MG53 alleviates airway inflammatory responses by regulating nuclear factor-κB pathway in asthmatic mice

Sijia Tan^a, Mengtian Li^a*, Xiao Xi Song^b

^aDepartment of Emergency, Xuzhou Central Hospital, Xuzhou, Jiangsu, China
^bDepartment of Ultrasound, Xuzhou Central Hospital, Xuzhou, Jiangsu, China

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KEYWORDS
airway resistance; asthma; MG53; nuclear factor-κB pathway; type 2 inflammation

Abstract
Background: Asthma is a common lung disease with increasing incidence and prevalence globally, thereby imposing a substantial global health and economic burden. Recently, studies have shown that Mitsugumin 53 (MG53) exhibits multiple biological functions and plays a protective role in a variety of diseases. However, the role of MG53 in asthma remained unknown; hence, in the present study we aimed to explore the functioning of MG53 in asthma.

Methods: Using ovalbumin and aluminum hydroxide adjuvant, an OVA-induced asthmatic animal model was constructed and administered with MG53. After establishing mice model, inflammatory cell counts and the levels of type 2 inflammatory cytokines were examined and histological staining of lung tissues were performed. The levels of key factors associated with the nuclear factor-κB (NF-κB) pathway were detected.

Results: Asthmatic mice displayed a remarkable accumulation of white blood cells, neutrophils, macrophages, lymphocytes, and eosinophils in bronchoalveolar lavage fluid, compared to control mice. MG53 treatment lowered the number of these inflammatory cells in asthmatic mice. The level of type 2 cytokines in asthmatic mice was higher than that in control mice, and was lessened by MG53 intervention. In asthmatic mice, airway resistance was elevated, which was reduced by MG53 treatment. In addition, inflammatory cell infiltration and mucus secretion were aggravated in the lung tissues of asthmatic mice, and both were attenuated by MG53 intervention. The levels of phosphorylated p65 and phosphorylated inhibitor of nuclear factor kappa-B kinase were elevated in asthmatic mice, but were downregulated by MG53 supplement.

Conclusion: The aggravated airway inflammation was observed in asthmatic mice; however, MG53 treatment suppressed airway inflammation by targeting the NF-κB pathway.

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Introduction

Asthma is a chronic, noncommunicable lung disease characterized by varying respiratory clinical manifestations and variable airflow limitations.1-3 Clinical manifestations of asthma occur when the small airways in the lungs become inflamed and narrow, leading to coughing, wheezing, shortness of breath, and chest tightness.1 Apart from adults, more and more children are also suffering with asthma.4 There is an increased risk of hospitalization, lost productivity, economic and health burden, and death in patients suffering from severe asthma.5 According to the World Health Organization (WHO), approximately 262 million people worldwide were affected by asthma in 2019 and 455,000 people died due to the disease.6 In spite of adequate treatment options being adopted, some asthma patients still have poor prognosis with the impairment of quality of life (QoL) and multiple complications. Thus, specific therapeutic targets for asthma treatment are required to reduce burden of the disease.

As a tripartite motif-containing (TRIM) protein, Mitsugumin 53 (MG53) belongs to the TRIM family.7 By maintaining cellular integrity, MG53 plays an important role in tissue regeneration and cell survival, as defects in repair of cell membranes contribute to a wide range of illnesses.7 Recently, more studies have shown that MG53 acts as a protective agent in the kidney and cardiac diseases.8,9 In addition, a wide range of human diseases, such as diabetes mellitus and lung cancer, are regulated by MG53. Further, studies have documented that MG53 is involved in a variety of physiological and pathological processes, such as the suppression of aberrant intracellular Ca2+ signaling, insulin resistance, inhibition of inflammation, suppression of cell apoptosis, reduction of oxidative stress, and wound healing.10-12 A previous study has demonstrated that knockout of MG53 led to more severe lung injury and lung dysfunction in acute lung injury murine model because of the impairment of cell membrane repair. However, role of MG53 in asthma has not been reported.13 This study aimed to investigate the function and molecular mechanism of MG53 in asthma, which could reveal novel opportunities for treating asthma.

Materials and Methods

Animal model of asthma

A total of 18 female C57BL/6 mice (5-6 weeks old, weighing 20±5 g) were purchased from Huafukang Bioscience Co. Inc. (Beijing, China), and kept under specific pathogen-free conditions. The Institutional Animal Care Committee of Xuzhou Medical University approved all animal experimentation procedures. Ethical approval for the study was obtained from the Ethics Committee of Xuzhou Medical University.

