Original Article

Therapeutic potency and possible mechanism of Wuhu decoction underlying asthmatic progression via Th1/Th2 imbalance

Yan Hu[1]^, Mengqing Wang[1], Yinhe Luo[2], Jing Xie[1], Luojia Jiao[3], Wei Tang[4], Yijue Deng[1], Bing Yao[1]

[1]Department of Pediatrics, The First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, China; [2]Teaching and Research Office of Chinese and Western Combination, Hunan University of Chinese Medicine, Changsha, China; [3]Department of Organizational Personnel Department, Hunan University of Chinese Medicine, Changsha, China; [4]Department of Acupuncture and Massage, The First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha, China

Contributions: (I) Conception and design: Y Hu, L Jiao; (II) Administrative support: Y Hu, L Jiao; (III) Provision of study materials or patients: Y Hu; (IV) Collection and assembly of data: Y Hu, M Wang, Y Luo, J Xie; (V) Data analysis and interpretation: L Jiao, W Tang, Y Deng, B Yao; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yan Hu, MM. Department of Pediatrics, The First Affiliated Hospital of Hunan University of Chinese Medicine, No. 95, Shaoshan Middle Road, Yuhua District, Changsha 410007, China. Email: aprah@hnucm.edu.cn; Luojia Jiao, MM. Department of Organizational Personnel, Hunan University of Chinese Medicine, No. 300, Bachelor Road, Yuelu District, Changsha 410208, China. Email: jiaoluojia92@126.com.

Background: Cough variant asthma (CVA) is a disease with no definitive diagnosis or pathogenic causes, and still lacks effective and safe treatment. Wuhu decoction is a traditional Chinese medicine with potential effects against CVA, of which underlying mechanism remains elusive. The aim of this study is to explore the therapeutic potential of Wuhu decoction against CVA.

Methods: The CVA mice model was established by ovalbumin (OVA) treatment. The airway hyperresponsiveness and remodeling were assessed. The pulmonary inflammatory injury was determined by counting of inflammatory cells and serum OVA-specific immunoglobulin E (IgE) in bronchoalveolar lavage fluid, as well as the amount of $CD4^+$, $CD8^+$ or $CD25^+$ T cells. The Th1/Th2 balance was evaluated by type specific cytokines. The level of antisense long non-coding RNA TRPM2 (lncTRPM2-AS) and its downstream targets were determined by mRNA and protein detection, respectively.

Results: Wuhu decoction could improve airway hyperresponsiveness and remodeling in OVA-induced asthmatic mice model by regulating Th1/Th2 balance and affecting pulmonary inflammatory injury. On the molecular level, Wuhu decoction significantly inhibited the expression of lncTRPM2-AS, which was found to be a critical modulator of Th1/Th2 balance. Meanwhile, overexpression of lncTRPM2-AS could abolish Wuhu decoction-mediated protection effects on OVA-induced asthmatic mice model.

Conclusions: This study discovered the protective function of Wuhu decoction against CVA and illustrated its molecular mechanism, highlighting the therapeutic application of Wuhu decoction for asthma treatment.

Keywords: Wuhu decoction; cough variant asthma (CVA); antisense long non-coding RNA TRPM2 (lncTRPM2-AS); Th1/Th2 balance

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^ ORCID: 0009-0000-7862-0021.

Introduction

Normally, asthma is a kind of disease with symptoms such as wheeze and cough. Cough variant asthma (CVA) is an atypical asthma that cough is the only or main symptom (1). It is prone to clinical missed diagnosis for patients with CVA since they have no wheezing, chest tightness or dyspnea (2). Nearly half of CVA patients will develop typical asthma if the symptoms are unable to be managed effectively (3). Chronic inflammation caused by asthma is a critical cause of airway remodeling, leading to incompletely reversible airway narrowing, bronchial hyperresponsiveness, airway edema and mucus hypersecretion (4,5). However, the available therapeutic treatments are limited. Although there are certain effects with the treatments such as antibiotics, glucocorticoids, and expectorants, patients suffer from side effects and symptoms that coincide with the recurrence of illness (6). Studies has shown that traditional Chinese medicine (TCM) has significant efficacy for CVA with decreased toxic and side effects (7,8). Therefore, it is highly desired to explore the therapy and mechanism with TCM for CVA treatments.

Helper T (Th) cells have been shown to play a critical role in the progress of asthma via immune dysfunction (9). Th cells are divided into four subtypes: Th1, Th2, Th17 cells and regulatory T cells (Tregs) (10). The most frequent abnormality in asthma is airway inflammatory response without specific cause (11), characterized by morphological changes in lung due to the secretion of inflammatory mediators and cytokine responses (11). It has been found that asthma was associated to disorder in differentiation and function of Th1 and Th2 cells, enhanced inflammatory cytokines and abnormal of immunomodulatory mechanism (12,13). Th1 cells mostly secrete interleukin (IL)-2, IL-12, and interferon γ (IFN-γ) which regulate cellular immunity, while Th2 cells secrete IL-4, IL-5, and IL-13 that regulate humoral immunity (14). These modulators regulate the level of immunoglobulin E (IgE) and immunoreaction (15). Restoring Th1/Th2 cytokines balance was a possible treatment for asthma (16). Intriguingly, cytokines produced by Th2 cells inhibits the production of cytokines by Th1 and natural killer cells. Meanwhile, Th1 cells can block the differentiation and proliferation of mastocytes, basophils, and eosinophils (EOSs), of which the activities are controlled by the synthesis of cytokines by Th2 cells (17). Moreover, the transcription factor Gata-binding protein 3 (GATA3) is shown to be inhibited by a Th1-specific transcription factor T-bet (18,19). In recent study, Th17 cells and Tregs are found to have a critical role in asthma (20). A study has shown that Tregs is important to the tolerance to asthma via suppressing the inflammatory response of Th2 cells (21). Th17 cells are characterized by the secretion of IL-17, which has a strong correlation with eosinophilic airway inflammation in asthma and could mediate airway neutrophilic inflammation that is related to the severity of asthma (21). A previous work shown that Th17 cells suppress Treg-mediated tolerance, and modulation of the Th17/Treg axis could improve airway inflammation (22).

