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Chronic Allergen Exposure Contributes to Steroid Resistance via Increased Phosphorylation of Glucocorticoid Receptors S226 and p38 MAPK in a Mouse Model of Asthma

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ABSTRACT

Chronic allergen exposure can significantly induce p38 mitogen-activated protein kinase (MAPK) activation in asthma. p38 MAPK is involved in steroid resistance through phosphorylation of glucocorticoid receptors (GR) at S226. This study aims to investigate whether chronic allergen exposure can induce steroid resistance and whether it is associated with p38 MAPK activation in asthma.

A mouse model of asthma was prepared by sensitizing and challenging mice with chronic ovalbumin (OVA) exposure. Key features of allergic asthma, encompassing bronchial hyperresponsiveness, pathology of lung tissues, cytokine profiles of inflammation in bronchoalveolar lavage fluid (BALF), and serum immunoglobulin (Ig)E concentration were evaluated. Furthermore, suppressive effects of corticosteroid on the splenocytes under stimulation of lipopolysaccharides, glucocorticoid receptor (GR) DNA binding ability of splenocytes, expression of GR α and phosphorylation of GR s226 in splenocytes, and p38 MAPK phosphorylation in splenocytes and lung tissues were determined.

Chronic OVA exposure substantially induced airway hypersensitivity, leading to increased inflammatory infiltration in lung tissues. Additionally, it resulted in elevated levels of interleukin (IL)-4, IL-5, and IL-6 in BALF, as well as heightened levels of IgE in serum. Furthermore, OVA exposure substantially enhanced p38 MAPK phosphorylation in lung tissues. It also weakened the suppressive impacts of corticosteroids on splenocytes, impaired the GR DNA binding ability, and led to an enhanced phosphorylated state of GR S226 and p38 MAPK in splenocytes.

Taken together, chronic allergen exposure contributes to steroid resistance in asthma, which is linked to an increased phosphorylated state of GR S226 and p38 MAPK.

Keywords: Asthma; Allergen; Glucocorticoid receptors; p38 mitogen-activated protein kinase; Resistance

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INTRODUCTION

Allergic asthma is a widespread pulmonary disorder characterized by airway inflammation and hyperresponsiveness.¹ Over 300 million individuals

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suffer from asthma across the globe.² Glucocorticoids are generally effective in inhibiting asthmatic airway inflammation. However, a subgroup of patients with asthma is unresponsive even to high doses of inhaled steroids and oral steroids. These patients are labeled with asthma with steroid resistance.³ Asthma with steroid resistance is biologically heterogeneous with distinct patterns including type 2-high or type 2-low airway inflammation. Both neutrophilic and paucigranulocytic airway inflammation contribute to the development of steroid resistance in type 2-low asthma.⁴ Type 2-high asthma with eosinophilic airway inflammation, comprising a majority of severe asthma, also encompasses a subgroup of patients with steroid resistance.⁵ However, the phenomenon of corticosteroid resistance in asthma with type 2 inflammation remains incompletely elucidated.

p38 MAPK, a conserved serine/threonine mitogen-activated protein kinase, exerts a crucial role in the mechanism of steroid resistance by phosphorylating the glucocorticoid receptor (GR) at S226 resulting in impaired translocation of GR to the nucleus.⁶ Chronic allergen exposure can significantly induce p38 MAPK activation with type 2 inflammation in patients with asthma.⁷ However, whether chronic allergen exposure can lead to glucocorticoid insensitivity in asthma with type 2 inflammation is still unknown. Thus, the aim of the research was to explore the impact of chronic allergen exposure on glucocorticoid sensitivity in asthma, with a specific focus on p38 MAPK.

MATERIALS AND METHODS

Experimental Animals

Twenty 6- to 8-week-old female BALB/c mice were procured from the Shanghai SLAC laboratory animal company. Mice were kept at a constant temperature of 22±2°C, and subjected to a 12-hour light/dark cycle. They were arbitrarily allocated into 2 groups (n=10 each): (1) the control group (saline), and (2) the model group (asthma).

