Thieme

Icariin Improves Glucocorticoid Resistance in a Murine Model of Asthma with Depression Associated with Enhancement of GR Expression and Function

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ABSTRACT

Icariin, a flavonoid glycoside isolated from *Epimedium brevicornum*, exerts a variety of biological activities. However, its effects on depression-induced glucocorticoid resistance in asthma and the underlying mechanisms have not been elucidated. In this study, a murine model of asthma with depression was established by exposure to ovalbumin combined with chronic unpredictable mild stress, and icariin was given orally during ovalbumin challenge and chronic unpredictable mild stress exposure. Depression-like behaviors were assessed by the open field test, forced swim test, and tail suspension test. The characteristic features of allergic asthma, including airway hyperreactivity, histopathology, inflammatory cytokine levels in bronchoalveolar lavage fluid, and immunoglobulin E and corticosterone levels in serum, were examined. Following splenocyte isolation *in vitro*, the inhibitory effects of corticosterone on the proliferation and cytokine secretion of splenocytes, glucocorticoid receptor DNA-binding activity, and expression of p-glucocorticoid receptor s226, glucocorticoid receptor α, and p-p38 mitogen-activated protein kinase in splenocytes were determined. We found that icariin had limited effects on depression-like behaviors, however, it markedly suppressed airway hyperresponsiveness, inflammatory infiltration in lung tissues, levels of interleukin-4, interleukin-5, and interleukin-6 in bronchoalveolar lavage fluid, and immunoglobulin E in serum. Furthermore, icariin improved the inhibitory effects of corticosterone on lipopolysaccharide-stimulated splenocytes, increased the glucocorticoid receptor expression and glucocorticoid receptor DNA-binding activity, and inhibited the phosphorylation of glucocorticoid receptors S226 and p38 mitogen-activated protein kinase. Taken together, icariin improved glucocorticoid resistance in a murine model of asthma with depression associated with enhancement of glucocorticoid receptor function and glucocorticoid receptor expression, and its effects on the glucocorticoid receptor function were related to decreased phosphorylation of glucocorticoid receptors S226 and p38 mitogen-activated protein kinase.

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* These two authors contributed equally to this work.

ABBREVIATIONS

AHR   airway hyperresponsiveness
BALF   bronchoalveolar lavage fluid
CCK-8   cell count kit-8
CUMS   chronic unpredictable mild stress
DEP   depression
Dex   dexamethasone
GR   glucocorticoid receptor
H&E   hematoxylin and eosin
IgE   immunoglobulin E
IL   interleukin
INF   interferon
MAPK   mitogen-activated protein kinase
OD   optical density
OFT   open field test
OVA   ovalbumin
Penh   enhanced pause

▶ **Fig. 1** Chemical structure of icariin.

Introduction

Asthma affects more than 300 million individuals around the world, and its global burden has increased by almost 30% in the past 20 years [1]. Individuals with asthma were reported to have a higher prevalence of depression than the general population [2]. Depression is closely related to poorer control and quality of life in adults with asthma, as well as a high rate of asthma exacerbation [3,4], which is attributed to increased airway inflammation aggravated by depression [5]. A diminished inhibitory action of endogenous glucocorticoid was involved in the pathogenesis of chronic stress-induced exacerbation of airway inflammation in patients with asthma [6]. A significant proportion of depressed patients exhibited steroid resistance with elevated levels of inflammatory biomarkers, as well as increased secretion of the stress hormone cortisol [7]. However, the mechanisms through which depression-induced steroid resistance in asthma have yet to be discovered.

p38 MAPK is a class of evolutionarily conserved serine/threonine mitogen-activated protein kinases, and it links extracellular signals, such as stress, to the intracellular machinery to influence a variety of cell responses [8]. The p38 MAPK-induced phosphorylation of GR S226 in cytoplasm can result in defective GR nuclear translocation, which leads to weakened GR function and thus steroid insensitivity [9]. Furthermore, activation of p38 MAPK has been shown to influence behaviorally relevant pathophysiologic activities, and p38 MAPK was found to be significantly activated in patients with depression [10]. Thus, p38 MAPK might be a good target for improvement of depression-induced steroid resistance in asthma. Indeed, a p38 MAPK inhibitor (SB203580) has been demonstrated to preferentially restore steroid sensitivity [11]. However, the adverse effects of the p38 MAPK inhibitor remain uncertain, and new drugs are still needed for asthma with steroid resistance that target p38 MAPK signaling with little side effects.

Epimedium brevicornum (Berberidaceae) Maxim has been widely used as a tonic herb to nourish the kidney Qi according to the famous Chinese medicine document Ben Cao Gang Mu [12].
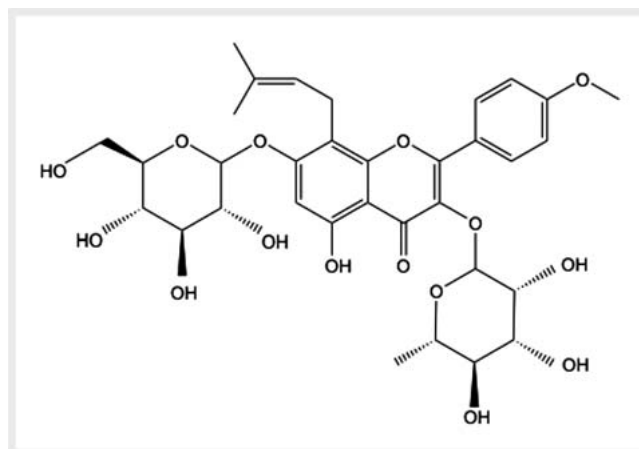
Icariin (▶ **Fig. 1**), a main active constituent of *E. brevicornum* Maxim, exerts a wide range of biological activities and pharmacological properties, including anti-inflammatory, immunomodulatory, estrogenic, and anti-osteoporotic effects [13–15]. Recently, icariin was demonstrated to effectively inhibit the activity of p38 MAPK *in vivo* and *in vitro* [16, 17]. However, its effects on depression-induced steroid resistance in asthma and the mechanisms involved have not been fully illustrated. Therefore, the objective of this study was to investigate the possible mechanisms of icariin action on steroid resistance in a murine model of asthma with depression, with particular emphasis on the p38 MAPK signaling pathway.