The traditional ancient Wuhu decoction has been widely used for the treatment of different diseases, including *Mycoplasma pneumoniae* pneumonia and asthma (23,24). One recent pharmacological study has demonstrated that Wuhu decoction has anti-inflammatory, immunomodulatory, and other effects (24). As the medicine recommended by the "Expert Consensus on Diagnosis and Treatment of Integrated Traditional Chinese and Western Medicine of *Mycoplasma* pneumonia in Children (formulated in 2017)", Wuhu decoction has been utilized to treat *Mycoplasma* pneumonia in Chinese children, demonstrating enhancement in efficacy and reduction in adverse reactions. Different administration methods of Wuhu decoction have been used in clinical practice, such as 300 mL/dose with 3 doses/day and 300 mL/dose with 1 dose/day (25). Adverse events have been reviewed for the combination treatment of Wuhu decoction and azithromycin, including abdominal pain, diarrhea, nausea, vomiting, allergy and rash (24). Wuhu decoction is shown to significantly protect against the development of bronchiolitis in typical asthma (26). In particular, previous reports have demonstrated that Wuhu decoction could inhibit the level of IL-13 and the deposition

Highlight box

Key findings

- Wuhu decoction could regulate Th1/Th2 balance.
- Wuhu decoction significantly inhibited the expression of antisense long non-coding RNA TRPM2 (lncTRPM2-AS).
- Wuhu decoction could improve airway hyperresponsiveness and remodeling in cough variant asthma (CVA).

What is known and what is new?

- Wuhu decoction has been widely used for the treatment of different diseases, including *Mycoplasma pneumoniae* pneumonia and asthma.
- Wuhu decoction could protect against CVA via regulating lncTRPM2-AS.

What is the implication, and what should change now?

- Wuhu decoction has therapeutic effect for asthma treatment.

of collagen fibers in lung tissue, leading to the improvement of airway remodeling in asthma and alleviation of asthma symptoms (23,27). Moreover, Wuhu decoction has been identified to improve the respiratory syncytial virus (RSV)-induced asthmatic symptoms by stimulating autophagy process of dendritic cells (DCs) in lung tissues. The However, the specific therapeutic effect and mechanism of Wuhu decoction on CVA are still unclear.

In this work, we demonstrated that Wuhu decoction could improve airway hyperresponsiveness and remodeling in ovalbumin (OVA)-induced asthmatic mice model by regulating Th1/Th2 balance and affecting pulmonary inflammatory injury. On the molecular level, Wuhu decoction significantly inhibited the expression of long non-coding RNA TRPM2 (lncTRPM2-AS), which was found to be a critical modulator of Th1/Th2 balance. Meanwhile, overexpression of lncTRPM2-AS could abolish Wuhu decoction-mediated protection effects on OVA-induced asthmatic mice model. Taken together, our study discovered the protective function of Wuhu decoction against CVA and illustrated its molecular mechanism, highlighting the therapeutic application of Wuhu decoction for asthma treatment. We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-804/rc).

Methods

OVA-induced asthmatic mice model

All animal experiments were conducted in accordance with the institutional guidelines of The First Affiliated Hospital of Hunan University of Chinese Medicine and the protocol was approved by the Ethics Committee of The First Affiliated Hospital of Hunan University of Chinese Medicine (No. LL2020072202). A small sample size (6 mice per group) was selected to provide basic evidence regarding the function and mechanism of Wuhu decoction *in vivo*. No criteria were set for including and excluding animals during the study. Animals were randomly divided into different groups using a computer based random order generator and confounders were not controlled. Investigators involved in data analysis were blind to experiments conduction. All mice were maintained on a regular diurnal lighting cycle (12:12 light: dark) with *ad libitum* access to food and water. Male Balb/c mice of 7–8 weeks old (Vital River, Beijing, China) were used for asthma mouse model established by OVA sensitization (Sigma Chemical, St. Louis, USA). Briefly, mice were intraperitoneally injected with 20 μg OVA and 100 μg aluminum hydroxide gel in 200 μL phosphate buffer saline (PBS) on the 1st and 2nd day. Then mice received atomization inhalation with OVA for 30 minutes from the 9th to 21st day. Wuhu decoction, dexamethasone (Dex) or normal saline was gastric administrated daily for the following 7 days before mice were sacrificed for further experiments. To overexpress lncTRPM2-AS, asthmatic mice were treated with nasal inhalation of its mimic plasma or negative control (4 mg/kg, purchased from Shanghai GenePharma, Shanghai, China) from the 9th to 21st day. No humane endpoints were included in the study.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were dissolved in TRIzol reagent (Invitrogen, Waltham, USA) for RNA extraction according to the manufacturer's protocol. After qualification, RNA was synthesized into complementary DNA (cDNA) by an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). qRT-PCR was performed using SYBR Green Supermixes (Bio-Rad, Hercules, USA) with GAPDH and U6 as the endogenous controls for normalization. Relative levels of RNA expression were normalized and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

After washed with cold PBS, cells were incubated with lysis buffer on ice for 30 min. The supernatant was collected and stored at −80 ℃ after centrifugation. A BCA assay kit (Beyotime Biotechnology, Shanghai, China) was used to determine protein concentrations. Protein samples were boiled and separated by 10% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), and transferring to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking with non-fat milk for 1 h, membranes were incubated overnight at 4 ℃ with the indicated primary antibodies from Cell Signaling Technology (Danvers, USA) at a 1:1,000 dilution, including IL-4 (#12227), IL-10 (#12163), GATA3 (#5852), IFN-γ (#8455), IL-2 (#12239), T-bet (#13232) and β-Actin (#3700). After washed, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:5,000 dilution (#7074 or #7076, Cell Signaling Technology) for 2 h. The signals were developed with an electrochemical luminescence detection kit (Cat. 32106, Pierce Biotechnology, Waltham, USA) and analyzed by ImageJ software.

Assessment of airway hyperresponsiveness

Airway mechanics was measured using forced oscillation technique by flexiVent, a small animal ventilator (SCIREQ®, Quebec, Canada). Parameters of airway hyperresponsiveness such as airway resistance (Rrs), elastance (Ers), Newtonian resistance (R_N), tissue elastance (H) and tissue damping (G) were assessed by a methacholine (MCh) (Sigma-Aldrich, St. Louis, MO, USA) challenge test with increasing doses of MCh (0–100 mg/mL).

Cell counting and cytokines detection in bronchoalveolar lavage fluid (BALF)

The mice were sacrificed and fixed after anesthesia. Sterile PBS was injected into the lung to collect the recovered fluid. After centrifuged at 500 ×g for 10 min at 4 ℃. The supernatant was collected and stored at −80 ℃ for subsequent experiments. Subgroups of blood cells were counted by Wright's staining (Beijing Solarbio Technology Co., Ltd., Beijing, China) after lysis of red blood cells. The quantification of $CD4^+$, $CD8^+$ or $CD25^+$ T cells were performed by flow cytometry. The testing of the concentration of soluble IL-4, IL-5, IL-2 or IFN-γ was conducted with a sandwich enzyme-linked immunosorbent assay (ELISA) kits (ThermoFisher, Waltham, USA) according to the manufacturer's instructions.

