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Inhibition of CREB promotes glucocorticoids action on airway inflammation in pediatric asthma by promoting ferroptosis of eosinophils

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Abstract

Background: Pediatric asthma is a common chronic disease of childhood with airway inflammation. Cyclic adenosine monophosphate response element binding protein (CREB) plays a significant role in the transcription of proinflammatory genes, but its role in pediatric asthma has remained unclear. Herein, we investigated the functions of CREB in pediatric asthma.

Methods: Eosinophils were purified from the peripheral blood of interleukin 5 (IL5) transgenic (IL5T) neonatal mice. The contents of CREB, long-chain fatty-acid-CoA ligase 4, transferrin receptor protein 1, ferritin heavy chain 1, and glutathione peroxidase 4 in eosinophils were examined by Western blot analysis. The viability of eosinophils, and the mean fluorescence intensity of Siglec F, C-C motif chemokine receptor 3 (CCR3), and reactive oxygen species were examined by flow cytometry. The concentration of iron in eosinophils was assessed by a commercial kit. The contents of malondialdehyde, glutathione, glutathione peroxidase, IL-5, and IL-4 were discovered by enzyme-linked-immunosorbent serologic assay. The C57BL/6 mice were randomly divided into four groups: sham, ovalbumin (OVA), OVA+Ad-shNC, and OVA+Ad-shCREB. The bronchial and alveolar structures were evaluated by hematoxylin and eosin staining. Leukocytes and eosinophils in the blood were measured using a HEMAVET 950. **Results:** The abundance of CREB in eosinophils was enhanced by CREB overexpression vector transfection, but reduced by short hairpin (sh)CREB transfection. Downregulation of CREB triggered the cell death of eosinophils. Knockdown of CREB could obviously contribute to ferroptosis of eosinophils. In addition, downregulation of CREB facilitated dexamethasone (DXMS, a type of glucocorticoid)-induced eosinophils death. Moreover, we established an asthma mouse model by OVA treatment. The CREB was upregulated in OVA group mice, but Ad-shCREB treatment obviously downregulated CREB level. Downregulation of CREB diminished OVA-induced asthmatic airway inflammation by reducing the number of inflammatory

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cells and the levels of proinflammatory factors. Downregulated CREB enhanced the anti-inflammatory effect of DXMS in OVA-induced mice.

Conclusion: Inhibition of CREB promoted the effect of glucocorticoids on airway inflammation in pediatric asthma through promoting ferroptosis of eosinophils.

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Introduction

Asthma is a chronic respiratory disease often caused by allergic inflammation.¹ Its clinical manifestations include wheeziness because of airway obstruction, coughing, chest stuffiness, and difficulty in breathing.² Pediatric asthma is a general term for a series of diseases with similar clinical characteristics, but differences in their manifestations, etiology, and pathophysiology.^{3,4} The World Health Organization (WHO) has estimated that approximately 30 billion people worldwide suffer from asthma, and nearly 2.5 million premature deaths take place due to asthma annually. The mortality rate of pediatric asthma range from 0 to 0.7 per 100,000 people.⁵ Allergic asthma, as one of the phenotypes of pediatric asthma, is associated with major eosinophilic inflammation.⁶

Eosinophils, a type of white blood cells, play an important role in the immune system. Eosinophils release a variety of chemical mediators that can contribute to inflammatory response. These mediators include cytokines, chemokines, and enzymes.⁷ In asthma, eosinophils accumulate in the airways and release inflammatory mediators that contribute to airway hyperresponsiveness, mucus production, and airway remodeling.⁸ Eosinophils are terminally differentiated granulocytes and are involved in the pathogenesis of various inflammatory reactions.⁷ Generally, eosinophils make up less than 5% of the white blood cells in the blood. In the absence of external stimulation, circulating eosinophils typically end physiologically by spontaneous apoptosis within 2-5 days.⁹ Conversely, the number of eosinophils increases in response to external stimuli. More and more studies have shown that the course of allergic airway inflammation depends not only on the recruitment of eosinophils but partly on the extension of the life span of eosinophils in inflamed tissues.¹⁰ Delayed apoptosis of eosinophils is a key mechanism for eosinophils to accumulate at the site of inflammation, thus delaying the resolution of airway inflammation.¹¹

Ferroptosis is a relatively new type of cell death characterized by lipid peroxidation of the cell membrane, decreased levels of intracellular glutathione (GSH), and accumulation of iron ions; all these characteristics distinguish it from other types of cell death, such as apoptosis and necrosis.¹² Recently conducted study has indicated that ferroptosis plays an important role in the pathogenesis of asthma, especially in relation to eosinophils.¹³ The study demonstrated that during asthma attacks in children, the oxidative stress molecules and excess iron ions released by eosinophils could cause oxidative damage and lipid peroxidation of the cell membrane, leading to cell death.¹³ Therefore, ferroptosis has become a hot research topic in the pathogenesis of pediatric asthma, and related studies can help us better understand the occurrence and development of asthma and provide

a theoretical basis for the development of new treatment strategies. Glucocorticoids (GC) is a commonly used anti-inflammatory therapy for children with asthma; this can effectively treat eosinophil-related diseases by directly inducing apoptosis of eosinophil and inhibiting pro-survival signals.¹⁴ However, the multipotent effect of corticosteroids limits the therapeutic use of GC, especially at high doses, as studies have established that high doses of GC can cause osteoporosis.¹⁵ In addition, omalizumab, a recombinant, humanized, monoclonal antibody against human immunoglobulin E, is also used in pediatric asthma in limited quantity.¹⁶ Therefore, current study focuses on the development of new drugs to prevent the recruitment and/or reduce the survival and activation of eosinophils.