Reagents and Materials

Lipopolysaccharide (LPS) and corticosterone were obtained from TargetMol (batch 131803), Boston, USA. Ovalbumin (OVA) and methacholine were procured from Sigma-Aldrich, Shanghai, China. Imject Alum adjuvant was procured by Thermo Fisher Scientific Co., Shanghai, China. ELISA kits including interleukin (IL)-

4, IL-5, IL-6, IL-13, IL-1β, tumor necrosis factor-alpha (TNF-α), interferon (IFN)-γ, and immunoglobulin (Ig)E were provided by eBioscience Co., San Diego, USA. The Cell Counting Kit-8 (CCK-8) assay utilized in the experiment was provided by Dojindo Laboratories, Japan. TransAM GR kits were obtained from Active Motif, USA. Antibodies for phosphorylated-p38 MAPK (Thr180/Tyr182), p38 MAPK, and phosphorylated-GR (S226) were provided by Abcam, Shanghai, China.

Model Establishment

A chronic asthma model was established according to a protocol reported previously.⁸ Briefly, each mouse was sensitized on days 0 and 7 with 0.2 mL of a mixture including 40 μg of OVA and 0.05 mL of alum adjuvant injected peritoneally. From day 14, the mice were exposed to inhalation of 1% OVA for 30 minutes every day for a duration of 5 successive weeks. Aerosolized normal saline was used instead of OVA in the saline group. The experimental procedure is outlined in Figure 1.

Evaluation of Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) was determined noninvasively with whole-body plethysmography systems (Buxco Research Systems, California, USA) following the last challenge with OVA. In brief, each mouse was managed in an airtight ventricle, and during the respiration cycle, the pressure variation was recorded. After 3 minutes of adaptation to reach a steady baseline, each mouse was given atomized saline or increasing doses of methacholine (6.25, 12.5, and 25 mg/mL). The Enhanced pause (Penh) was used to show variation in airway resistance.⁹

Serum Collection and Analysis

Following the evaluation of AHR, blood samples were obtained from the mice after administering complete anesthesia with sodium pentobarbital. Subsequently, the blood samples were subjected to centrifugation at a speed of 1500g for 10 minutes. The resulting serum was carefully collected. The determination of IgE levels was performed later by ELISA following the producer's protocols.

Preparation of Bronchoalveolar Lavage Fluid and Cytokine Evaluation

Bronchoalveolar lavage fluid (BALF) was obtained through the process of lung lavage 2 times using 1 mL

of saline solution. After centrifugation, the supernatant was separated and stored for subsequent analysis. The levels of cytokines, including IL-4, IL-5, IL-13, IFN- γ , IL-6, IL-1 β , and TNF- α , in the BALF were evaluated by enzyme-linked immunosorbent assay (ELISA) following protocols provided by the producer.

Lung Tissue Pathology Examination

Following BALF performance, pulmonary lobes were collected and inflated with 4% neutral-buffered formalin. After being sliced into 4- μ m-thick sections, lung tissues were stained with hematoxylin and eosin (H&E). Lung tissue pathology was evaluated by 3 observers independently using Image-Pro Plus software. The severity of airway inflammation was assessed by utilizing a scoring strategy as reported previously.¹⁰

Splenocytes Preparation and Glucocorticoid Sensitivity Assay

Splenocytes were isolated according to the methods described previously to evaluate the sensitivity of splenocytes' response to corticosterone^[11]. Splenocytes were cultured in triplicate at a density of 2.5×10^5 cells/well, which were administered with different doses of corticosterone (0.05 to 5 μ M) and activated with 1 μ g/mL LPS in 96-well plates. After 18 hours of incubation, cell culture suspensions were collected for subsequent cytokine evaluation. Subsequently, IL-6 in the suspensions was assessed by ELISA. After a 48-hour culture period, cell viability was evaluated by the Cell Counting Kit-8 analysis. The cell viability was calculated as a proportion of the optical density of the control.

GR Function Assessment

Splenocytes were cultured with dexamethasone (1×10^{-7} M) for 2 hours. The nuclear extraction of splenocytes was obtained according to the methods described previously^[12]. TransAM GR kits were utilized to determine GR-DNA binding ability as a representative measure of GR function. In brief, nuclear extracts, each containing 20 μ g of protein, were added to 96-well plates. Following the incubation for 1 hour, a primary anti-GR antibody was added and incubated for another 1 hour. Finally, a peroxidase-conjugated secondary antibody was added to the wells and incubated for another hour. After the addition of the substrate, the color absorbance at 450 nm was used to measure the GR function.