Results

As shown in ▶ **Fig. 2**, the asthma-DEP group displayed depressive behavior, as evidenced by increased duration of immobility in both the forced swim test ($p < 0.01$) (▶ **Fig. 2a**) and the tail suspension test ($p < 0.05$) (▶ **Fig. 2b**) when compared to the single asthma group and saline control group. These results suggested that 4 weeks of CUMS induced depression-like behaviors. Furthermore, the time in the center area in the OFT test was significantly reduced in the asthma-DEP group compared to the saline control group. However, the results of the above behavioral tests were not significantly changed after administration with icariin, thereby illustrating that icariin had limited effects on the depression-like behaviors in this study.

To evaluate the effects of icariin on OVA-induced AHR, the Penh was used as an indicator of airway responsiveness at baseline and following delivery of increasing concentrations of inhaled methacholine (6.25 to 25 mg/mL). As shown in ▶ **Fig. 3**, mice in the asthma-DEP group displayed increased Penh when compared to mice in both the single asthma group and saline control group ($p < 0.01$), thereby demonstrating that AHR was aggravated by depression induced by CUMS. The oral administration of icariin at 25, 50, and 100 mg/kg and Dex caused an obvious reduction in Penh to methacholine at 6.25, 12.5, and 25 mg/mL compared to mice in the asthma-DEP group ($p < 0.01$).
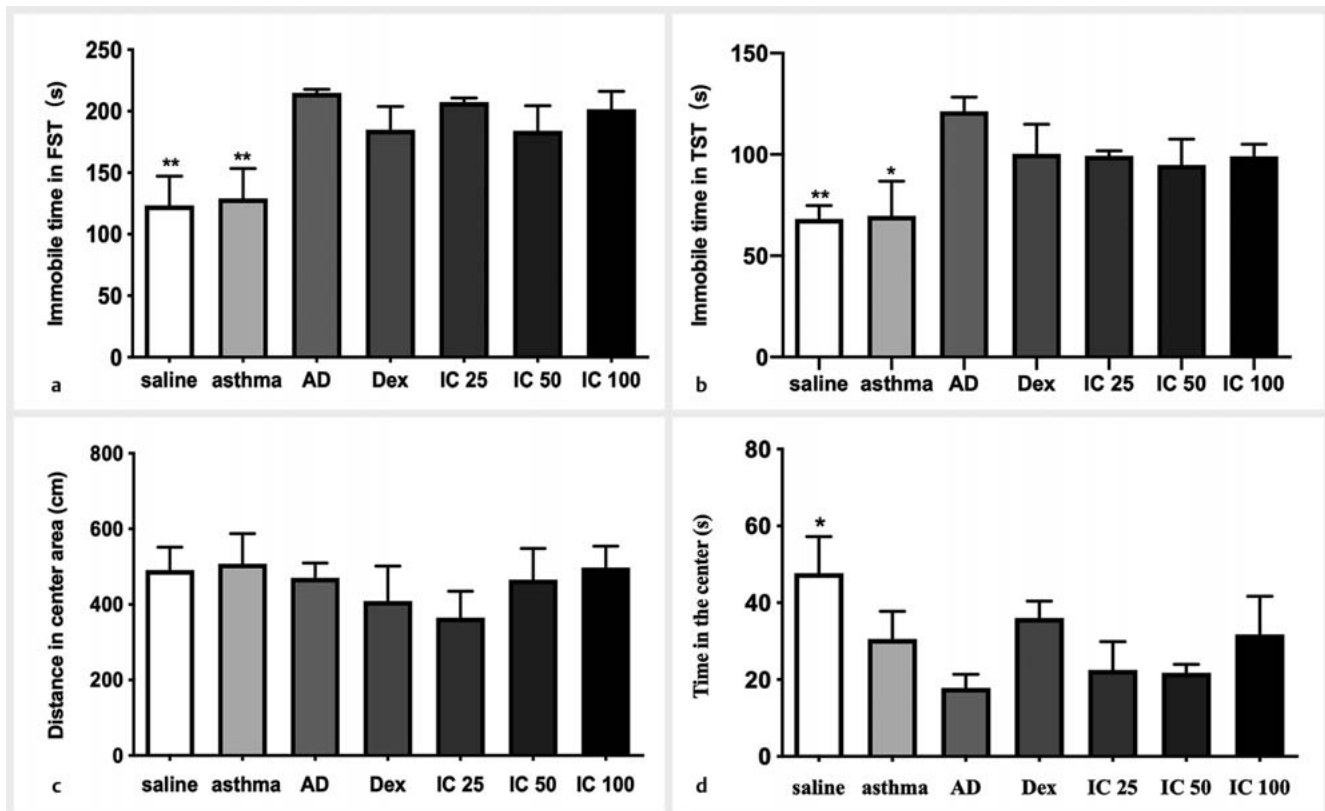
▶ **Fig. 2** Behavioral assessment of depression-like symptoms. **a** The immobility time in the forced swimming test (FST). **b** The immobility time in the tail suspension test (TST). **c** The travel distance in the center zone in the open field test. **d** The time in the center in the open field test. Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC_{25}, IC_{50}, IC_{100}: the low, median, and high dosage of icariin groups treated with 25, 50 and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. All data are expressed as the mean ± SEM (n = 6–8 each group); *p < 0.05 and **p < 0.01 versus asthma in depression mice.