Immunohistochemistry

After washed, the lung tissues were fixed and embedded in paraffin, followed with sectioning for hematoxylin-eosin (H&E) staining, periodic acid-Schiff (PAS) staining and Masson staining (Beijing Solarbio Technology Co., Ltd.). Immunofluorescence staining was used to investigate the pathological changes of lung. For sections stained with H&E, the degree of inflammatory cells infiltration was evaluated via the following scoring system based on how many inflammatory cells around the airway: 0, no inflammatory cells; 1, a few inflammatory cells; 2, a ring of inflammatory cells (1–2 cell-layers); 3, a ring of inflammatory cells (3–5 cell-layers); 4, a ring of inflammatory cells (>5 cell-layers).

Separation of $CD4^+$ T cells

Peripheral blood was collected from mice or healthy volunteers for the isolation of peripheral blood mononuclear cells (PBMCs) using lymphocyte separation medium (Sangon, Shanghai, China). After washed by PBS, the $CD4^+$ T cells were separated through a magnetic activated cell sorting (MACS) system (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, cells were mixed with solution [2 mmol/L ethylenediaminetetraacetic acid (EDTA), 20 mL/L fetal bovine serum (FBS) in PBS] and added with CD4 microbeads. After incubation for 20 min, the mixture was centrifugated at 1,000 rpm for 10 min. After the resultant supernatants were removed, cell sediments were resuspended in 2 mL of solution and transferred into LS column. After washed and centrifugated, the cells collected were $CD4^+$ T cells.

Fluorescence in situ hybridization (FISH)

FISH assay was conducted following the protocol of ViewRNA Tissue Assay Kit (#QVT0700, ThermoFisher, Waltham, USA) with probe against lncTRPM2-AS being used. Fixed and pretreated tissue slides were hybridized in probe solution containing probe for 2 h, followed by two wash steps with wash buffer. For signal amplification, the PreAmplifier Mix solution was added and incubated for 1 h, followed by two wash steps using wash buffer and incubation for 1h with PreAmplifier Mix solution. After two wash steps with wash buffer, Label Probe Mix was added and incubated for 1 h. Slides were washed twice with wash buffer followed by incubation with Hoechst solution (#P0133, Beyotime, Shanghai, China) in PBS for 30 min at room temperature. After washing with PBS, images were taken via confocal imaging system and quantified by ImageJ software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t*-test. All *in vitro* experiments were performed at least three times. No exclusions occurred. Data were presented as the mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.

Results

Wuhu decoction improved airway hyperresponsiveness and remodeling in OVA-induced asthmatic mice model

To evaluate the effect of Wuhu decoction on asthma, OVA-

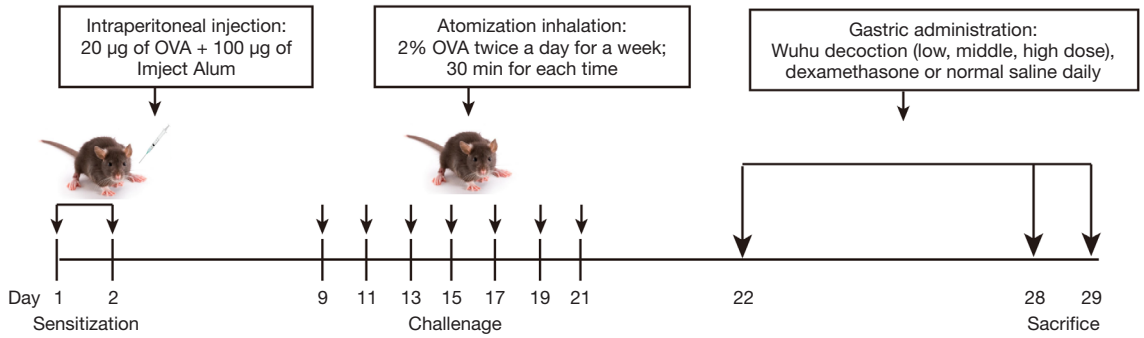

Figure 1 Flow chart for establishment of the modeling process of asthmatic mice exposing with OVA. OVA, ovalbumin.

induced asthmatic mice model was established as shown in *Figure 1*. Wuhu decoction (0.2 mL) at 5 mL/kg (Wuhu-L), 10 mL/kg (Wuhu-M) and 50 mL/kg (Wuhu-H) were administrated respectively, and Dex was used as a positive control for the treatment of asthma. The dose range of Wuhu decoction was selected based on a previous study (26), which is also under the range of clinical practice based on body surface area conversion between humans and mice. The hyperresponsiveness was assessed by testing the total respiratory system Rrs, Ers, R_N, G and H after treated with different concentration of MCh. As shown in *Figure 2A-2E*, OVA treatment significantly induced hyperresponsiveness, which was rescued by the combined treatment of Wuhu decoction at middle or high dose or Dex. Moreover, airway mucus secretion and collagen deposition after treatments were determined by PAS staining and Masson staining. As shown in *Figure 2F-2I*, OVA treatment dramatically increased the positive area of PAS staining and Masson staining, which was not affected by the low dose treatment of Wuhu decoction but significantly reduced in the group of Wuhu-M, Wuhu-H and Dex. Taken together, these results indicated that Wuhu decoction, especially at high dose, could improve airway hyperresponsiveness and remodeling in OVA-induced asthmatic mice model.

Wuhu decoction alleviated pulmonary inflammatory injury in OVA-induced asthmatic mice model