Western Blot Analysis

The protein lysis buffer was utilized to extract total protein from both splenocytes and lung tissues. After transferring the proteins onto a nitrocellulose membrane, the immunoblot was incubated overnight with the subsequent antibodies and their respective dilutions: phosphor-GR ser226 (1:1000), GR α (1:1000), phosphorylated-p38 MAPK Thr180/Tyr182 (1:1000), p38 MAPK (1:1000), and GAPDH (1:2000). Afterward, the membrane underwent incubation with secondary antibodies conjugated with HRP at a dilution of 1:1000. The immunoblots were developed using enhanced chemiluminescence (ECL). The protein bands were visualized and quantified utilizing the ImageQuant LAS 4000 ECL System.

Statistical Analysis

The data are displayed as the mean \pm standard error of the mean. Statistical evaluation was conducted using Prism version 6 (GraphPad Software, San Diego, CA). Student's t-test was used to assess differences when the data followed a normal distribution. Otherwise, the Mann-Whitney U test was employed instead. $p < 0.05$ was considered statistically significant.

RESULTS

Chronic Allergen Exposure Induced AHR and Type 2 Airway Inflammation

In Figure 2A, it is shown that chronic OVA challenge resulted in increased airway AHR in asthmatic mice. This is demonstrated by the increased airway resistance measured by Penh upon methacholine stimulation compared to the control group ($p < 0.01$). Figure 2B depicts lung histopathology observed using hematoxylin and eosin staining. A significant accumulation of inflammatory cells around the airways is evident, as indicated by an elevated inflammatory score ($p < 0.01$). Furthermore, Figure 2C presents the impact of OVA exposure on various markers of inflammation. OVA exposure elevated the levels of TNF- α , IL-4, IL-5, and IL-6 ($p < 0.01$), while reducing IFN- γ levels in the BALF ($p < 0.05$). Additionally, OVA exposure markedly upregulated the level of serum IgE ($p < 0.01$).

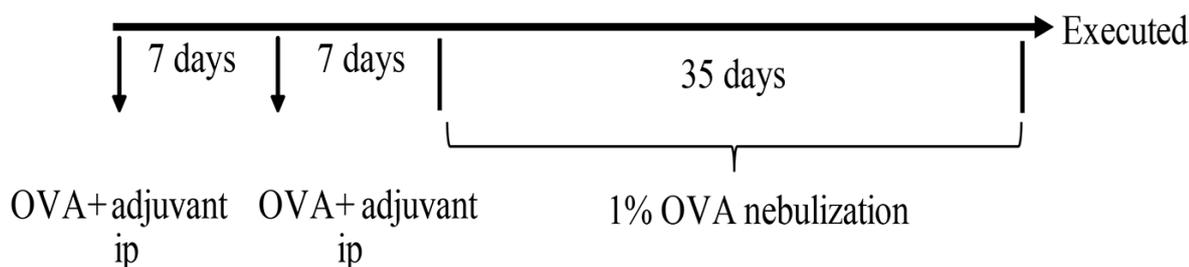


Figure 1. Experimental protocols. Mice in the asthma group were subjected to ovalbumin (OVA) sensitization and chronic challenge. Mice were sensitized twice and challenged with 1% OVA every day for 5 consecutive weeks. Normal saline was given instead in the saline control group.

Chronic Allergen Exposure Increased p38 Phosphorylation in Lung Tissues

P38 activation was associated with type 2 airway inflammation, thus p38 phosphorylation was evaluated in lung tissues. We found that after OVA exposure, the phosphorylation level of p38 was elevated in lung tissues in this asthma model in comparison with the saline control (Figure 3).

Chronic Allergen Exposure Impaired the Steroid Sensitivity of Splenocytes in Vitro

P38 activation contributes to steroid resistance, and therefore the effects of allergen exposure on steroid resistance were determined in vitro. Figure 4 distinctly illustrates the dose-dependent suppression of corticosterone on splenocyte proliferation. The suppressive impacts of corticosterone on splenocyte proliferation in the asthma group were significantly reduced in comparison to the saline control, which indicated that the asthma group displayed impaired steroid sensitivity. Furthermore, the effects of corticosterone at concentrations of 0.05 and 5 μ M on IL-6 were also markedly impaired in the model group ($p < 0.01$).