Cyclic adenosine monophosphate response (cAMP) element binding protein (CREB) is a ubiquitous transcription factor that promotes the expression of various target genes by binding to conserved cAMP response elements in its promoter region. Multiple studies have shown that CREB directly binds to the promoter of proto-oncogenes to play a cancer-promoting role.¹⁷ It has been determined in a recent report that CREB directly binds to the promoter region of glutathione peroxidase 4 (GPX4), stimulating the activity of lung adenocarcinoma cells and inhibiting potential ferroptosis.¹⁸ In addition, CREB activation enhances airway smooth muscle cell proliferation.¹⁹ CREB activation promotes Th2 response and Th2/regulatory T cells (Treg) imbalance in an asthmatic mouse model, thereby exacerbating asthma.²⁰ Therefore, CREB can play an important role in asthmatic airway disease and its ferroptosis process. However, few studies are reported on CREB and eosinophils.

This study aimed to investigate the functions of CREB in pediatric asthma. Eosinophils were purified from the peripheral blood (PB) of interleukin 5 (IL5) transgenic (IL5T) neonatal mice. The asthma mouse model was constructed by ovalbumin (OVA) treatment. The content of CREB in eosinophils and lung tissues of asthma mice were examined. Moreover, the influence of CREB on the effect of dexamethasone (DXMS, a type of glucocorticoid) on eosinophils and airway inflammation in pediatric asthma was discovered. Our findings have important implications for developing new strategies for treating pediatric asthma with GC.

Materials and Methods

Transgenic animal

The IL5T neonatal mice (transgenic mice that overexpressed IL5 in T cells) were obtained from Changsheng Biology Co. (Changchun, China). All tests on mice were executed according to the Guide for the Care and Use of

Laboratory Animals.²¹ The animal research complied with the directions of the Animal Ethical and Welfare Committee of Zhejiang Provincial People's Hospital, Affiliated People's Hospital, China (approval No. IACUC-A2022121302).

Purification of eosinophils

Eosinophils were purified from the PB of IL5T neonatal mice. PB was taken from the retroorbital sinus into the tubes having ethylenediaminetetraacetic acid (EDTA; BD Biosciences, San Jose, CA). Mice were sacrificed by the overdose of anesthetic. An ammonium chloride buffer (BD Biosciences) was applied to lyse red blood cells. Then, the PB samples were coped with surface antibodies to SiglecF (E50-2440; BD Biosciences) and CCR3 (Jo73E5; BioLegend, San Diego, CA), and separated with a fluorescence-activated cell sorter. The stained cells were analyzed by a flow cytometer using the BD FACSCanto II system with the BD FACSuite Clinical software (BD Biosciences). Prior to flow cytometry, cells were resuspended in phosphate-buffered saline (PBS) comprising 2% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO), 2-mM EDTA (Sigma-Aldrich), 1- μ g/mL propidium iodide (PI; Sigma-Aldrich) to exclude dead cells. Flow cytometric evaluations were accomplished with the FlowJo software. For DXMS treatment, the eosinophils were coped with 1.6-mM DXMS (Sigma-Aldrich) for 24 h before cell transfection. Control cells were treated with the same volume of PBS.

Cell transfection

CREB overexpression vector (CREB) and control, and short hairpin RNA (shRNA)-binding CREB (shCREB) and control (shNC) were purchased from Sangon Biotech (Shanghai, China). Lipofectamine 2000 (SITRAN-RO; Sigma-Aldrich) was applied to conduct transfection of eosinophils conforming to the directions. After transfection, the eosinophils were further analyzed by flow cytometry.

Western blot analysis

Western blot analysis was performed according to Rahmati et al. (2021, 2023).^{22,23} Proteins were separated from eosinophils and lung tissues by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (GVWP02500; Sigma-Aldrich). After blocking and hatched with primary antibodies at 4°C overnight, the membrane was exposed to goat anti-rabbit immunoglobulin G (IgG; ab205718; 1:2500; Abcam, Cambridge, MA) for 1 h. The protein bands were evaluated by enhanced chemiluminescence (ECL)-plus reagent (32132X3; Invitrogen, Carlsbad, CA). Following were the primary antibodies: anti-CREB (ab32515; 1:1000; Abcam), anti-long-chain fatty acid-CoA ligase 4 (ACSL4; ab155282; 1:1000; Abcam), anti-transferrin receptor protein 1 (TFR1; ab214039; 1:1000; Abcam), anti-ferritin heavy chain 1 (FTH1; 3998; 1:1000; CST, Danvers, MA), anti-GPX4 (ab125066; 1:1000; Abcam), and anti- β -actin (ab8226; 1:1000; Abcam).

Flow cytometry

After diverse treatments, 2×10^4 eosinophils were cultivated in 6-well plates. Then, an Annexin V-FITC/PI kit (Sigma-Aldrich) was used to stain eosinophils in dark at 37°C. Subsequently, a flow cytometer (BD Biosciences) was applied to evaluate the number of viable cells. Annexin V-FITC/PI cells were considered as viable eosinophils. To assess the content of reactive oxygen species (ROS), eosinophils were resuspended in binding buffer (Sigma-Aldrich) and exposed to 20- μ M 2,7-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich) at 37°C for 0.5 h. The samples were reaped on MACSQuant Analyzer 10 and evaluated by using the FlowJo software.

Iron assay

To assess the concentration of iron in eosinophils, an iron assay kit (ab83366; Abcam) was used conforming to the directions for specific experimental procedures.

Enzyme-linked immunosorbent serological assay (ELISA)

After diverse treatments, the eosinophils were lysed, and the lysate was centrifuged for evaluating the contents of malondialdehyde (MDA; ab118970; Abcam), GSH (ab112132; Abcam), and GPX4 (M0937; SenBeiJia, Nanjing, China) using the ELISA kits. Besides, the PB samples and alveolar lavage fluid (ALF) were homogenized and centrifuged to obtain supernatant. The contents of IL-5 (ab204523; Abcam) and IL-4 (ab100710; Abcam) were evaluated by applying test procedures instructed on ELISA kits.