Regarding the critical role of inflammation in the progress of asthma, we further evaluated the effect of Wuhu decoction on pulmonary inflammatory injury. The number of inflammatory cells in BALF of mice were determined. As shown in *Figure 3A*, the total number of inflammatory cells in the OVA-induced asthma mice, including lymphocytes, EOSs and neutrophils, was significantly enhanced compared to control group. Intriguingly, when Wuhu decoction (middle or high dose) or Dex was added, the number of these inflammatory cells was obviously rescued in comparison with OVA treatment alone. Meanwhile, since Th2 cytokines could regulate IgE production, the level of OVA-specific IgE in serum was also tested. As shown in *Figure 3B*, OVA treatment strongly induced the production of IgE in serum. While after the treatment of Wuhu decoction or Dex, the level of IgE got significantly decreased. Then the pathological changes of lung tissues in mice were observed. The results of H&E staining indicated that the bronchial and lung tissue of mice in control group were of complete and normal structure, without infiltration of inflammatory cells (*Figure 3C*). On the contrary, lung tissues from OVA-treated mice showed increased mucus in the airway, irregular cilia arrangement, a large number of inflammatory cells around the airway, and decreased number of alveoli (*Figure 3C*). All these pathological alters were significantly improved by the treatment of Wuhu decoction (middle or high dose treatment) or Dex (*Figure 3C*). The statistical analysis identified that the airway inflammation score of asthma mice was dramatically increased compared to the control group. However, the scores of Wuhu decoction (middle or high dose) or Dex group were significantly lower than that of asthma group (*Figure 3D*). Moreover, compared with the control group, the number of $CD8^+$ T cells increased significantly, the ratio of $CD4^+/CD8^+$ T cells decreased, and the number of $CD4^+$ T cells and $CD4^+CD25^+$ T cells decreased dramatically (*Figure 3E,3F*). All of these changes were significantly rescued by the treatment of Wuhu decoction (middle or high dose) or Dex compared to the asthma group (*Figure 3E,3F*). These data strongly suggested that Wuhu decoction improved pulmonary inflammatory injury in asthma mice.

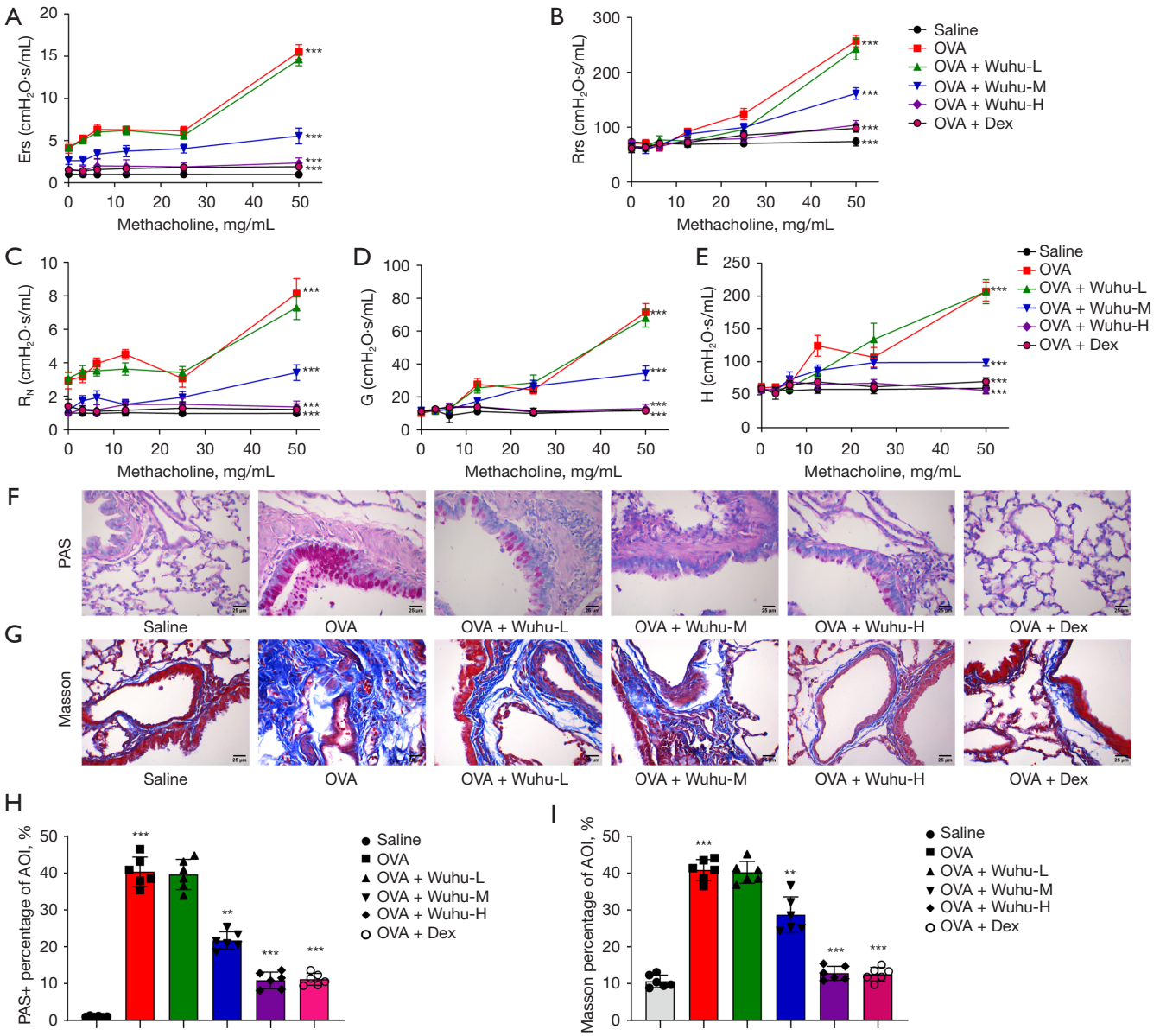

Figure 2 Assessment of Wuhu decoction on airway hyperresponsiveness and remodeling of OVA-induced asthmatic mice model. (A-E) Assessment of airway hyperresponsiveness of OVA-induced asthmatic mice after indicated treatments. Representative images (F) and statistic results (H) of PAS staining of lung tissues after indicated treatments. Magnification, 400×; scale bar, 25 μm. Representative images (G) and statistic results (I) of Masson staining of lung tissues after indicated treatments. Magnification, 400×; scale bar, 25 μm. N=6. **, P<0.01; ***, P<0.001. Ers, elastance; Rrs, respiratory resistance; R_N, Newtonian resistance; G, tissue damping; H, tissue stiffness; OVA, ovalbumin; Wuhu-L, Wuhu decoction at 5 mL/kg; Wuhu-M, Wuhu decoction at 10 mL/kg; Wuhu-H, Wuhu decoction at 50 mL/kg; Dex, dexamethasone; PAS, periodic acid-Schiff; AOI, average optical intensity.