Chronic Allergen Exposure Reduced GR-DNA Binding Ability and Increased Phosphorylated Status of GR S226 and p38 MAPK in Vitro

To investigate the potential mechanisms underlying the steroid resistance induced by OVA exposure, GR-DNA binding ability was assessed in vitro. As depicted in Figure 5, the asthma group demonstrated a substantial decrease in GR-DNA binding ability in comparison with the saline control group. This suggests that chronic

allergen exposure may impair GR function. To investigate the potential cause of decreased GR DNA binding ability, we examined the phosphorylated status of GR at S226. Increased phosphorylation at this site can inhibit GR nuclear translocation, leading to decreased GR-DNA binding ability. The research indicated that there was a considerable increase in the phosphorylated status of GR S226 in splenocytes from the asthma group compared with the control ($p < 0.01$). This suggests that OVA exposure may promote the phosphorylated status of GR S226, thereby compromising GR function. Furthermore, the activation of p38 MAPK signaling was assessed since it has been implicated in phosphorylating GR S226. Figure 5 demonstrates that p38 MAPK phosphorylation was remarkably elevated in splenocytes of the asthma group ($p < 0.01$). Increased p38 MAPK phosphorylation may contribute to the observed GR S226 phosphorylation and impaired GR-DNA binding ability. It should be noted that reduced GR expression can also contribute to steroid insensitivity. However, the study did not detect a noteworthy difference in GR expression in splenocytes between the two groups. These findings suggest that chronic allergen exposure may induce steroid resistance in splenocytes by impairing GR function, potentially through the phosphorylation of GR S226 and the activation of p38 MAPK signaling.