Creation of OVA-induced mouse model

Thirty C57BL/6 mice (male, 3 days old) were randomly divided into the following six groups (five mice in each group): sham, OVA, OVA+Ad-shNC, OVA+Ad-shCREB, DXMS+OVA, and DXMS+OVA+Ad-shCREB. The mice were fed in 12/12 h light/dark condition. During the whole experiment, the newborn mice were fed with mother's milk. After 2 days of adaptation, except for the sham group, the mice were sensitized on day 5 and day 10 by intraperitoneal (i.p.) injection of OVA (10 μ g; Sigma-Aldrich) and then treated with 3% sprayed OVA for 10 min every day until day 20. The sham group mice were treated with normal saline. For DXMS treatment,²⁴ the DXMS+OVA group and DXMS+OVA+Ad-shCREB group mice were treated with 5- μ g/kg DXMS (i.p. injection; Sigma-Aldrich) one day prior to the first sprayed OVA treatment, which lasted for five successive days.

Downregulation of CREB

The pAdeasy-shCREB adenovirus (Ad-shCREB) and control (Ad-shNC) were constructed by GenePharma (Shanghai, China). Two days prior to the first sensitization, the

OVA+Ad-shNC group and OVA+Ad-shCREB group mice received i.p. injection of Ad-shNC or Ad-shCREB.

Immune cell count assay

The blood was collected from the retroorbital plexus at 24 h after the last atomized OVA treatment. The number of leukocytes and eosinophils in the blood were discovered using HEMAVET 950 (Drew Scientific Inc., Oxford, UK).

Collection and analysis of alveolar lavage fluid

Airway inflammatory response was estimated at 24 h after the last sprayed OVA treatment. ALF was collected via transporting 0.8-mL cold PBS (Sigma-Aldrich) through endotracheal intubation and soft suctioning of the fluid. Lung lavage was carried out for three times.

Histologic assessment

The specific steps taken were according to the report of Bostani et al.²⁵ The lung tissues were fixed in 4% paraformaldehyde (30525-89-4; Sigma-Aldrich) for 14 h and embedded in paraffin (Sigma-Aldrich). The samples were sectioned (5 μ m), dewaxed, dehydrated, and stained with hematoxylin and eosin (H&E; H3136 and 318906; Sigma-Aldrich). The images were obtained through a light microscope (Leica, Wetzlar, Germany) at \times 200 magnification.

Statistical assay

All experiments were repeated for three times. Statistical analyses were accomplished using SPSS 22.0 (SPSS Inc., Chicago, IL). The data were presented as mean \pm SD. Paired comparison was evaluated using Student's *t*-test. Multiple comparison was conducted by applying analysis of variance (ANOVA). $P < 0.05$ was considered as statistically significant.

Results

Inhibition of CREB-induced eosinophils death

To examine the functioning of CREB on eosinophils, we isolated eosinophils from the PB of IL5T neonatal mice. As displayed in Figure 1A, the expression of CREB in eosinophils was enhanced by CREB overexpression vector transfection but reduced by shCREB transfection ($P < 0.05$). These results confirmed that overexpression and knockdown of CREB were effective. Moreover, we discovered that the viability of eosinophils (Figure 1B, $P < 0.05$) and the mean fluorescence intensity (MFI) of Siglec F and CCR3 (Figure 1C, $P < 0.05$) were ameliorated by CREB upregulation but attenuated by CREB knockdown. Therefore, these results revealed that downregulation of CREB triggered the cell death of eosinophils.

Inhibition of CREB promoted ferroptosis of eosinophils

Next, we analyzed the effects of CREB on ferroptosis of eosinophils. The results showed that the levels of iron (Figure 2A, $P < 0.05$), MDA (Figure 2B, $P < 0.05$), reactive oxygen species (ROS; Figures 2C and 2D; $P < 0.05$) attenuated by upregulated CREB but augmented by downregulated CREB. Moreover, the contents of GSH and GPX4 were enhanced after CREB overexpression but reduced by shCREB transfection (Figure 2B, $P < 0.05$). In addition, the contents ACSL4 and TFR1 in eosinophils were apparently declined in the CREB group but augmented in the shCREB group (Figure 2E, $P < 0.05$). On the contrary, the levels of FTH1 and GPX4 were boosted by CREB upregulation but reduced by silencing CREB (Figure 2E, $P < 0.05$). Hence, we revealed that downregulation of CREB could obviously contribute to ferroptosis of eosinophils.

Inhibition of CREB promoted DXMS-induced eosinophils death

We analyzed the influence of CREB on anti-inflammatory effect of DXMS. We found that the viability of eosinophils was reduced by DXMS treatment in shNC and shCREB groups (Figure 3A, $P < 0.05$). Interestingly, shCREB transfection could enhance the inhibitory effect of DXMS on the viability of eosinophils. Besides, the levels of iron (Figure 3B, $P < 0.05$), ROS (Figures 3C and 3D, $P < 0.05$), ACSL4, and TFR1 (Figure 3E, $P < 0.05$) were increased but levels of FTH1 and GPX4 (Figure 3E, $P < 0.05$) were reduced by DXMS treatment in shNC and shCREB-transfected eosinophils. Meanwhile, silencing CREB reinforced the relevant regulatory effect of DXMS on eosinophils. These outcomes suggested that downregulation of CREB facilitated DXMS-induced eosinophils death.