Evaluation of Wuhu decoction on Th1 and Th2-specific cytokines levels in BALF of asthma mice

Since the Th1/Th2 imbalance was critical for the development of asthma, their specific cytokine levels in BALF of mice were also determined. As shown in *Figure 4A*, the Th2 cytokines IL-4 and IL-5 were significantly increased by OVA treatment compared to control group, which were then decreased to the normal levels after the

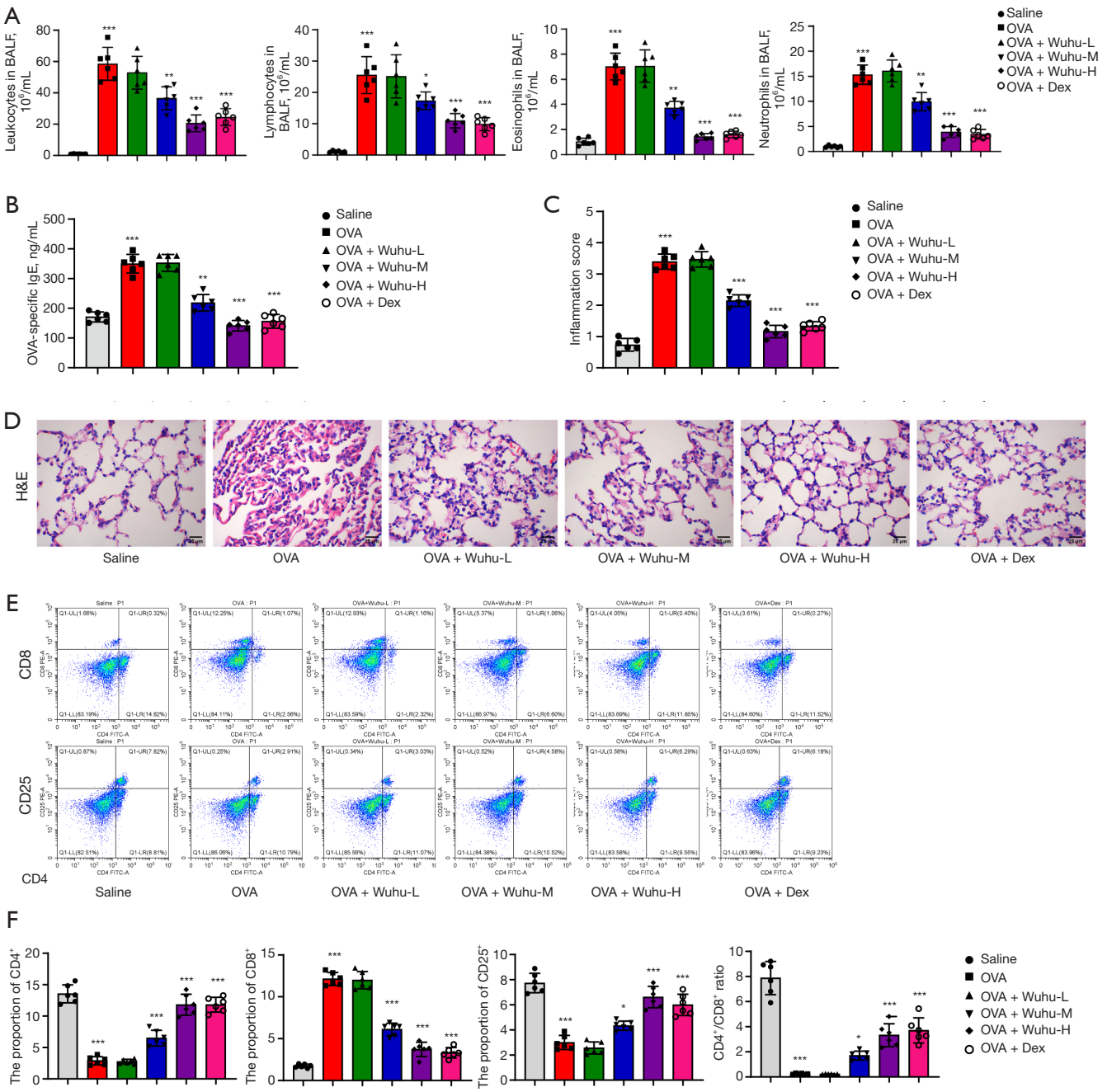

Figure 3 Assessment of Wuhu decoction on pulmonary inflammatory injury of OVA-induced asthmatic mice model. (A) Count of inflammatory cells in BALF from OVA-induced mice after indicated treatments. (B) Detection of serum OVA-specific IgE in BALF after indicated treatments. (C,D) Representative images of H&E staining (D) for lung tissues from mice after indicated treatments and inflammation score (C) based on H&E staining of lung tissues for mice after indicated treatments. Magnification, 400×; scale bar, 25 μm. Flow cytometry (E) and statistic results (F) of $CD4^+$, $CD8^+$ or $CD25^+$ T cells in the blood of mice after indicated treatments. N=6. *, P<0.05; **, P<0.01; ***, P<0.001. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; IgE, immunoglobulin E; Wuhu-L, Wuhu decoction at 5 mL/kg; Wuhu-M, Wuhu decoction at 10 mL/kg; Wuhu-H, Wuhu decoction at 50 mL/kg; Dex, dexamethasone; H&E, hematoxylin-eosin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

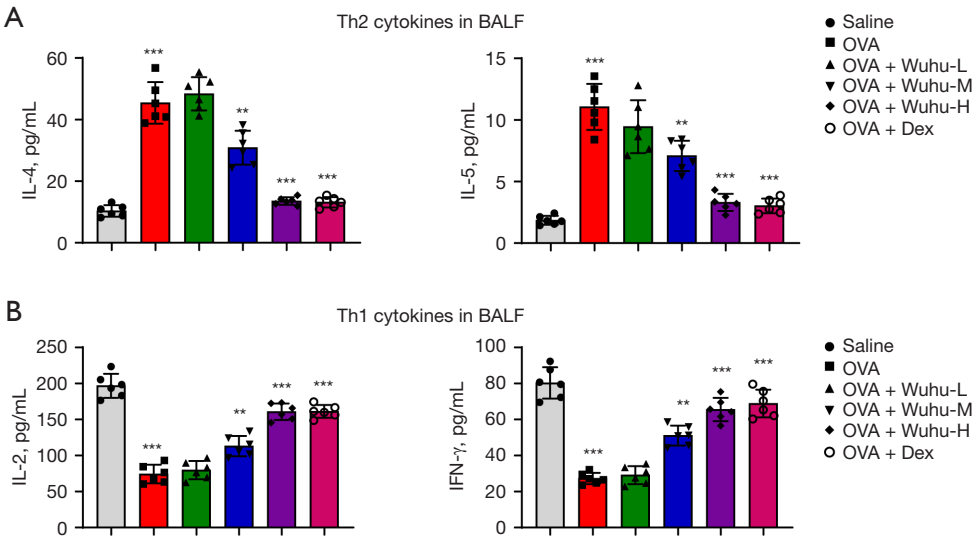

Figure 4 Evaluation of Wuhu decoction on Th1 and Th2-type cytokines in BALF of OVA-induced asthmatic mice model. (A) Detection of Th2-type cytokines in BALF of each group. (B) Detection of Th1-type cytokines in BALF of each group. N=6, **, P<0.01; ***, P<0.001. BALF, bronchoalveolar lavage fluid; IL, interleukin; OVA, ovalbumin; Wuhu-L, Wuhu decoction at 5 mL/kg; Wuhu-M, Wuhu decoction at 10 mL/kg; Wuhu-H, Wuhu decoction at 50 mL/kg; Dex, dexamethasone.