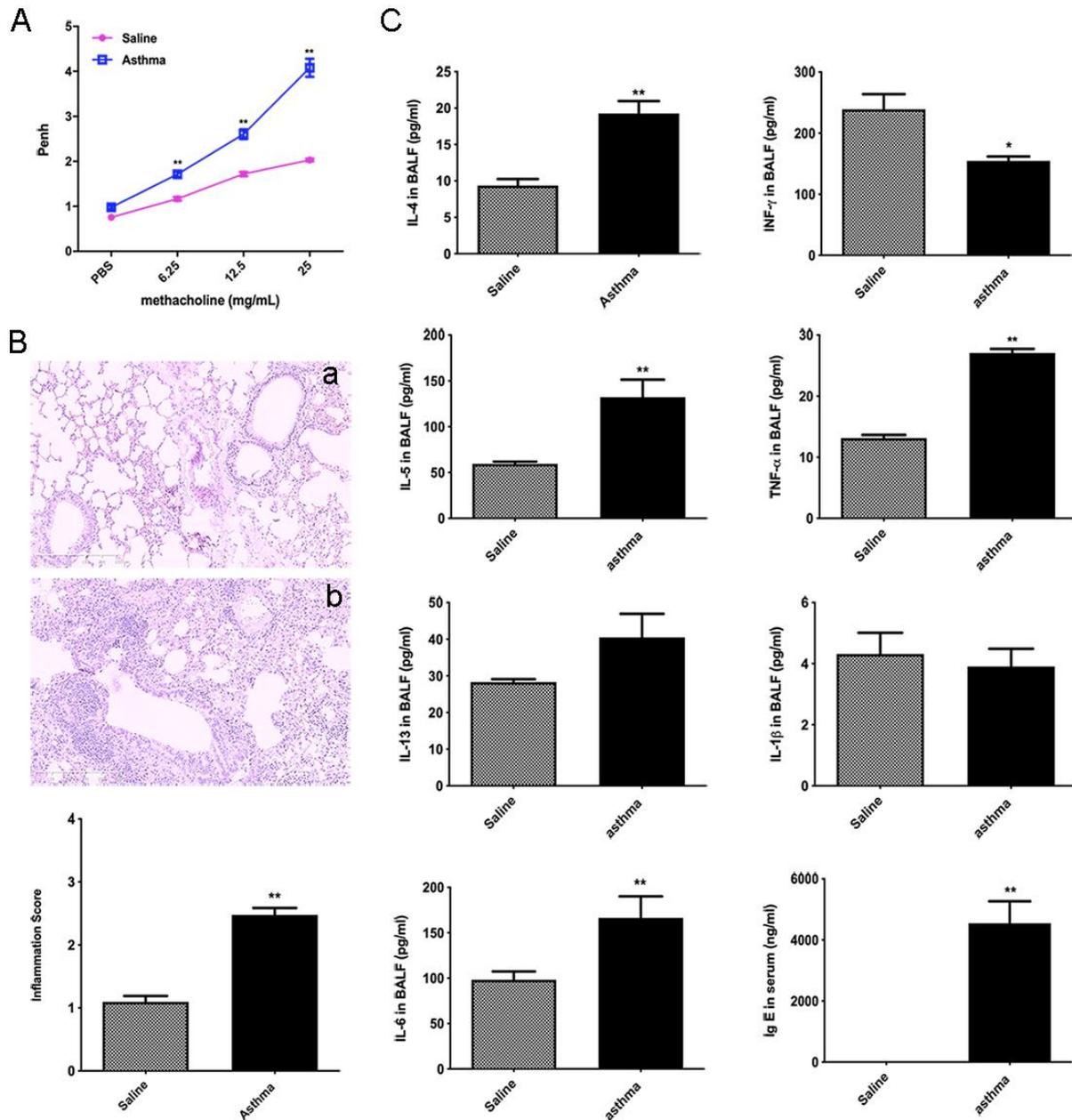


Figure 2. Chronic allergen exposure induced airway hyperresponsiveness (AHR) and airway inflammation. **A.** Mice in the asthma group with chronic allergen exposure exhibited AHR to methacholine. **B.** Chronic allergen exposure-induced airway inflammation in lung tissues evaluated by hematoxylin and eosin. Lung tissue slices were observed under a microscope (100 \times). Representative pictures of each group are presented as stated below: (a) saline. (b) asthma. The score represents the severity of airway inflammation. **C.** Chronic allergen exposure elevated type 2 cytokines in bronchoalveolar lavage fluid (BALF) and immunoglobulin (Ig)E in serum. BALF was obtained by conducting lung lavage twice with 1 mL saline, and the supernatant was collected after centrifugation at 500g for 10 minutes. Levels of cytokines, including interleukin (IL)-4, IL-5, IL-13, interferon (IFN)- γ , IL-6, IL-1 β , and tumor necrosis factor-alpha (TNF- α) in BALF, as well as serum IgE were quantified using ELISA. All data are presented as mean \pm SEM (n = 8-10 for each group). * p <0.05 and ** p <0.01 versus mice in the saline control group.

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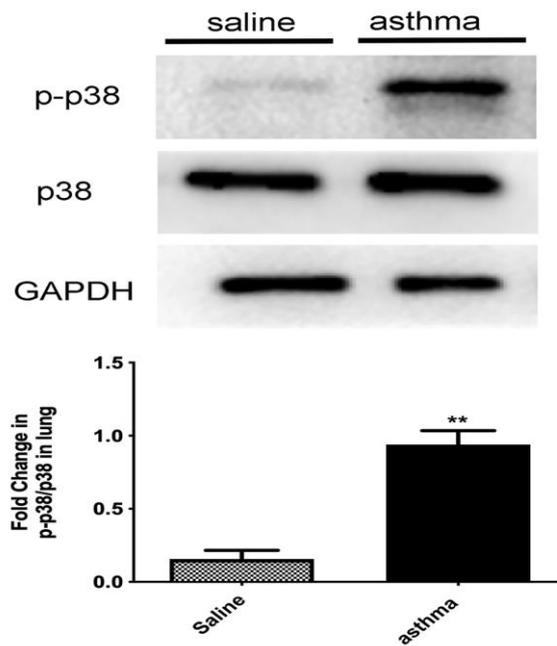


Figure 3. Chronic allergen exposure increased phosphorylation of p38 in lung tissues. A Western blot was performed to evaluate the phosphorylation level of p38 mitogen-activated protein kinase (MAPK) in lung tissues. The band intensity of phosphorylated p38 was standardized to the corresponding total protein. Data are presented as means \pm SEM (n = 6 in each group). ** $p < 0.01$ versus the saline control.

