as the main or only clinical symptom. The recurrent episodes of CVA may cause further impairment of lung function and small airway function, resulting in classic asthma [14]. While the exact pathogenesis of CVA remains incompletely understood, it is widely believed to be closely associated with airway inflammation, remodeling, hyperresponsiveness, and epigenetic factors, with eosinophil infiltration being a prominent pathological feature of chronic airway inflammation

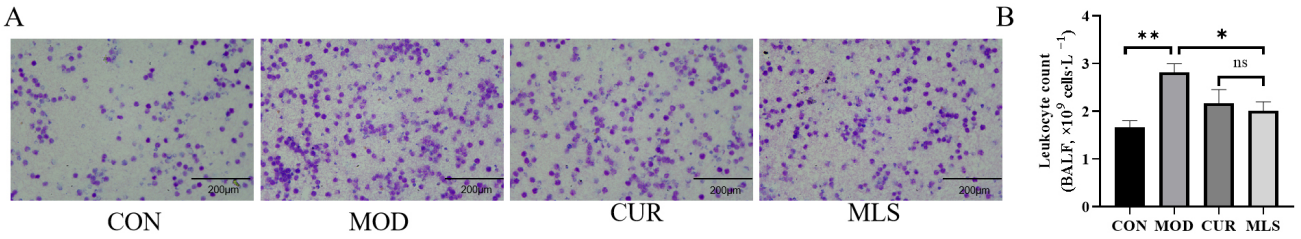

Fig. 4. Effect of curcumin on inflammatory cell counts in alveolar lavage fluid of CVA rats. (A) Rat alveolar lavage fluid stained by Rachel's stain (purplish red: nuclei of visual normal cells; light blue/no staining: nuclei of visual leukocytes). (B) Number of leukocytes. ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$. n = 8. Scale bar = 200 μm.

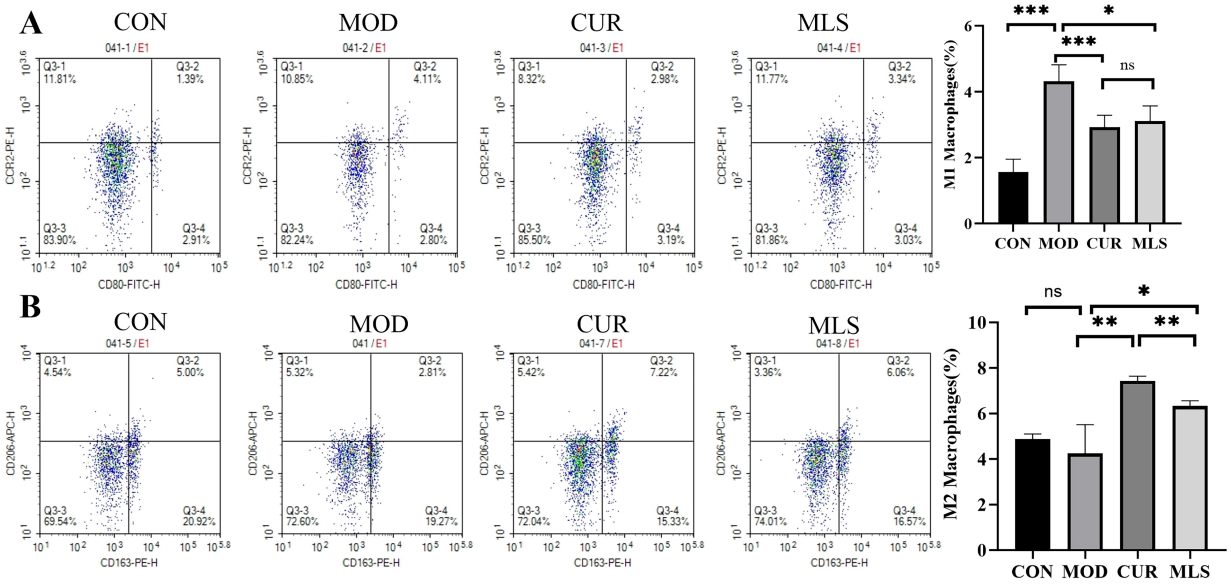

Fig. 5. Effect of curcumin on macrophage polarization in CVA rats. (A) Detection of M1-type macrophage markers by flow cytometry. (B) Detection of M2-type macrophage markers by flow cytometry. ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n = 8.

[15]. The current first-line drugs for the clinical treatment of CVA suffer from drawbacks such as significant adverse effects, poor patient tolerance, and high relapse rates upon discontinuation, thus limiting their clinical utility. CUR, the primary active compound in turmeric, possesses diverse pharmacological activities. Studies have indicated its potential to ameliorate inflammatory diseases by modulating macrophage polarization and function within the context of inflammation-related conditions [16,17]. Building upon this knowledge, the present study was undertaken to investigate the potential therapeutic effects of CUR in a rat model, with MLS serving as a positive control.