In murine experiments, there were three groups, which included control group, OVA group, and OVA+MG53 group. To build an allergic asthmatic mouse model (OVA group; n = 6), 500-μL sterile saline mixture containing 20-μg ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) and 200-μg aluminum hydroxide (Sigma-Aldrich) was injected intraperitoneally (i.p.) into mice on day 0 and day 14 for sensitization. On days 25-31, the mice were subjected to an atomization environment using an ultrasonic atomizer with 1% OVA for 30 min daily for stimulation.14,15 For MG53 intervention (OVA+MG53 group; n = 6), asthmatic mice were given 6-mg/kg MG53 1 h prior to atomization on days 25-31. In the control group (n = 6), mice were intraperitoneally injected with 500 μL of saline on day 0 and day 14 and were exposed to saline using an ultrasonic atomizer on days 25-31.

Measurement of airway resistance

Airway resistance was performed 24 h after ultimate challenge using the flexiVent ventilator (Scireq, Montréal, Canada) as described by Johnson et al. and Gupta et al. Responses of the respiratory system to methacholine chloride (Sigma-Aldrich) delivered into the inspiratory line of flexiVent ventilator at increasing concentrations (3.12-25 mg/mL) were used to determine fluctuations in airway resistance.

Assessment of bronchoalveolar lavage fluid (BALF)

After establishing the mouse model, BALF was collected through intratracheal instillation with 1-mL cold sterile saline and repeating for three times. BALF was immediately centrifuged at 2000 rpm for 5 min. The quantity of inflammatory cells in BALF was measured using a NucleoCounter (ChemoMetec, Gjødevang, Denmark). The collected supernatant was stored at −80°C.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from tissues using Trizol reagent (TaKaRa Biotechnology, Dalian, China) according to manufacturer’s instructions.16 Complementary DNA (cDNA) was generated by using PrimerScript RT reagent kit (TaKaRa Biotechnology). RT-PCR was performed with CFX96 Real-time PCR detection system (Bio-Rad, Redmond, WA). Relative gene expression was calculated by applying delta-delta CT (2[-DeltaDeltaCt]) method and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).17

Enzyme-linked immunosorbent serological assay (ELISA)

The protein levels of interleukin (IL)-4, IL-5, and IL-13 in BALF were examined by using commercial ELISA kit (Abcam, Cambridge, MA, UK) according to the manufacturer’s instructions.18 Sensitivity for IL-4, IL-5, and IL-13 were 1.0, 1.2, and 1.2 pg/mL, respectively.

Histological staining

Histological examination of the mice was performed 48 h after the last OVA challenge. The mouse lung tissues were fixed with 4% paraformaldehyde, embedded in paraffin.
blocks, and sectioned to 4.5-μm thickness. Then, hematoxylin and eosin (H&E) and periodic acid-schiff (PAS) stainings were performed on the sections as previously described.\textsuperscript{14,21} Stained sections were analyzed under a light microscope (Olympus, Tokyo, Japan).\textsuperscript{22,23}

**Western blot analysis**

The tissue homogenates were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors (ServoBio, Wuhan, China). The protein concentrations of supernatant were determined by Bicinchoninic acid protein detection kit (ServoBio).\textsuperscript{24} Proteins were loaded and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and equal amounts were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were incubated with primary antibodies for 24 h. After incubation, the membranes were treated with secondary antibodies for 1 h. Then the blots were exposed with enhanced chemiluminescence (ECL) chromogenic solution (ServoBio).\textsuperscript{25,26} The detailed information of antibodies used in Western blot analysis is present in Table 2.

**Statistical analysis**

All data analysis and visualization were performed by the GraphPad Prism software (version 9.0). There were at least three experimental replicates in this study. The Kolmogorov-Smirnov or Shapiro-Wilk test was applied to determine normality and homogeneity of variance distribution. Student’s t test or one-way analysis of variance was applied for comparisons. P < 0.05 was considered statistically significant.

**Results**

**MG53 reduces number of inflammatory cells in BALF in OVA-induced asthma**

In order to elucidate the role of MG53 in asthma, we first quantified the number of multiple immune cell populations in BALF of different murine groups (Figure 1). The results showed that OVA-induced asthmatic mice exhibited a significant accumulation of white blood corpuscles (WBC; Figure 1A), neutrophils (Figure 1B), macrophages (Figure 1C), lymphocytes (Figure 1D), and eosinophils (Figure 1E) in BALF. MG53 treatment markedly decreased the infiltration of inflammatory cells, such as WBCs, neutrophils, macrophages, lymphocytes, and eosinophils (Figure 1A-1E) in BALF. These findings suggested that MG53 was effective in reducing inflammation in asthma.