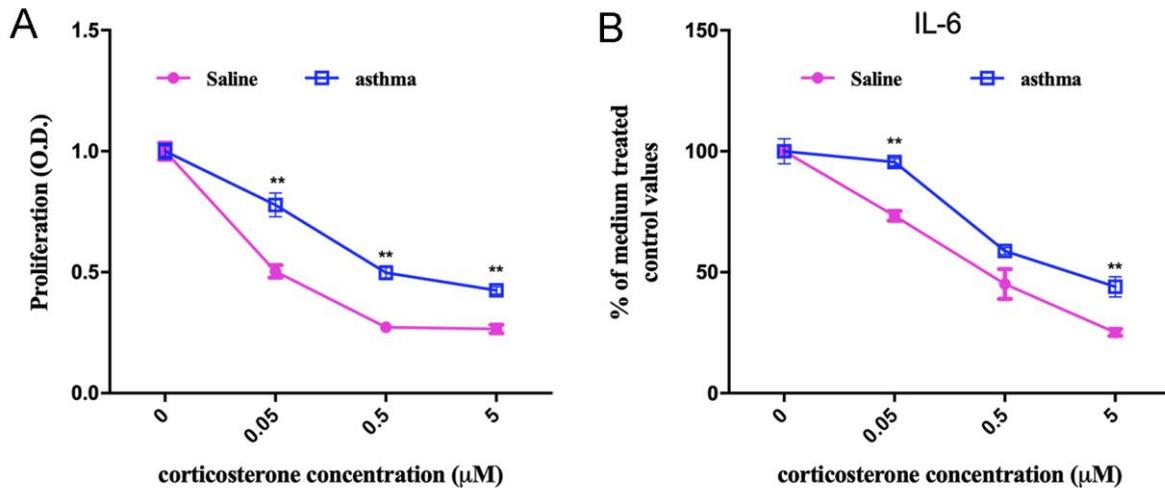


Figure 4. Chronic allergen exposure impaired the steroid sensitivity of splenocytes in vitro. (A) Proliferation response of splenocytes to corticosterone. Splenocytes were cultured in triplicate at a density of 2.5×10^5 cells/well. The cells were exposed to different concentrations of corticosterone (0.05 to 5 μ M) and stimulated with 1 μ g/mL lipopolysaccharide (LPS) in 96-well plates. After 48 hours of culture, Cell Counting Kit-8 assay was used to determine cell viability. (B) interleukin (IL) -6 secretion response of splenocytes to corticosterone. Splenocytes were cultured in triplicate at a density of 2.5×10^5 cells/well in a volume of 300 μ L/well, treated with different concentrations of corticosterone (0.05 to 5 μ M) and stimulated with 1 μ g/mL LPS in 96-well plates. Cell supernatants were obtained after 18 h of incubation, and the levels of IL-6 were assessed using ELISA. ** $p < 0.01$ compared to the saline control.