As shown in ▶ **Fig. 4**, lung tissues were further examined after H&E staining to confirm the inhibitory effects of icariin on airway inflammation. When compared to the single asthma group and saline control group, mice in the asthma-DEP group displayed aggravated airway inflammation, as evidenced by the increased inflammatory score of H&E staining (p < 0.01). However, oral administration of icariin at 50 and 100 mg/kg and Dex dramatically attenuated the inflammation infiltrated around the airways and blood vessels (p < 0.05 and p < 0.01, respectively).

As shown in ▶ **Fig. 5**, mice in the asthma-DEP group showed remarkable airway inflammation with increased levels of IL-4, IL-5, IL-13, IL-6, and TNF-α (p < 0.05), and a decreased level of INF-γ in BALF (p < 0.05), as well as a higher level of antigen-specific IgE in serum (p < 0.01) when compared to mice in the saline control group. Furthermore, mice in the asthma-DEP group displayed higher levels of IL-4 and IL-5 in BALF compared to the single asthma group (p < 0.05 and p < 0.01, respectively). However, after the oral administration of icariin or Dex, levels of IL-4, IL-5, and IL-6 in BALF and IgE in serum were dramatically reduced (p < 0.05 and p < 0.01, respectively).

The results presented in ▶ **Figs. 5h** and **6** demonstrate the beneficial effects of icariin on steroid resistance. ▶ **Fig. 5h** shows that the corticosterone level in the group of asthma-DEP was sig-

nificantly higher than that in the single asthma group and saline control group (p < 0.01), whereas Dex and icariin at doses of 50 and 100 mg/kg remarkably inhibited corticosterone production (p < 0.01 and p < 0.05, respectively). ▶ **Fig. 6** clearly shows the dose-dependent inhibition of the steroid on splenocyte function in the saline group. The inhibitory effects of corticosterone were markedly abolished on splenocyte proliferation and cytokine secretion in the asthma-DEP group when compared to both the saline and single asthma groups (p < 0.01 and p < 0.05, respectively). Treatment with icariin, but not Dex, facilitated the responsiveness of splenocytes to corticosteroid, as evidenced by icariin at doses of 50 and 100 mg/kg, which markedly ameliorated steroid sensitivity in the inhibition of splenocyte proliferation and cytokine secretion of IL-6 and TNF-α (p < 0.01 and p < 0.05, respectively).

To determine whether the beneficial effects of icariin on glucocorticoid sensitivity were attributed to the improved GR function or GR expression, GR DNA-binding activity and GR expression in splenocytes were evaluated. As shown in ▶ **Fig. 7**, splenocytes in the asthma-DEP group displayed significantly lower GR DNA-binding activity and decreased GR expression when compared to the single asthma group and the saline control group (p < 0.01 and p < 0.05, respectively). However, administration with icariin at

doses of 50 and 100 mg/kg remarkably enhanced GR DNA-binding activity and total GR expression in splenocytes.

Increased GR S226 phosphorylation can result in the inhibition of GR nuclear translocation, thereby inducing reduced GR DNA-binding activity. p38 MAPK signaling has been previously shown to play an important role in regulating GR S226 phosphorylation. Therefore, the phosphorylation of GR S226 and p38 MAPK in splenocytes was further determined. As shown in ▶ **Fig. 7**, GR S226 phosphorylation was significantly increased in splenocytes in the asthma-DEP group when compared to the single asthma and saline control groups ($p < 0.01$ and $p < 0.05$, respectively). The p38 MAPK phosphorylation was significantly increased in the asthma-DEP group compared to the saline group ($p < 0.01$), but when compared to the single asthma group, no obvious change of p38 MAPK phosphorylation was observed. However, icariin at doses of 50 and 100 mg/kg remarkably inhibited phosphorylation of GR S226 and p38 MAPK in splenocytes ($p < 0.05$).

Discussion

The present study revealed that depression aggravated airway inflammation and steroid resistance in a murine model of asthma. Notably, administration with icariin dramatically improved airway inflammation and steroid sensitivity, which was associated with its enhancing GR function and GR expression. Its beneficial effects on the GR function could possibly be attributed to the inhibition of phosphorylation of GR S226 and p38 MAPK.