Inhibition of CREB alleviated OVA-induced pathological state in mice

We discovered the effects of CREB on OVA-induced asthma in mice. The mice were randomly divided into four groups: sham, OVA, OVA+Ad-shNC, and OVA+Ad-shCREB. The results showed that the level of CREB in lung tissue was evidently boosted in the OVA group mice, compared to the sham group. After i.p. injection of Ad-shCREB, the level of CREB was markedly decreased, compared to the OVA+Ad-shNC group, thereby suggesting that Ad-shCREB obviously diminished CREB abundance in lung tissues (Figure 4A, $P < 0.05$). The result of histologic assessment confirmed that in both OVA and OVA+Ad-shNC groups, bronchial and alveolar structures were disordered and missing, and the inflammatory cell infiltration around bronchial mucosa was increased, compared to the sham group. After Ad-shCREB co-treatment, the inflammatory cell infiltration was apparently diminished (Figure 4B). In addition, the number of leukocytes and eosinophils in the blood (Figure 4C, $P < 0.05$) and the contents of IL-5 and IL-4 in PB and ALF (Figure 4D, $P < 0.05$) were increased after OVA treatment whereas

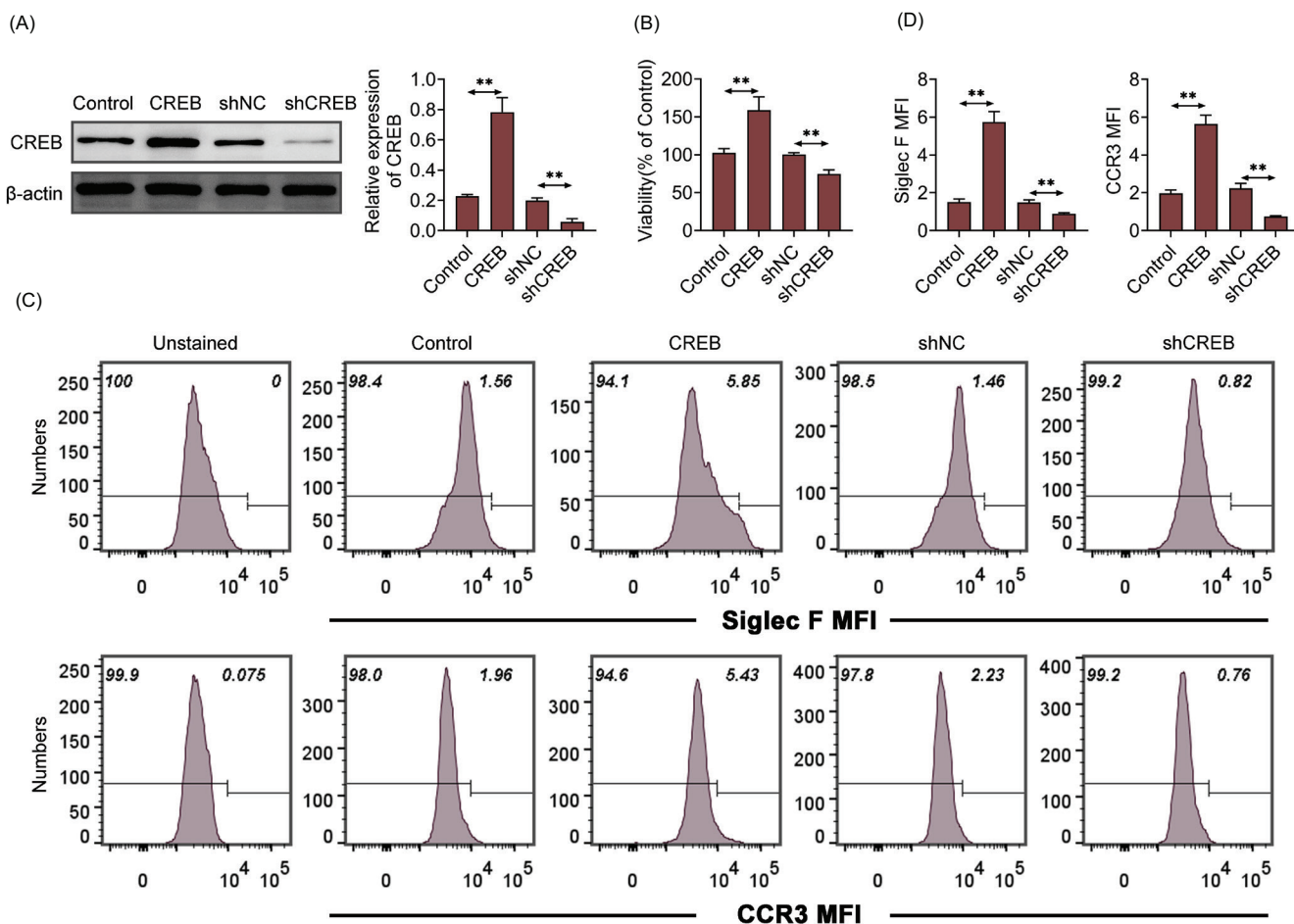


Figure 1 Inhibition of CREB reduced eosinophils viability and mean fluorescence intensity of Siglec F and CCR3. (A) The content of CREB in eosinophils was examined by Western blot analysis. (B) The viability of eosinophils was assessed by flow cytometry. (C) The mean fluorescence intensity of Siglec F and CCR3 was examined by flow cytometry. ** $P < 0.01$. The data were presented as mean \pm SD.

this increase was diminished by Ad-shCREB co-treatment. Therefore, downregulation of CREB diminished OVA-induced asthmatic airway inflammation by declining both number of inflammatory cells and levels of proinflammatory factors.

Inhibition of CREB reinforced the regulatory effect of DXMS in OVA mice

Finally, we explored the influence of CREB on anti-inflammatory effect of DXMS in OVA mice. We found that DXMS treatment could reduce OVA-induced alveolar inflammatory cell infiltration and structural damage, and Ad-shCREB co-treatment significantly enhanced the anti-inflammatory effect of DXMS (Figure 5A). Moreover, DXMS treatment significantly inhibited the improved effect of OVA induction on the number of leukocytes and eosinophils in the blood (Figure 5B, $P < 0.05$) and the levels of IL-5 and IL-4 in PB and ALF (Figure 5C, $P < 0.05$). Co-treatment of Ad-shCREB improved the inhibition effect of DXMS. Hence, these results demonstrated that down-regulated CREB reinforced the anti-inflammatory effect of DXMS in OVA-induced mice.