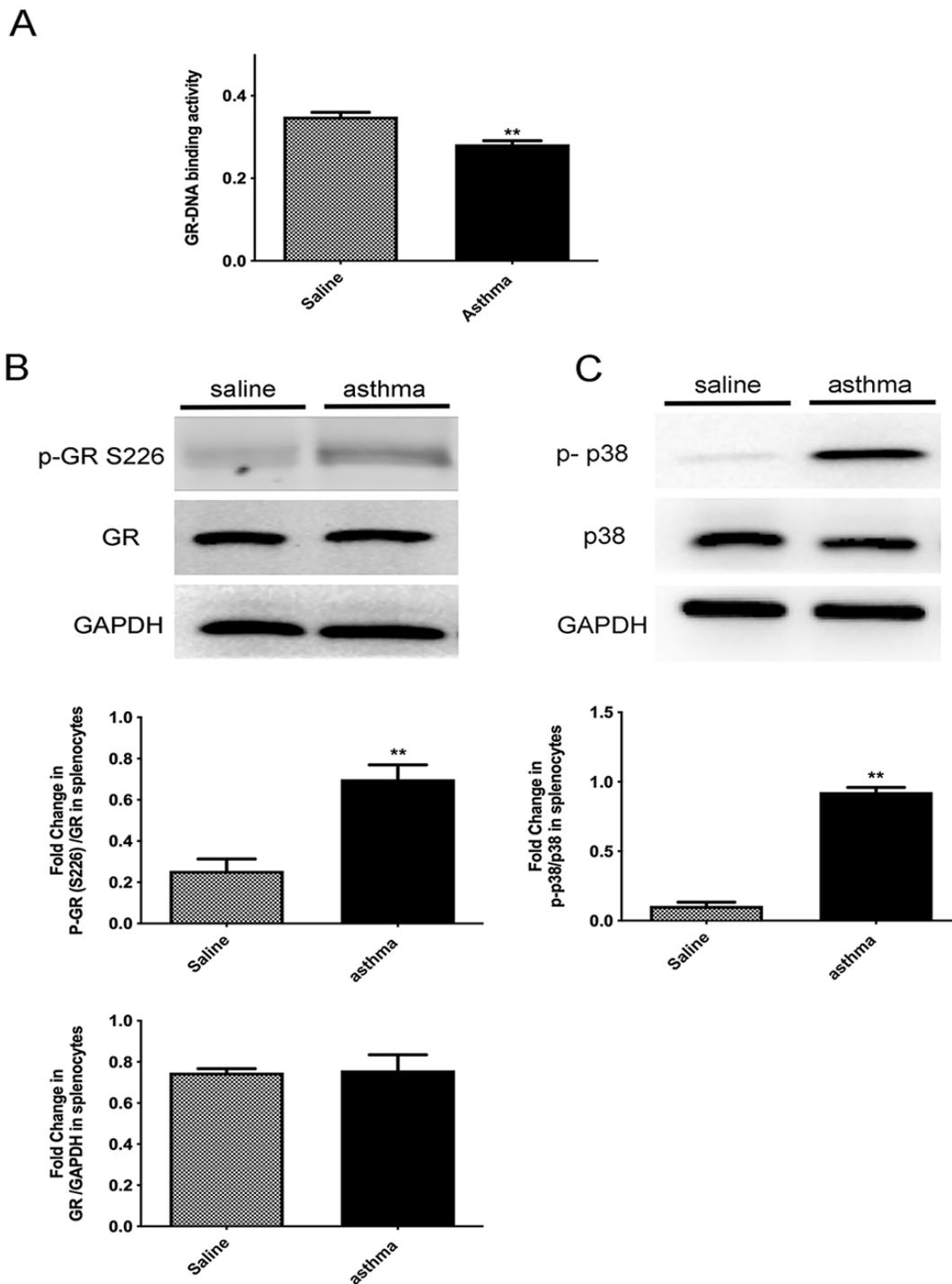


Figure 5. Chronic allergen exposure decreased glucocorticoid receptors (GR)- deoxyribonucleic acid (DNA) binding ability and increased the phosphorylated status of GR S226 and p38 mitogen-activated protein kinase (MAPK). (A) Assessment of GR-DNA binding ability. TransAM GR-DNA binding kit was used to determine the GR-DNA binding ability in splenocytes. (B and C) The phosphorylated states of GR S226 and p38 MAPK, as well as total GR expression, were determined by Western blot assay. Data are presented as means \pm SEM (n = 6 in each group). ** $p < 0.01$, versus the saline control.

DISCUSSION

The present study identified an association between chronic allergen exposure and the development of steroid resistance, along with the proposed involvement of p38 MAPK activation. The findings demonstrated that chronic allergen exposure induced allergic type 2 inflammation in the asthma mouse model. Additionally, it was observed that the mice exposed to chronic allergen displayed impaired steroid sensitivity and reduced GR-DNA binding ability. Further analysis revealed that chronic OVA exposure led to significant phosphorylation of p38 MAPK and GR S226. These molecular changes were associated with the observed steroid resistance. In accordance with these findings, it is proposed that prolonged exposure to allergens may increase the risk of developing steroid resistance. These findings contribute to a better understanding of the potential risks associated with long-term exposure to allergens and highlight the importance of managing steroid resistance in asthma treatment strategies.

The involvement of p38 MAPK activation is evidenced to be crucial in the pathogenesis of allergic type-2 inflammation in patients with asthma. Previous research findings have highlighted that inhibiting p38 activation can effectively suppress the inflammatory response mediated by eosinophils and CD4⁺ T cells in allergic asthma models.¹³ In this particular study, a chronic asthma model was used, which involved repeated challenges with OVA over a period of 5 weeks. The results obtained from this model confirmed that chronic exposure to OVA induced significant AHR, increased airway inflammation, and elevated levels of cytokines such as IL-4, IL-5, IL-6, and TNF- α in the BALF. Additionally, there was an elevation in serum IgE levels, further confirming the presence of allergic type 2 inflammation. Moreover, the study revealed that p38 MAPK was markedly activated in the lung tissues after chronic OVA exposure. This finding provides additional evidence supporting the notion that chronic allergen exposure leads to the development of type 2 inflammation with concomitant engagement of the p38 MAPK pathway in the specific asthma model utilized in the study.