Patients with asthma often experience depression and vice versa [2]. Depression can decrease the control of asthma symptoms and increase the asthma exacerbations rate [4, 18]. In this study, OVA exposure combined with CUMS remarkably increased immobility duration in both the forced swim test and tail suspension test compared to single OVA exposure, indicating that CUMS successfully induced depression-like behaviors. As compared to the single asthma group, mice in the asthma-DEP group displayed more severe AHR, increased inflammatory cell infiltrates in the airways in histopathological examination, and higher IL-4 and IL-5 levels in the BALF, indicating that depression could aggravate airway inflammation. Our results showed that icariin had little effects on the depression-like behaviors. However, icariin significantly alleviated AHR, infiltration of inflammatory cells in airways, and levels of inflammatory cytokines in BALF, consistent with reduced levels of OVA-specific IgE in serum, which contributed to the anti-inflammatory effects of icariin in mice with asthma and depression.

It is now recognized that chronic inflammation in asthma is not just attributed to an exposure to provocative stimuli, leading to excess airway inflammation, but also to insufficient engagement of endogenous pro-resolving mediators [19]. Endogenous glucocorticoid exerts as a key anti-inflammatory mediator in asthma to restrain and resolve allergic inflammation [20]. However, a diminished inhibitory action of endogenous glucocorticoid, namely steroid resistance, was suggested to be involved in the pathogenesis of chronic stress-induced exacerbation of airway inflammation [6]. In this study, when compared to the saline control group, the corticosterone level in serum in the asthma-DEP group was significantly increased, however, the inhibitory effects of corticosterone
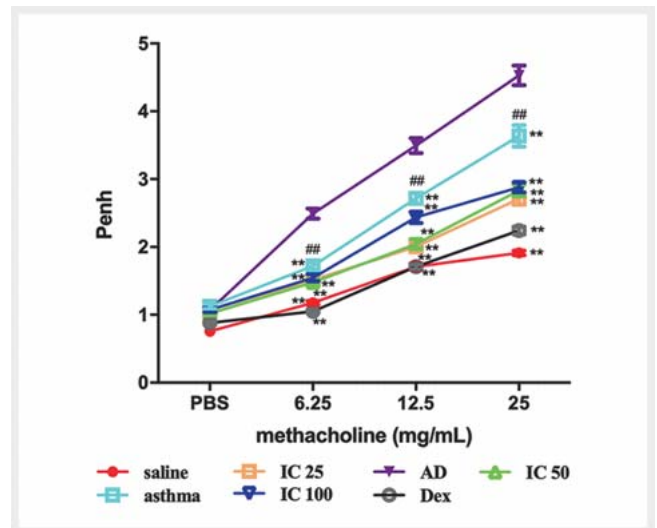
▶ **Fig. 3** Icariin suppressed methacholine-induced airway hyper-responsiveness (AHR). Airway responsiveness to aerosolized methacholine was evaluated by Buxco's whole-body barometric plethysmography in awake unrestrained mice. Mice were nebulized with PBS followed by increasing doses (6.25 to 25 mg/mL) of methacholine. The enhanced pause (Penh) index of airway hyper-reactivity was used as an indicator of changes in airway resistance. Treatment with icariin at 25, 50, and 100 mg/kg and dexamethasone (Dex) (0.5 mg/kg) caused a marked decrease in Penh compared to mice in the asthma with depression group ($p < 0.01$). Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC_{25}, IC_{50}, IC_{100}: the low, median, and high dosage of icariin groups treated with 25, 50, and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. Data are expressed as means ± SEM (n = 8–10 in each group); $^{**}p < 0.01$ versus asthma with depression group.

in the asthma-DEP group were significantly abolished on both splenocyte proliferation and cytokine secretion. These results suggest that OVA exposure combined with CUMS contributes to the glucocorticoid resistance. Furthermore, there was still statistical significance between the asthma-DEP group and the single asthma group in terms of the inhibitory effects of corticosterone, which indicates that CUMS exposure might aggravate glucocorticoid resistance. However, this study showed that administration with icariin at 50 and 100 mg/kg significantly ameliorated hypercortisolemia and enhanced inhibitory effects of corticosterone on splenocyte proliferation and cytokine production, which suggests that icariin could improve steroid sensitivity in an asthma model with depression. However, the mechanisms through which icariin ameliorates steroid insensitivity have not been fully elucidated.