treatment of Wuhu decoction (middle or high dose) or Dex. On the other hand, the Th1 cytokines IL-2 and IFN-γ levels were dramatically inhibited in BALF of asthma mice, which were rescued by Wuhu decoction (middle or high dose) or Dex (*Figure 4B*). Above all, these results demonstrated that Wuhu decoction could improve the asthma development by rescuing the Th1/Th2 imbalance.

Wuhu decoction regulated Th1/Th2 imbalance via mediating lncTRPM2-AS

To investigate the molecular mechanism of Wuhu decoction on OVA-induced asthma, we then focused on the long non-coding RNAs (lncRNAs) that have been reported to play a role in the development of asthma. $CD4^+$ T cells were isolated from OVA-induced asthmatic mice with or without high dose Wuhu decoction treatment. The expression levels of 9 lncRNAs were measured and two of them were found to be significantly downregulated by Wuhu decoction, especially lncTRPM2-AS (*Figure 5A*). Meanwhile, when compared with control group, lncTRPM2-AS was dramatically upregulated in $CD4^+$ T cells from OVA-treated mice (*Figure 5B*). To further explore the function of lncTRPM2-AS, we isolated $CD4^+$ T cells from the PBMC of healthy donors and manipulated its expression by overexpression or knockdown, as shown in *Figure 5C*. Intriguingly, the expression of Th2 cytokines IL-4 and IL-10 and transcription factor GATA3 in both mRNA and protein levels were shown to be positively regulated by lncTRPM2-AS (*Figure 5D,5E*). However, the Th1 cytokines IFN-γ and IL-2 and transcription factor T-bet were found to be negatively mediated by lncTRPM2-AS (*Figure 5F,5G*). These results illustrated that Wuhu decoction was able to inhibit the expression of lncTRPM2-AS, which could stimulate the Th2 differentiation of $CD4^+$ T cells.

Reinforced lncTRPM2-AS abolished Wuhu decoction-mediated protection effects on OVA-induced asthmatic mice model

To demonstrate the relation of Wuhu decoction and lncTRPM2-AS *in vivo*, the negative control or lncTRPM2-AS mimics was nasally administrated in OVA-treated mice. The pathological changes of lung tissues in mice were observed by H&E staining, PAS staining and Masson staining. As shown in *Figure 6A,6B*, overexpression of lncTRPM2-AS significantly rescued the therapeutic effects of Wuhu decoction on OVA-induced asthma of mice. Meanwhile, the OVA-induced serum IgE and Th2 cytokines were inhibited by Wuhu decoction and then restored by upregulation