**MG53 decreases the levels of inflammatory cytokines in OVA-induced asthma**

Asthma is featured as type 2 inflammation with aberrant production of IL-4, IL-5, and IL-13 cytokines.\textsuperscript{27} The levels of type 2 cytokines in both BALF and lung tissues by detected by ELISA (Figure 2). The levels of IL-4, IL-5, and IL-13 in both lung tissues (Figure 2A) and BALF (Figure 2B) were dramatically increased in OVA-induced asthma mice, compared to control mice. The administration of MG53 reversed increase in these inflammatory mediators (Figures 2A and 2B). Thus, MG53 supplement inhibited type 2 inflammation that could suppress the development of asthmatic diseases.

**MG53 alleviates airway resistance and airway remodeling in OVA-induced asthma**

Airway resistance was examined 24 h after the final challenge to determine the therapeutic effects of MG53 to treat asthma. In response to increasing doses of methacholine chloride (0–25 mg/mL), airway resistance was elevated in asthmatic mice, compared to control mice, which was reduced by MG53 treatment (Figure 2A). In order to determine whether MG53 improved airway remodeling by histopathological intervention, we conducted a histopathological examination of lung tissues (Figures 2B and 2C). After OVA exposure, inflammatory

### Table 1 Primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>IL-4</td>
<td>(F) 5’-GGCTCTCAACCCCCAGCTAGT-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-GCCGATGATCTCTCTCAAGTGAT-3’</td>
</tr>
<tr>
<td>IL-5</td>
<td>(F) 5’-GCAATGAGACGATGAGGCTTC-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-GCCCCTGAAAGATTTCTCCAATG-3’</td>
</tr>
<tr>
<td>IL-13</td>
<td>(F) 5’-TGAGCAACATCACACAAGACC-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-GGCTTTGCCGTTACAGAGG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(F) 5’-ACAACCTTGGATAGCTGGAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-GCCATCACGCCCACAGTTTC-3’</td>
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</table>

### Table 2 Primary antibodies used in Western blot analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Concentration</th>
<th>Reference</th>
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<tbody>
<tr>
<td>p65</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>8242</td>
<td>Cell Signaling Technology (Boston, MA, USA)</td>
</tr>
<tr>
<td>p-p65</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>3033</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>IκBα</td>
<td>Mouse</td>
<td>1:1000</td>
<td>4814</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>p-IκBα</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>2859</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>1:2000</td>
<td>3700</td>
<td>Cell Signaling Technology</td>
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</tbody>
</table>
Figure 1  MG53 reduces the number of inflammatory cells of BALF in OVA-induced asthma. The number of (A) WBC, (B) neutrophils, (C) macrophages, (D) lymphocytes, and (E) eosinophils was assessed in the BALF of control, OVA, and OVA with MG53 treatment groups. \( \ast \ast \ast P < 0.001 \) versus the OVA group.

Figure 2  MG53 decreases the levels of inflammatory cytokines in OVA-induced asthma. (A) RT-PCR shows the relative mRNA expression levels of IL-4, IL-5, and IL-13 of the lung tissues in control, OVA, and OVA with MG53 treatment groups. (B) The protein levels of IL-4, IL-5, and IL-13 of BALF in the control, OVA, and OVA with MG53 treatment groups. \( \ast \ast \ast P < 0.001 \) versus the OVA group.
MG53 alleviates inflammatory in asthmatic mice

Discussion

As a common lung disease, the incidence and prevalence of asthma are rising globally. Previous studies have demonstrated that MG53 plays a protective role in various inflammatory diseases. However, the role of MG53 in asthmatic diseases remained unclear. Our study showed that MG53 inhibited airway inflammation by inactivating the NF-κB signaling pathway in asthmatic mice.