Discussion

In this study, we discovered that the level of CREB in eosinophils was enhanced by CREB overexpression vector transfection but reduced by shCREB transfection. We revealed that downregulation of CREB triggered the cell death of eosinophils. Downregulation of CREB obviously contributed to ferroptosis of eosinophils. In addition, downregulation of CREB facilitated DXMS-induced death of eosinophils. Moreover, we established an asthma mouse model by OVA treatment. The CREB was upregulated in OVA group mice, and Ad-shCREB treatment obviously downregulated CREB level. Downregulation of CREB diminished OVA-induced asthmatic airway inflammation by reducing the number of inflammatory cells and the levels of proinflammatory factors. Downregulated CREB reinforced the anti-inflammatory effect of DXMS in OVA-induced mice. Therefore, we confirmed that inhibition of CREB promoted the effect of GC on airway inflammation in pediatric asthma by promoting ferroptosis of eosinophils.

Asthma has affected 30 billion people globally, and is quite common in children.²⁶ Pediatric asthma affects approximately 14% of children, and in about 15% of children

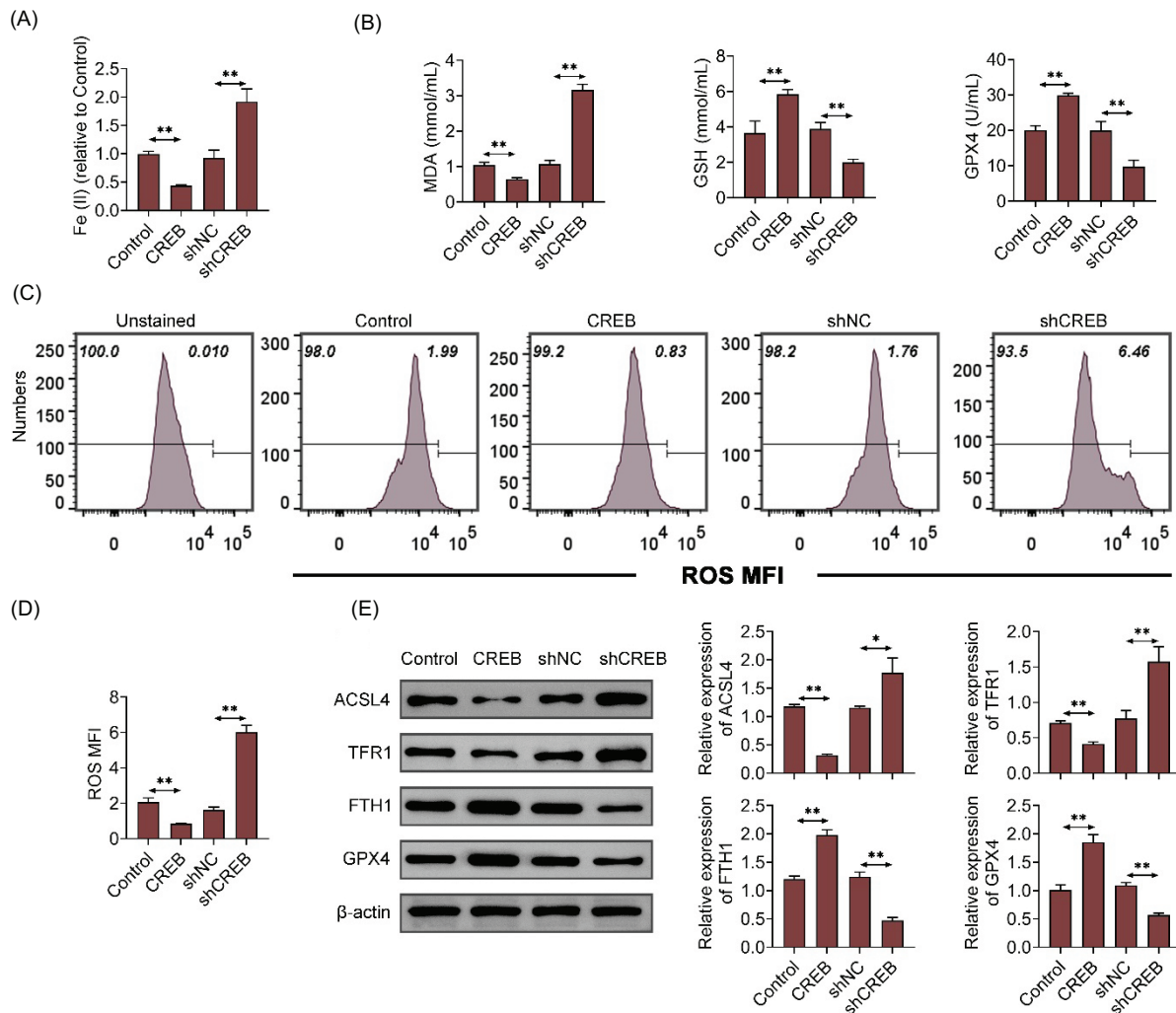


Figure 2 Inhibition of CREB promoted ferroptosis and oxidative stress of eosinophils. (A) The concentration of iron in eosinophils was assessed by using commercial kits. (B) The contents MDA, GSH, and GPX were discovered by ELISA. (C and D) The mean fluorescence intensity of ROS in eosinophils was evaluated by flow cytometry. (E) The contents of ACSL4, TFR1, FTH1, and GPX4 in eosinophils were examined by Western blot analysis. * $P < 0.05$, ** $P < 0.01$. The data were presented as mean \pm SD.

with respectable management compliance for a year, asthma is still not fully controlled.^{27,28} Pediatric asthma is caused by multiple factors, such as infections, smoke, allergens, hormones, exercise, genetic mutations, and eosinophilia.^{2,29-31} In current clinical practice, GC is frequently utilized to treat pediatric asthma, but the therapeutic effect is limited.³² With the development of molecular therapy, targeted gene therapy is attracting more attention.³³ We explored the functions of CREB in pediatric asthma.