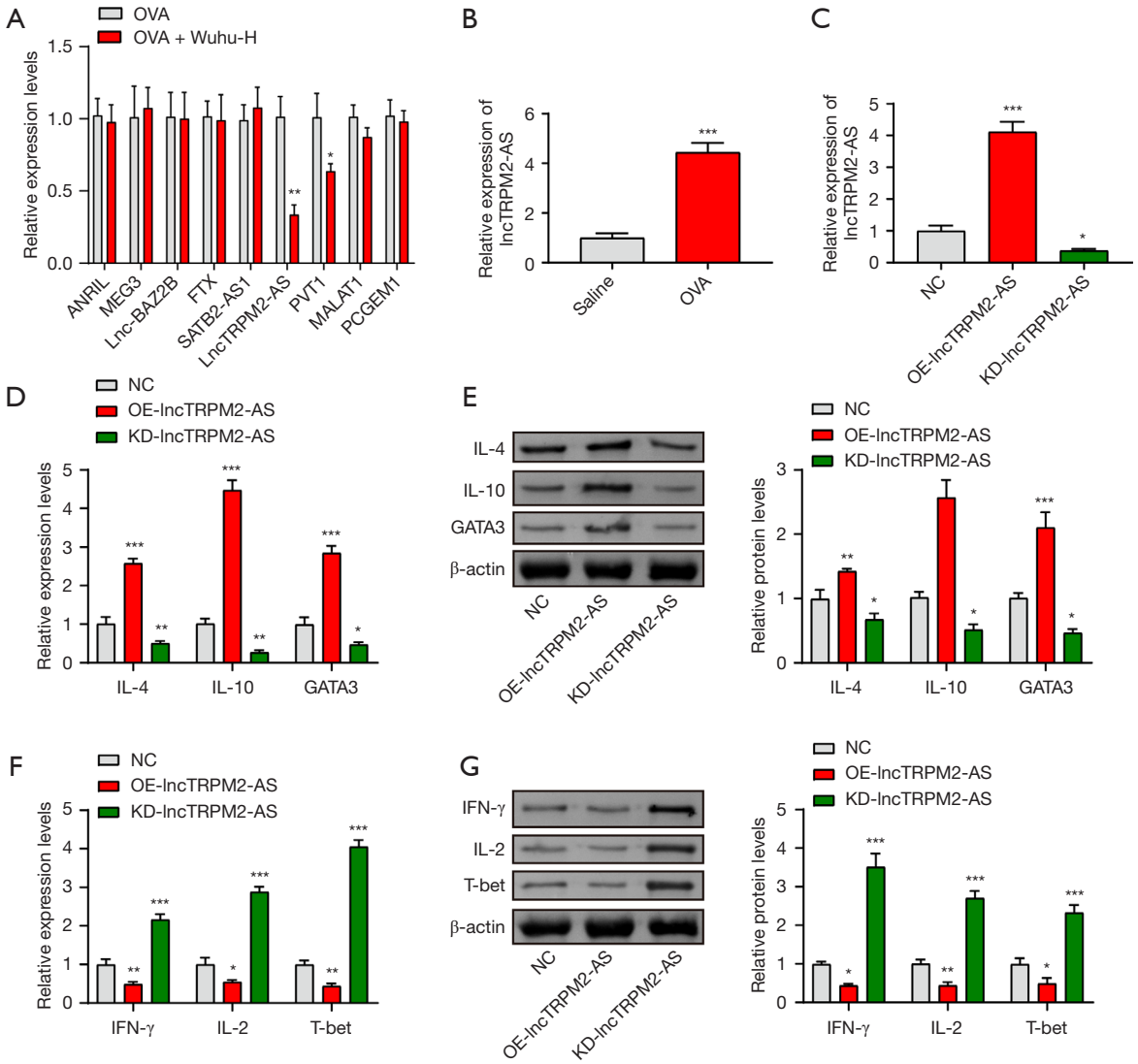

Figure 5 Wuhu decoction regulated Th1/Th2 and T-bet/GATA3 ratios via lncTRPM2-AS. (A) Relative expression of lncRNAs after OVA + Wuhu decoction treatment in comparison with OVA alone. (B) Expression level of lncTRPM2-AS after OVA treatment in comparison with saline treatment. (C) Expression level of lncTRPM2-AS in $CD4^+$ T cells after indicated treatments. mRNA (D) and protein (E) levels of IL-4, IL-10 and GATA3 in cells after indicated treatments. mRNA (F) and protein (G) levels of IFN-γ, IL-2 and T-bet in cells after indicated treatments. N=3. *, P<0.05; **, P<0.01; ***, P<0.001. OVA, ovalbumin; Wuhu-H, Wuhu decoction at 50 mL/kg; lncTRPM2-AS, antisense long non-coding RNA TRPM2; NC, negative control; OE, overexpression; KD, knock down; IL, interleukin; IFN, interferon; Th1, help T type 1 cell; Th2, help T type 2 cell; Th1/Th2, Th1 to Th2 cell amount ratio; T-bet/GATA3, T-bet to GATA3 expression ratio.

of lncTRPM2-AS (*Figure 6C,6D*). On the contrary, the decreased Th1 cytokines by Wuhu decoction were significantly stimulated by lncTRPM2-AS (*Figure 6E*). On the molecular level, the expression of lncTRPM2-AS in BALF was determined by FISH staining and real-time PCR. As shown in *Figure 6F,6G*, OVA treatment significantly increased the expression of lncTRPM2-AS in BALF, which was inhibited by Wuhu decoction and then restored by the overexpression of lncTRPM2-AS. In sum, our data indicated that lncTRPM2-AS was the downstream target that responsible for Wuhu decoction-mediated protection effects on OVA-induced asthmatic mice model.

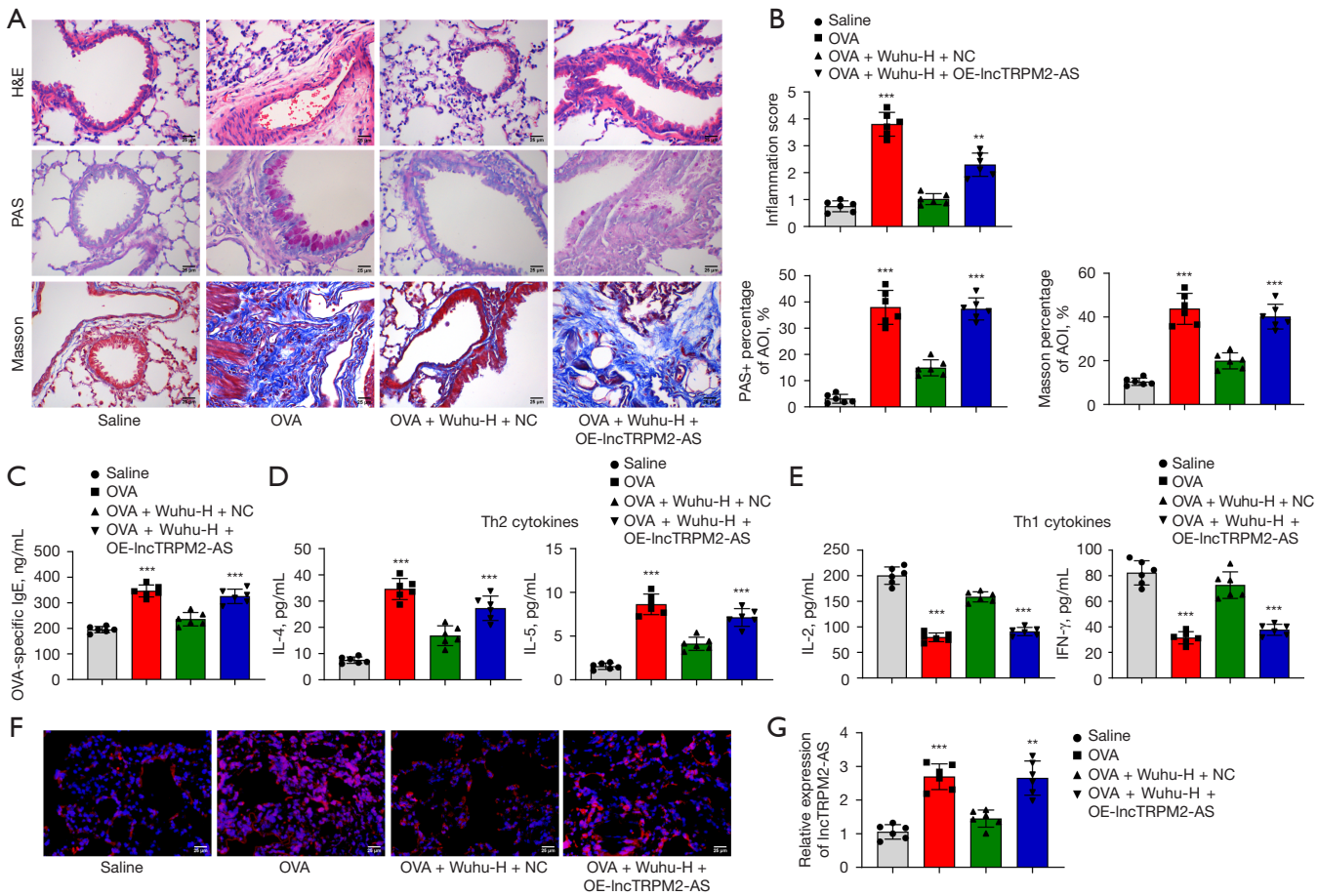

Figure 6 Reinforces lncTRPM2-AS abolished Wuhu decoction-mediated protection effects on OVA-induced asthma. Representative images (A) and statistic results (B) of H&E staining, PAS staining and Masson staining of lung tissues from mice with indicated treatments. Magnification, 400×; scale bar, 25 μm. (C) Quantification of serum OVA-specific IgE in BALF after indicated treatments. Th2-type (D) and Th1-type (E) cytokines levels from mice with indicated treatments. Images (F) and statistic results (G) of lncTRPM2-AS expression in lung tissues from mice after indicated treatments. ISH staining. N=6. **, P<0.01; ***, P<0.001. H&E, hematoxylin-eosin; PAS, periodic acid-Schiff; OVA, ovalbumin; Wuhu-H, Wuhu decoction at 50 mL/kg; NC, negative control; OE, overexpression; lncTRPM2-AS, antisense long non-coding RNA TRPM2; AOI, average optical intensity; IgE, immunoglobulin E; IL, interleukin; IFN, interferon; Th1, help T type 1 cell; Th2, help T type 2 cell; BALF, bronchoalveolar lavage fluid; ISH, in situ hybridization.