Glucocorticoid exerts its effects by binding to a single cytoplasmic GR, followed by efficient GR translocation to the nucleus and GR DNA-binding under normal signal transduction [21, 22]. Therefore, GR function and GR expression were evaluated to investigate the mechanism of icariin on glucocorticoid resistance in asthma, and GR function was reflected by evaluation of GR DNA-binding activity in nuclear fractions of splenocytes. When compared to the single asthma group, mice in the asthma-DEP group displayed further impaired GR DNA-binding activity and
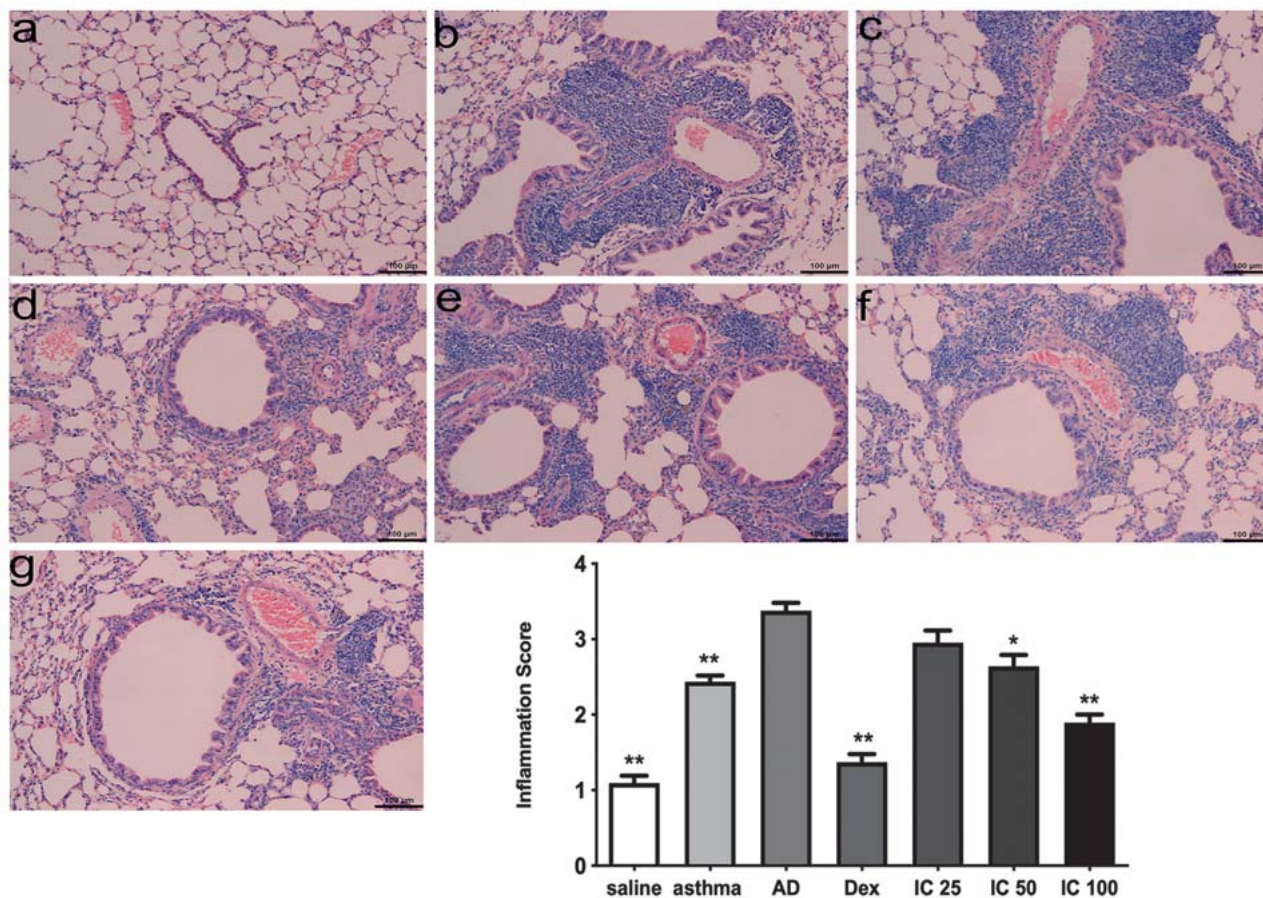
▶ **Fig. 4** Icariin attenuated airway inflammation in lung tissues. Lung tissue slices were stained with H&E and then observed under a microscope (100 ×). The inflammation score represents the severity of inflammatory cell infiltration in the airway. Representative photomicrographs of each group (n = 8 per group) are shown as follows: (**a**) saline, (**b**) asthma, (**c**) asthma with depression, (**d**) dexamethasone (Dex; 0.5 mg/kg/day), (**e**) icariin 25, (**f**) icariin 50, and (**g**) icariin 100. Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC_{25}, IC_{50}, IC_{100}: the low, median, and high dosage of icariin groups treated with 25, 50, and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. The size of the scale bars is 100 µm. Data are expressed as means ± SEM (n = 10 in each group); $^*p < 0.05$ and $^{**}p < 0.01$ versus the asthma with depression group.

decreased total GR expression in this study, which indicates that depression could further impair GR function and expression. Icariin at doses of 50 and 100 mg/kg remarkably improved GR DNA-binding activity and increased GR expression in splenocytes. A previous study showed that hypercortisolemia could lead to decreased GR mRNA levels in patients with mood disorders [23]. In the present study, icariin alleviated hypercortisolemia, which therefore might be associated with its enhancement of GR expression. The phosphorylation of GR S226 contributes to the inhibition of GR nuclear translocation, thereby resulting in reduced GR DNA-binding activity. When compared to the single asthma group, mice in the asthma-DEP group displayed significantly increased GR S226 phosphorylation. Thus, the impaired GR function aggravated by depression might be associated with the increased GR S226 phosphorylation. However, icariin at doses of 50 and 100 mg/kg remarkably inhibited GR S226 phosphorylation, which possibly contributed to its effects on the GR DNA-binding activity in splenocytes.

The activation of p38 MAPK can lead to the GR S226 phosphorylation in the cytoplasm, thus resulting in reduced GR DNA binding [24]. In this study, phosphorylation of p38 MAPK was significantly increased in the asthma-DEP groups compared to the saline group, indicating the possible role of p38 MAPK pathway activation in the increased phosphorylation of GR S226. However, phosphorylation of p38 MAPK was significantly inhibited by icariin treatment in splenocytes. Therefore, our findings indicate that the effects of icariin on glucocorticoid resistance were associated with suppression of the phosphorylation of GR S226 and p38 MAPK.