Eosinophils play an important role in asthma and other allergic diseases. The number of eosinophils increases greatly during asthma attacks and releases a variety of disease-causing factors, such as interleukin-5 (IL-5), IL-13, IL-4, and IL-9. These factors lead to airway inflammation, and increase mucus secretion, airway remodeling, and other pathophysiological changes in asthma.⁸ Targeting eosinophils is a novel approach for treating severe asthma. Several related drugs, such as eosinophil inhibitors and anti-IL-5 antibodies, are already in clinical trials.³⁴ The goal of these drugs is to reduce the number and function of eosinophils, thereby reducing asthma. Studies have shown

that targeting eosinophils has potential efficacy in reducing asthma attacks, lowering hormone doses, improving lung function, and reducing emergency room treatment.⁷ However, more research is needed to determine the long-term efficacy and safety of these treatments.

The current studies have found that eosinophils have become the preferred target for treating asthma.³⁵ In clinical studies, some immunotherapy drugs targeting eosinophils (mepolizumab and reslizumab) have been found to be effective in treating severe asthma.⁸ Eosinophils play an important role in regulating inflammatory response in allergic asthma.³⁴ Suppressing the body's inflammatory response is essential to prevent severe lung tissue damage caused by asthma. It has been found that eosinophils at the inflammatory site have a long lifespan, which may lead to slower clearance of inflammatory cells, further inhibiting the regression of airway inflammation.¹³ Recently, therapy targeting eosinophils has become a hot topic in asthma-related research. Treatments that promote death of eosinophils could have positive implications for controlling allergic asthma.³⁶

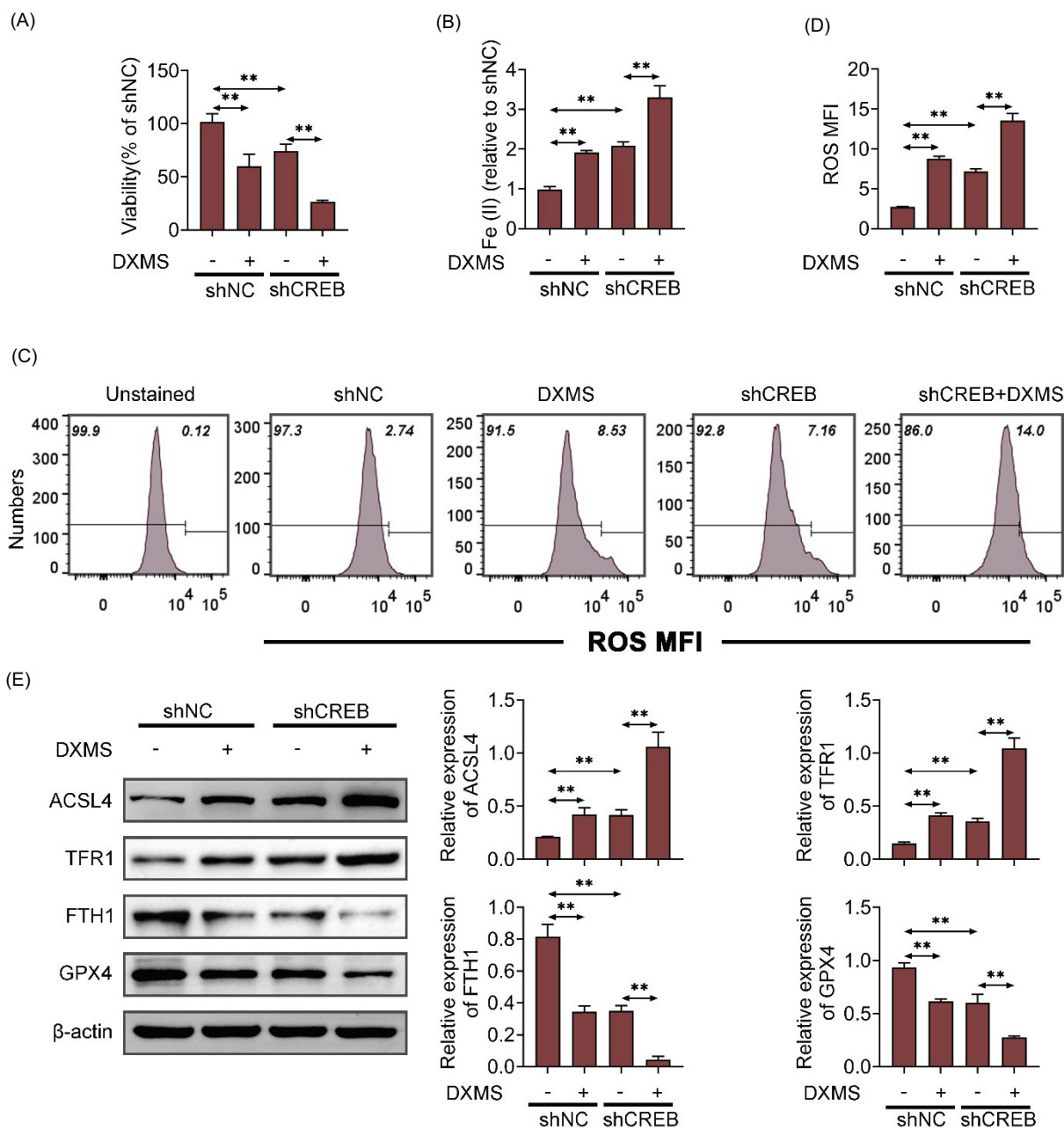


Figure 3 Inhibition of CREB promoted DXMS-induced ferroptosis and oxidative stress of eosinophils. (A) The viability of eosinophils was evaluated by flow cytometry. (B) The concentration of iron in eosinophils was measured by using the commercial kit. (C and D) The mean fluorescence intensity of ROS in eosinophils was assessed by flow cytometry. (E) The levels of ACSL4, TFR1, FTH1, and GPX4 in eosinophils were inspected by Western blot analysis. $^{**}P < 0.01$. The data were presented as mean \pm SD.