Asthma often causes lung damage, and curcumin has been shown to be effective in protecting against lung damage. Chunmei Wang *et al.* [18] showed that curcumin effectively protected against lipopolysaccharide-induced acute lung injury. In this experiment, the pathological results showed that CUR administration significantly ameliorated the abnormal proliferation of lung tissue structures and inflammatory cell infiltration in CVA rats. This suggests that CUR has a significant protective effect on lung injury in CVA rats and can reduce lung lesions. Acetylcholine visualizes airway hyperresponsiveness, and elevated airway hyperresponsiveness plays a significant role in the development of classic asthma from CVA, which may raise the likelihood of conversion [19]. Cough hypersensitivity is associated with factors such as airway inflammation, which is one of the pathogenic mechanisms and an important feature of asthma [20]. This study demonstrated that the treatment of CUR considerably decreased airway resistance and airway sensitivity in CVA rats. The above results suggest that CUR can effectively alleviate airway hyperresponsiveness and chronic airway inflammation and reduce inflammatory infiltration, protecting lung tissue.

CVA is considered a chronic nonspecific inflammatory disease of the airways stemming from immunodeficiency. Chronic airway inflammation represents the principal characteristic of CVA, involving various inflamma-

tory cells such as inflammatory mediators like cytokines that collectively contribute to airway inflammation [21]. IL-10, IL-5, and TNF-α are pivotal factors in promoting airway inflammation and are instrumental in asthma development. Additionally, matrix metalloproteinase MMP-9 is closely linked to airway inflammation, as it fosters the activation of inflammatory factors, thereby intensifying the inflammatory response [22–24]. Our findings indicate that CUR administration significantly reduces the levels of pro-inflammatory factors while concurrently elevating the levels of the anti-inflammatory factor. Moreover, CUR administration leads to a significant reduction in eosinophil and leukocyte counts. These outcomes suggest that CUR mitigates the infiltration of inflammatory cells by modulating the balance of inflammatory factors, thereby effectively mitigating the airway inflammatory response in CVA rats.

In recent years, the theory of dysregulation of macrophage polarization balance has introduced new perspectives for exploring the pathogenesis of airway inflammation in asthma. Macrophages, a group of immune cells with high plasticity and diversity, are classified into two main phenotypes: M1-type (classically activated) and M2-type (alternatively activated). M1 macrophages primarily secrete pro-inflammatory mediators like TNF-α, contributing to bacteriostasis, host defense, and anti-tumor responses, while M2 macrophages secrete IL-10 and other anti-inflammatory factors associated with tissue remodeling and suppressing inflammation [25]. The immune mechanisms involving macrophages may hinge on the balance between M1 and M2 macrophage types, and dysregulation of this balance can impact the development of inflammatory diseases, including CVA [26]. Studies have reported the overlapping mediators between M2 polarization and LPS tolerance including IL-10, A20, IRG1, miR-221, and MerTK [27]. Macrophages play an important role in the proinflammatory response, resolution of inflammation, and tissue healing [28]. A study by Feng CN *et al*. [29] found that the inflammatory factor IL-24 is highly expressed in allergic asthma mice and exacerbates asthma disease progression through macrophage polarization. Our findings demonstrate that CUR administration significantly increases M2-type macrophage levels and decreases M1-type macrophage levels, suggesting that CUR alleviates airway inflammation in CVA rats by modulating macrophage polarization toward the anti-inflammatory M2 phenotype.

Conclusions

In conclusion, CUR has been demonstrated to be effective in mitigating OVA-induced airway inflammation in CVA rats by modulating macrophage polarization towards the anti-inflammatory M2 phenotype, leading to a consequent reduction in airway inflammation, hyperresponsiveness, and lung tissue injury. Although the study successfully observes curcumin's capacity to modulate the M1/M2 macrophage ratio, it does not thoroughly investigate the underlying molecular mechanisms. Future research should, therefore, focus on delving into the specific signaling pathways through which curcumin regulates macrophage polarization, with particular emphasis on pathways such as nuclear factor kappa-B (NF-κB), Janus kinase/signal transducer and activator of transcriptions (JAK/STAT), or mitogen-activated protein kinases (MAPK) signaling, which are pivotal in the inflammatory response.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author.

Author Contributions

HY, JX, and MQW designed the research study. JX, MQW, XT, and HW performed the research. HY, XT, and KR provided help and advice on the experiments. HW and KR wrote the first draft and analyzed the data. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee of The First Hospital of Hunan University of Chinese Medicine (CM743900).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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