Taken together, this study suggests that icariin alleviated airway inflammation by improving endogenous glucocorticoid sensitivity, which, in addition to the increase of GR expression, was partially attributed to the enhancement of GR function associated with the decreased phosphorylation of GR S226 and p38 MAPK.
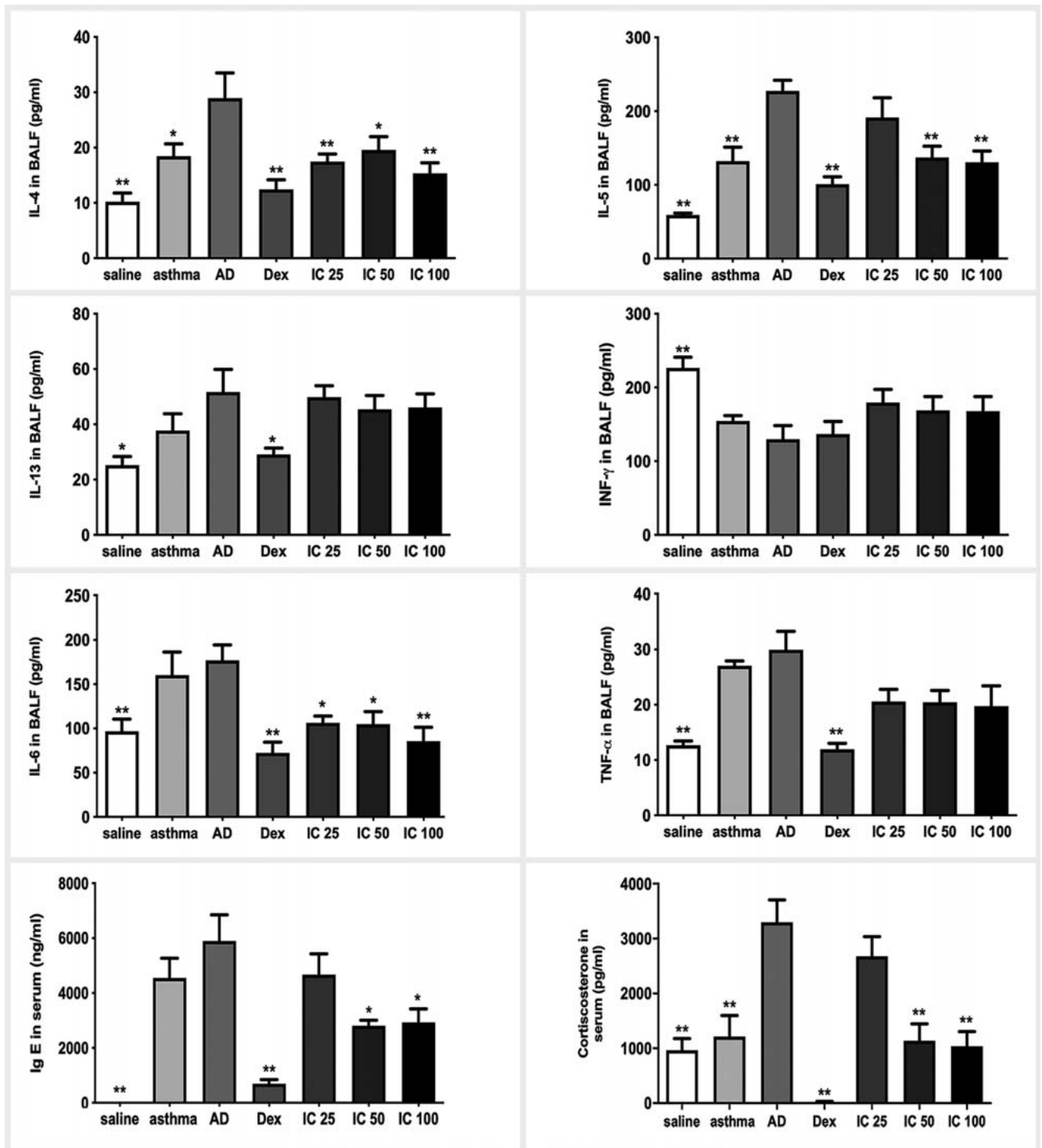
▶ **Fig. 5** Icariin inhibited the secretion of inflammatory cytokines in BALF and IgE and corticosterone in serum. Secreted levels of IL-4, IL-5, IL-13, INF-γ, IL-6, and TNF-α cytokines in BALF and serum antigen-specific IgE and corticosterone levels were measured by ELISA. Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC$_{25}$, IC$_{50}$, IC$_{100}$: the low, median, and high dosage of icariin groups treated with 25, 50, and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. Data are expressed as means ± SEM (n = 10 in each group); *p < 0.05 and **p < 0.01 versus the asthma with depression group.
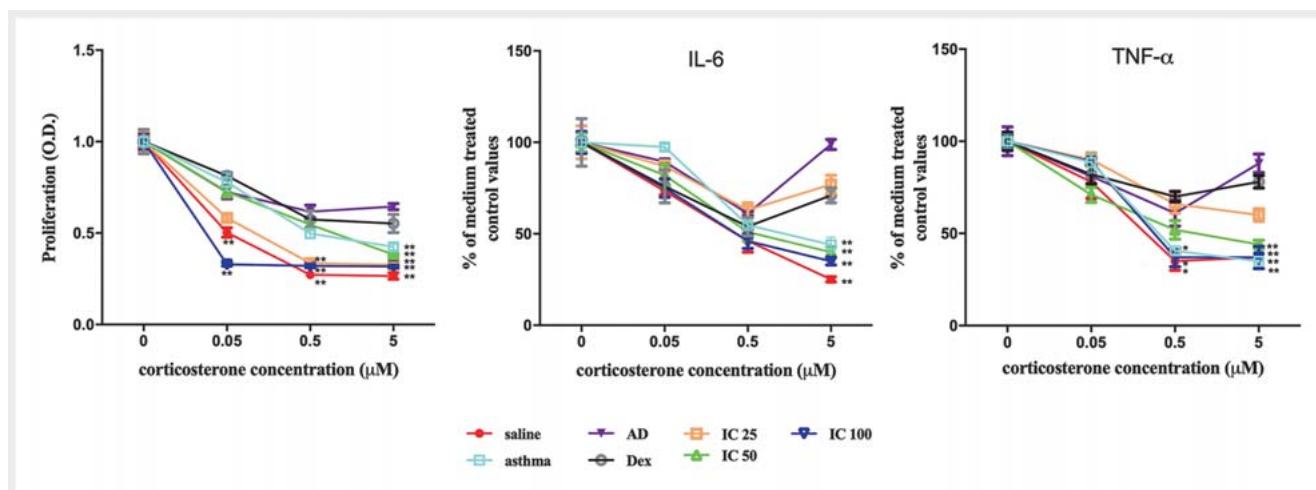
▶ **Fig. 6** Icariin facilitated the inhibitory effects of corticosterone on LPS-stimulated splenocytes. Data are presented as the percentage of cell viability or cytokine concentration of control values treated without corticosterone. Treatment of icariin facilitated corticosteroid responsiveness in splenocytes. The doses of 50 and 100 mg/kg of icariin markedly improved glucocorticoid sensitivity in the inhibition of splenocyte proliferation and inflammatory cytokine secretion. Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC_{25}, IC_{50}, IC_{100}: the low, median, and high dosage of icariin groups treated with 25, 50, and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. Data are expressed as means ± SEM (n = 6 in each group); $*p < 0.05$ and $**p < 0.01$ versus the asthma with depression group.