CREB is an important transcription factor that regulates various cellular processes, such as proliferation and migration.^{19,37,38} Koopmans et al. confirmed that CREB-binding protein/ β -catenin modulated growth and extracellular matrix remodeling of airway smooth muscles.³⁹ Moreover, Koga et al. revealed that CREB could modulate TNF- α -induced GM-CSF secretion in lung fibroblasts.⁴⁰ A study conducted by Wu et al. demonstrated that CREB activation enhanced airway smooth muscle cell proliferation and played an important role in airway remodeling in asthma.⁴¹ In the present study, we found that the level of CREB in eosinophils was enhanced by CREB overexpression

vector transfection but reduced by shCREB transfection. We revealed that downregulation of CREB triggered the cell death of eosinophils.

ACSL4, TFR1, FTH1, GPX4, and MDA are the molecules associated with oxidative stress and iron metabolism.⁴² ACSL4 is a membrane-bound esterase that catalyzes the esterification of fatty acids with coenzyme A on cell membrane. Increased expression of ACSL4 leads to the generation of peroxidized lipids, which can increase ROS production. TFR1 is an iron carrier protein that is involved in iron uptake and metabolism, and its expression regulates iron levels and ROS production. FTH1 is an iron protein that

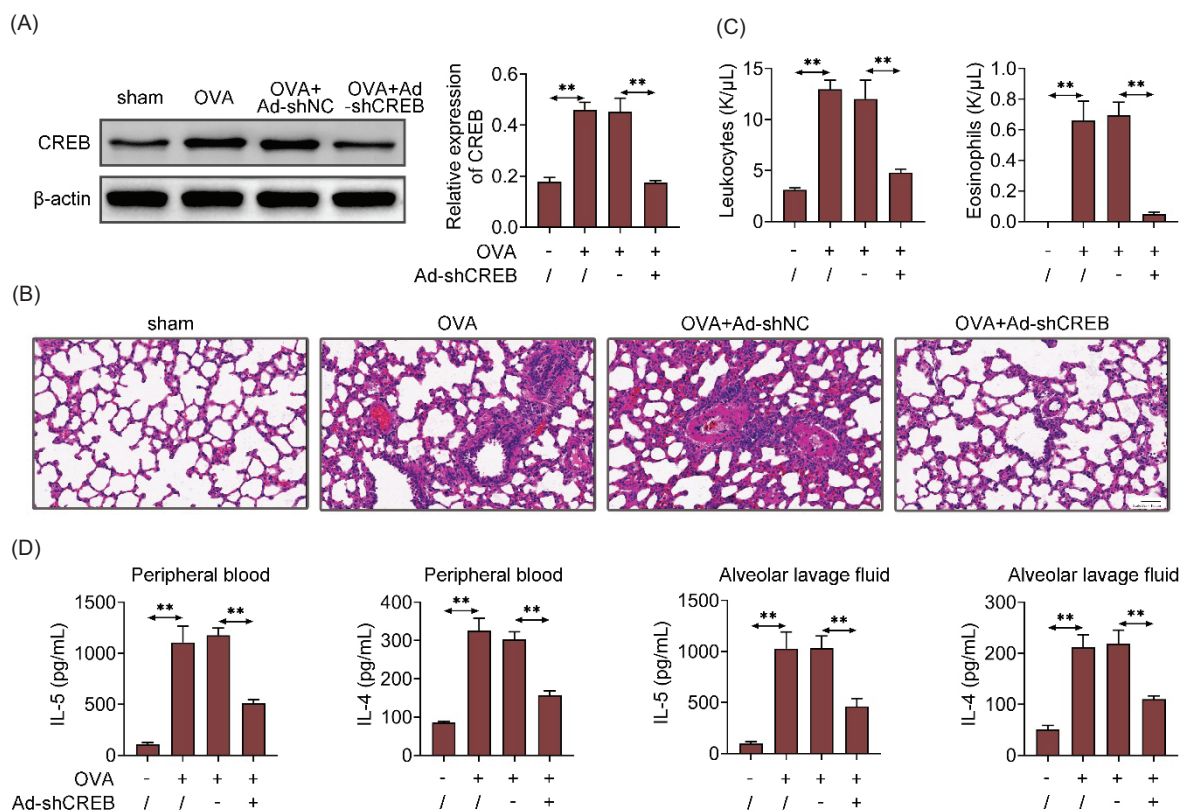


Figure 4 Inhibition of CREB alleviated OVA-induced pathological state in mice by declining the quantity of inflammatory cells and the levels of proinflammatory factors. (A) The content of CREB in eosinophils was examined by Western blot analysis. (B) The bronchial and alveolar structures were evaluated by H&E staining ($\times 200$). (C) The quantity of leukocytes and eosinophils in the blood was measured using HEMAVET 950. (D) The contents of IL-5 and IL-4 were examined by ELISA. ** $P < 0.01$. The data were presented as mean \pm SD.

binds and store free iron, protecting cells from oxidative stress caused by iron overload. GPX4 is a glutathione peroxidase that reduces harmful peroxidized lipids to harmless lipids and reduces ROS production. MDA is an oxidative stress marker and is one of the metabolites of peroxidized lipids which reflects the degree of cellular oxidative stress.⁴²

Chen et al. confirmed that CREB was involved in modulating ferroptosis of lung fibroblasts (HT1080 cells).⁴³ Furthermore, Xiong et al.⁴⁴ and Hu et al.⁴⁵ revealed that CREB participated in regulating ferroptosis of neuronal cells and liver cells. We found that knockdown of CREB could obviously promote ferroptosis of eosinophils. Besides, downregulation of CREB facilitated DXMS-induced eosinophils death. These results were in accordance with the results demonstrated by Chen et al.⁴³ and Xiong et al.⁴⁴

Wu et al.¹³ found that ferroptosis-inducing agents (FINS) promoted eosinophils death, and reinforced the anti-inflammatory effect of GCs in the management of allergic disorders. Their studies suggested that combined ferroptosis-apoptosis treatment could act as an operative management for allergic airway inflammation. In the present study, we established an asthma mouse model by OVA treatment. The CREB was upregulated in the OVA group, and the Ad-shCREB treatment obviously downregulated the CREB level. Downregulation of CREB attenuated OVA-induced asthmatic airway inflammation by reducing the number of inflammatory cells and the levels of proinflammatory factors.