Previous studies suggest that TH1/TH17-mediated responses and neutrophil-dominated airway inflammation contribute to steroid resistance in type 2-low asthma. However, it is still unclear whether chronic allergen exposure will induce steroid resistance in

asthma characterized by type 2-high inflammation, and the possible mechanism involved is also not fully elucidated. Recently, it was shown that the p38 MAPK pathway is significantly activated in asthma patients with steroid resistance, and activation of p38 MAPK has been proposed as a potential biomarker for identifying steroid-resistant asthma patients.^{6,14} As illustrated above, p38 MAPK was significantly activated in lung tissues in this study. Therefore, we investigated whether the asthma model used in the research exhibited steroid resistance by assessing the impacts of corticosterone on splenocytes. The findings of the study revealed that chronic OVA exposure impaired the suppressive impacts of corticosterone on splenocyte proliferation *in vitro*. Additionally, the impacts of glucocorticoids on the secretion of the cytokine IL-6 from splenocytes were also significantly compromised. These findings collectively indicate that chronic allergen exposure contributes to the formation of steroid resistance in the specific asthma model employed in the study.

Under normal conditions, when glucocorticoids bind to the GR in the cytoplasm, the receptor is efficiently translocated to the nucleus. In the nucleus, the GR can bind to glucocorticoid-responsive elements or other promoters in the DNA, regulating gene expression.¹⁵ Therefore, impaired GR function or decreased GR expression has been identified as contributing factors to steroid resistance.¹⁶ To further elucidate the potential mechanism underlying allergen exposure-induced steroid resistance, the present study investigated GR function and expression in splenocytes. The findings revealed significant impairment in GR function in splenocytes in the asthma group. Nevertheless, no apparent disparity in total GR expression was observed in splenocytes between the two groups. These results indicated that the steroid resistance induced by chronic OVA exposure in the asthma model could be attributed to impaired GR function rather than alterations in total GR expression. These results strengthen the significance of GR function in mediating the response to glucocorticoids and suggest that impaired GR function may contribute to the observed steroid resistance in the asthma model used in the study. It further supports the notion that regulating GR activity and function could be a potential therapeutic approach to overcome steroid resistance in asthma.

GR phosphorylation at S226 is associated with reduced nuclear translocation of the GR, ultimately resulting in impaired activity of GR-DNA binding.¹⁷

Our study demonstrated a significant increase in phosphorylation of GR S226 in the asthma group. This finding suggests that the impaired GR function observed in the asthma model may be attributed to increased phosphorylation of GR S226. Notably, previous research has demonstrated a significant correlation between p38 MAPK activation and GR S226 phosphorylation.¹⁸ To further elucidate the mechanisms underlying the OVA-exposure-induced steroid resistance in the type 2 inflammation asthma model, the researchers evaluate the p38 MAPK signaling in splenocytes. The results revealed marked activation of p38 MAPK in splenocytes from the asthma group. These findings indicated that the observed steroid resistance induced by allergen exposure may be related to the enhanced phosphorylated status of GR S226 and activation of p38 MAPK. This signaling pathway may represent a key mechanism underlying the impaired GR function and subsequently reduced responsiveness to glucocorticoid treatment.

In conclusion, our work indicates that chronic allergen exposure could induce steroid resistance in a mouse model of asthma characterized by type 2 airway inflammation. The impaired function of the GR and dysregulated signaling through p38 MAPK contribute to reduced responsiveness to corticosteroid treatment. Additional investigation is needed to clarify the precise molecular interactions and signaling pathways involved in this process. This knowledge may facilitate the formulation of novel therapeutic strategies that can effectively address steroid resistance in asthma and improve treatment outcomes for affected individuals.

STATEMENT OF ETHICS

All animal experiments were conducted according to the Chinese guidelines for experimental animal welfare and approved by the local Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (Permit No. IACUC-20190909-08; December 2020). The study was carried out in accordance with the ARRIVE guidelines.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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