Discussion

Inhaled glucocorticoids are one of the main modern clinical treatments together with bronchodilators 2 agonists and leukotriene receptor antagonists for CVA. However, unsatisfactory results often occur due to the side effects and though efficacy is found within a short period, symptoms resurface after the treatment (6). As cough is the only or main disorder of CVA with no obvious lung signs, there are a lot of cases with underdiagnosis or misdiagnosis in clinic, leading to many of CVA evolving into typical asthma (28). Consequently, early diagnosis and treatment would be helpful against the progression of CVA. TCM has been used in the treatment of CVA for a long time with solid theoretical and clinical experience (29). As one of the famous TCMs, the effectiveness of Wuhu decoction on CVA has been previously explored. It has been shown that Wuhu decoction could decrease the airway hyperresponsiveness in RSV-induced asthmatic mice and improve the symptoms of airway inflammation by reducing the infiltration of airway inflammatory cells (26). Meanwhile, it could also inhibit airway collagen deposition and goblet epithelia hyperplasia,

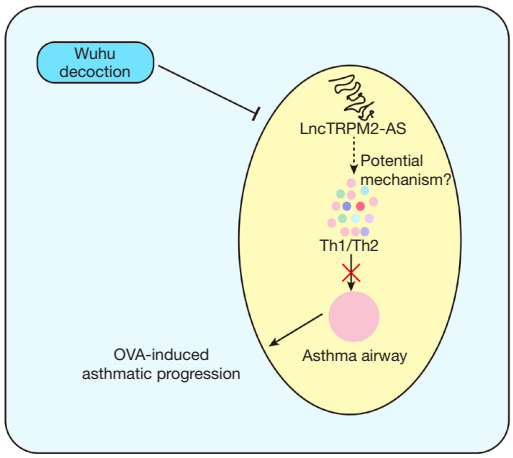

Figure 7 A diagram summarizing therapeutic efficiency of Wuhu decoction in disrupting asthmatic progression. LncTRPM2-AS, antisense long non-coding RNA TRPM2; OVA, ovalbumin; Th1, help T type 1 cell; Th2, help T type 2 cell.

improving airway remodeling. Consistent with those findings, we identified that treatment with Wuhu decoction, especially at high dose level, could significantly alleviate the airway hyperresponsiveness and facilitate airway remodeling in the OVA-induced asthmatic mice model. In particular, Wuhu decoction significantly reduced the infiltration of inflammatory cells in pulmonary tissue and increased the ratio of $CD4^+/CD8^+$ T cells and the level of $CD25^+$ T cells, indicating that it could protect against pulmonary inflammatory injury induced by OVA treatment. Th1/Th2 cell imbalance is considered as a critical pathogenesis leading to airway inflammation in asthma. Excessive proliferation of Th2 cells with increased secretion of inflammatory factors such as IL-4 and IL-5 causes bronchial mast cell degranulation, histamine release and eosinophil infiltration (30-32). This work demonstrated that Wuhu decoction could obviously inhibit the level of Th2 related cytokines and increase that of Th1 related cytokines., restoring the Th1/Th2 cell balance.

LncRNAs belong to the family of non-coding RNAs with more than 200 nucleotides, that are able to modulate various physiological functions and affect different diseases development (33). Several lncRNAs are found to be dysregulated in asthma and involved in airway allergic inflammation (34). For instance, lncPVT1 could regulate the growth of airway smooth muscle cells and release of IL-6 in the asthma patients (35). LncRNA PCGEM1 is a regulator of several metabolic pathways that distinguish early osteoarthritis from late-stage one and lowly expressed in the serum samples of asthma patients with protective effects in children with CVA (36-39). To explore the potential downstream target of Wuhu decoction, we analyzed the expression levels of different lncRNAs and identified lncTRPM2-AS that was significantly downregulated by Wuhu decoction. Previous research has demonstrated that lncTRPM2-AS is upregulated in asthmatic patients and inhibition of lncTRPM2-AS strongly suppresses macrophage proliferation and cytokine production via regulating TRM21-dependent TRPM2 ubiquitination (40). Our results found that lncTRPM2-AS was significantly upregulated in $CD4^+$ T cells isolated from OVA-induced asthmatic mice. Moreover, lncTRPM2-AS was proved to facilitate Th2 differentiation via regulating T-bet/GATA3 ratio in human isolated $CD4^+$ T cells. Most importantly, when lncTRPM2-AS was restored in OVA-induced asthmatic mice, the protective effects by Wuhu decoction against OVA-induced asthma was significantly impaired, demonstrating that Wuhu decoction alleviated the airway hyperresponsiveness and facilitated airway remodeling via inhibiting lncTRPPM2-AS. There were still limitations in our work that need to be further addressed in future studies, such as no unbiased screening to identify downstream targets of Wuhu decoction other than lncTRPM2-AS that involved in OVA development.

Conclusions

Taken together, our findings demonstrated that Wuhu decoction could alleviate airway hyperresponsiveness and improve airway remodeling in OVA-induced asthmatic mice model by regulating Th1/Th2 balance and affecting pulmonary inflammatory injury (*Figure* 7). On the molecular level, Wuhu decoction significantly inhibited the expression of lncTRPM2-AS, which was found to be a critical modulator of Th1/Th2 balance. Meanwhile, overexpression of lncTRPM2-AS could abolish Wuhu decoction-mediated protection effects on OVA-induced asthmatic mice model. Our study discovered the protective function of Wuhu decoction against CVA and illustrated its molecular mechanism, highlighting the therapeutic application of Wuhu decoction for asthma treatment.

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None.

Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were conducted in accordance with the institutional guidelines of The First Affiliated Hospital of Hunan University of Chinese Medicine and the protocol was approved by the Ethics Committee of The First Affiliated Hospital of Hunan University of Chinese Medicine (No. LL2020072202).

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