Downregulated CREB reinforced the anti-inflammatory effect of DXMS in OVA-induced mice. These results were comparable to the results described by Wu et al.¹³ For the first time, we found that inhibition of CREB could help DXMS in treating pediatric asthma. This study enhanced our understanding of the molecular mechanism of asthma and provided a new idea for treating asthma. The present research has some limitations. We only investigated the effects of CREB on eosinophils and in asthma mouse models, and the findings need further confirmation in clinical practice.

Conclusion

In summary, our findings demonstrated that inhibition of CREB promoted the effect of DXMS on airway inflammation in pediatric asthma by promoting ferroptosis of eosinophils. In the future studies, we would develop corresponding CREB inhibitor combined with GC to treat pediatric asthma. This study suggested that CREB is a potential target for treating pediatric asthma.

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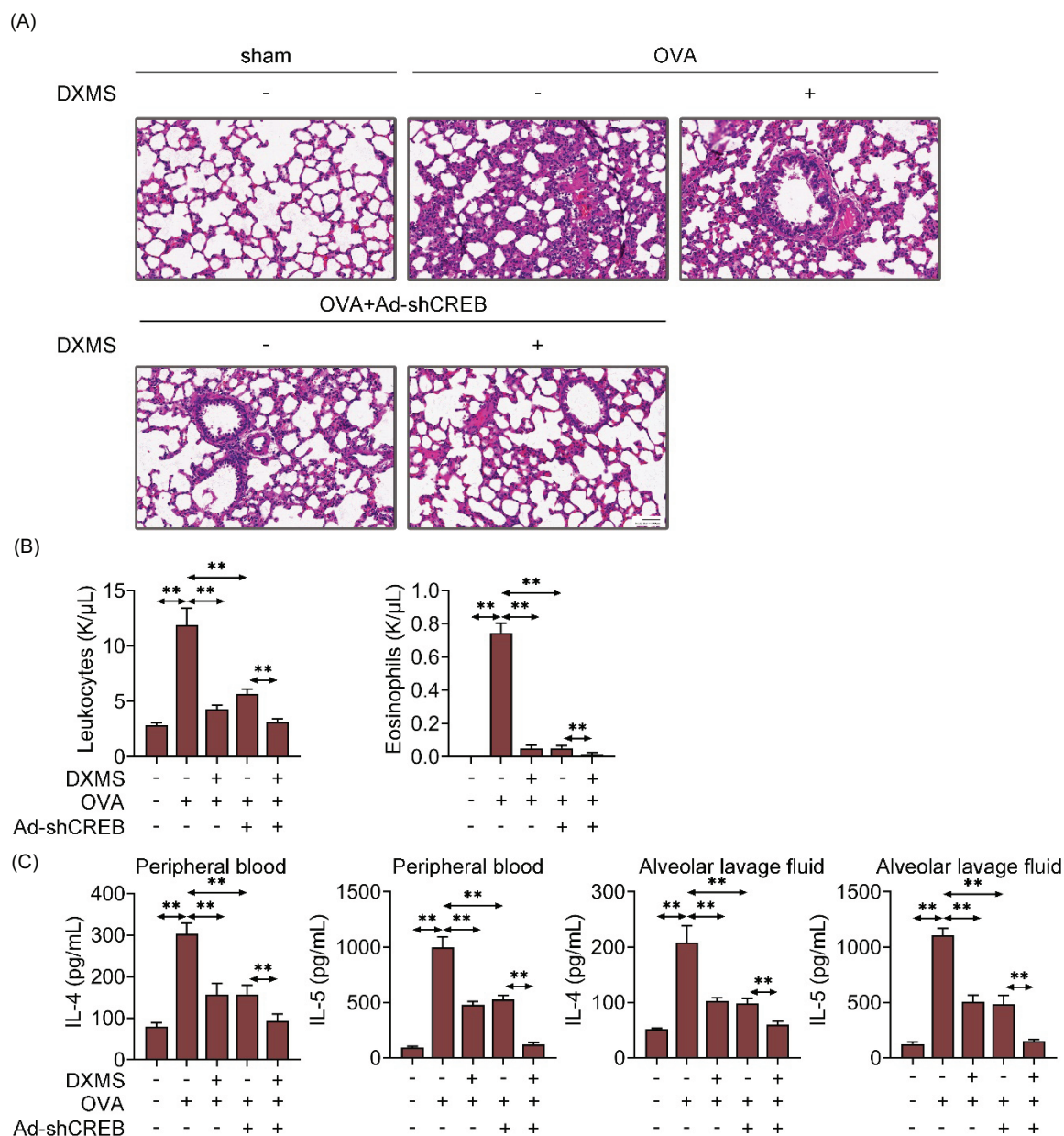


Figure 5 Inhibition of CREB reinforced the anti-inflammatory effect of DXMS in OVA mice. (A) The bronchial and alveolar structures were appraised by H&E staining ($\times 200$). (B) The quantity of leukocytes and eosinophils in the blood was examined by HEMAVET 950. (C) The contents of IL-5 and IL-4 were examined by ELISA. $^{**}P < 0.01$. The data were presented as mean \pm SD.

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 there was no conflict of interest to disclose.

Author Contributions

Tong Yu and Yiping Yu designed the study, completed the experiment, and supervised data collection. Yingyu Ma analyzed and interpreted the data. Guoqing Chen prepared the manuscript for publication and reviewed its draft. All authors read and approved the final manuscript.

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