Materials and Methods

Animals

Seventy specific pathogen-free male BALB/c mice (aged 6 weeks, weighed 20–22 g) were purchased from Shanghai SLAC Laboratory Animal Company. Animals were maintained in a temperature-controlled environment ($22 \pm 2\,°C$) on a 12-h light-dark schedule with free access to food and water under pathogen-free conditions. Mice were randomly assigned into the following seven groups (n = 10 per group): (1) normal saline control group (saline), (2) single asthma group (asthma), (3) asthma with depression group (AD), (4) dexamethasone (Dex) group (0.5 mg/kg), (5) low-dose icariin group (25 mg/kg, IC_{25}), (6) median-dose icariin group (50 mg/kg, IC_{50}), and (7) high-dose icariin group (100 mg/kg, IC_{100}). All experimental protocols were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (permit number: SYXK-2018-0012; December 30, 2018).

Reagents and materials

Icariin (purity 98%), corticosterone, and LPS were purchased from TargetMol. OVA (grade V) and methacholine were provided by Sigma-Aldrich. Injectable Dex sodium phosphate was obtained from Tianjin Pharmaceutical Group Xinzheng Company. Imject alum adjuvant, a formulation of aluminum hydroxide and magnesium hydroxide, was provided by Thermo Fisher Scientific Company. ELISA kits for IL-4, IL-5, IL-6, IL-13, TNF-α, interferon (INF)-γ, and OVA-specific IgE were purchased from eBioscience Company. The CCK-8 assay was obtained from Dojindo Laboratories. TransAM GR kits were purchased from Active Motif. Antibodies for phosphorylated p38 MAPK (Thr180/Tyr182, Cat. no. 4511), p38 MAPK (Cat. no.8690), and phosphorylated GR (S226, Cat.

no.97 285) were obtained from Cell Signaling Technology, and GR alpha (ab3580) was purchased from Abcam.

Model establishment and drug administration

Animals were sensitized and challenged by OVA according to a modified protocol for the asthma model as described previously [25]. In brief, mice were immunized on days 0 and 7 with a peritoneal injection of 0.2 mL of sterile saline containing 40 µg of OVA and 0.05 mL of alum adjuvant. One week after sensitization, mice were challenged with aerosolized 1% OVA for 30 min/day for 5 consecutive weeks from day 14. For mice in the asthma-DEP group and treatment groups, mice were further subjected to CUMS from day 21 for 4 weeks. The stress conditions applied in this experiment included a wet pad for 8 h, ice-water bath for 5 min at 6 °C, constraint for 1 h, tilted cage (30°) for 3 h, exposed to light overnight, noise for 3 h, deprivation of water and food for 12 h, and tail clamping for 1 min. Two types of stress inducers were randomly selected and applied each day. From days 21 to 48, mice in the treatment groups were administrated icariin at doses of 25, 50, and 100 mg/kg or Dex at 0.5 mg/kg/day once a day by oral gavage. Mice in the saline control group were challenged and treated with normal saline instead. The protocols for sensitization, challenge, stress exposure, and drug administration are summarized in ▶ **Fig. 8**.