MG53 is a TRIM family protein specifically expressed in cardiac and skeletal muscles and performs multiple biological functions. It has been documented that MG53 has a protective effect against the pathogenesis of multiple inflammatory diseases. MG53 predominantly contributes to cardioprotection in cardiac diseases through regulating several cell signaling pathways, such as phosphatidylinositol 3-kinase-protein kinase B (PI3K) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathways. Moreover, accumulating evidence showed that systemic cell infiltration and mucus secretion were aggravated in lung tissues but were attenuated by MG53 intervention (Figures 2B and 2C). This result reflected a significant improvement in airway resistance and airway remodeling by MG53 intervention.

MG53 suppresses the activation of nuclear factor-κB (NF-κB) pathway in OVA-induced asthma

The activity of NF-κB pathway was assessed in the asthma model. The relative levels of phosphorylated p65 (p-p65) and phosphorylated inhibitor of nuclear factor kappa-B kinase (p-IκB) were significantly elevated, while the levels of total IκB kinase were reduced in lung tissues of asthmatic mice (Figure 4), suggesting the activation of NF-κB pathway in asthmatic mice. MG53 supplement attenuated the increased p-p65 and p-IκB in asthmatic mice (Figure 4), implying that MG53 treatment weakened the activity of NF-κB pathway.

Figure 3 MG53 alleviates airway resistance and airway remodeling in OVA-induced asthma. (A) Airway resistance was measured after challenge with different dose of methacholine chloride in the control, OVA, and OVA with MG53 treatment groups. (B) H&E staining shows the histological characteristics of lung tissues in the control, OVA, and OVA with MG53 treatment groups. (C) PAS staining shows mucins staining of lung tissues in the control, OVA, and OVA with MG53 treatment groups. \( \text{**} P < 0.001 \) versus the OVA group.

Figure 4 MG53 suppresses the activation of NF-κB signaling pathway in OVA-induced asthma. The relative protein levels of phosphorylated p65, phosphorylated IκB kinase, and total IκB kinase were measured by Western blot analysis in the lung tissues of the control, OVA, and OVA with MG53 treatment groups. \( \text{**} P < 0.01 \) and \( \text{***} P < 0.001 \) versus the OVA group.
administration of MG53 protein reduced the adverse effects of a variety of injury insults on the brain, kidneys, etc.\textsuperscript{7} It is unclear whether MG53 impacts inflammation in asthma. In this study, using an OVA-induced asthmatic mouse model, we discovered that MG53 treatment could reduce the number of inflammatory cells, levels of inflammatory cytokines, and airway remodeling. These findings suggest that MG53 plays a protective role in asthmatic mice.

The NF-κB pathway has been widely recognized as a canonical proinflammatory signaling pathway because of its involvement in the production of proinflammatory chemokines, cytokines, and adhesion molecules,\textsuperscript{29} all of which are crucial in the pathogenesis of asthma, neuroinflammation, corneal inflammation, and other chronic inflammatory diseases, such as rheumatoid arthritis.\textsuperscript{30,34} The emergence of anti-inflammatory drugs targeting the NF-κB pathway has drawn considerable interest.\textsuperscript{31}

Our results demonstrated that induction of OVA elevated the levels of p-p65 and p-κB, which reflected the activation of NF-κB signaling pathway in asthmatic mice. However, MG53 intervention markedly lessened the levels of p-p65 and p-κB, suggesting that MG53 inhibited the NF-κB signaling pathway in OVA-induced asthmatic mice. These results suggested that MG53 relieved lung inflammation in asthmatic mice through regulating the NF-κB pathway.

Limitations

Some limitations were discovered to this study. The major limitation of this study was the lack of clinical human tissue samples, which could further confirm the effect of MG53 on asthma. Although animal experiments could closely mimic the mechanism of asthma condition, the consistency would be enhanced if further verification could be conducted in clinical settings by utilizing human samples in research. The role of MG53 in different diseases, however, is still debated. Although our results demonstrated that MG53 played a protective role in asthmatic mice, another report indicated that MG53 could lead to insulin resistance in diabetes. Therefore, the potential of MG53 as a clinically relevant therapeutic drug needs further investigation.

Conclusion

Our results revealed that MG53 suppressed airway inflammation via regulating the NF-κB pathway in asthmatic mice, implying that MG53 is a promising therapeutic target to treat asthma.

Availability of Data and Materials

All data generated or analyzed in this study are included in this published article. The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Competing Interests

The authors stated that they had no conflict of interest to declare.

Author Contributions

All authors contributed to conception and designing of the study. Material preparation and experiments were performed by Sijia Tan. Data collection and analysis were done by Mengtian Li. The first draft of the manuscript was prepared by Xiaoxi Song. All the authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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