Open field test

The OFT was applied to assess the spontaneous movement of mice. As previously described [26], the test system consisted of a square arena (50 cm length × 50 cm width × 50 cm depth) with a digital camera positioned directly above the center of the field. Each animal was gently placed in the center of the cages and tracked for 5 min. Locomotor activity, including travel distance
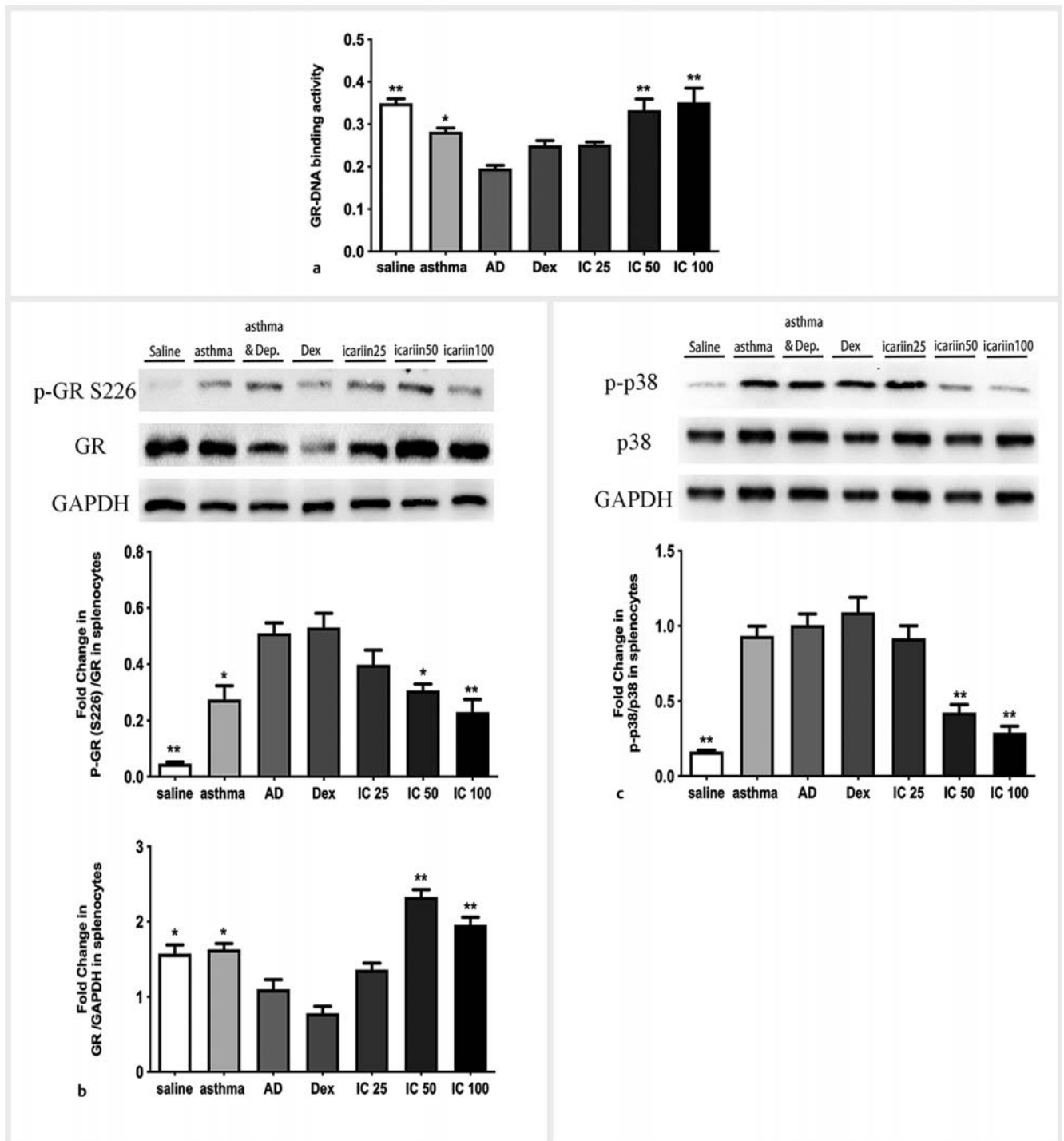
▶ **Fig. 7** Icariin enhanced the DNA-binding activity of the glucocorticoid receptor (GR) and GR expression and inhibited the phosphorylation of GR S226. **a** Evaluation of GR DNA-binding activity. Splenocytes from each group were isolated and incubated in the presence of dexamethasone (10^{-7} M) for 1 h *in vitro*. After preparation of nuclear extracts from splenocytes, GR nuclear translocation was then quantitated by the TransAM GR DNA-binding ELISA kit. **b, c** Western blotting analysis was performed to investigate GR S226 phosphorylation and GR expression in splenocytes. **c** Western blotting analysis was performed to investigate p38 MAPK phosphorylation in splenocytes. The expression of these proteins was quantified and represented as the band intensity of phosphorylated proteins normalized to the relevant total proteins. Asthma: single asthma group, AD: asthma with depression group, Dex: dexamethasone group. IC_{25}, IC_{50}, IC_{100}: the low, median, and high dosage of icariin groups treated with 25, 50, and 100 mg/kg, respectively. The asthma with depression group is a control for the treatment groups. Data are expressed as means ± SEM (n = 6 in each group); $^*p < 0.05$, $^{**}p < 0.01$ versus the asthma with depression group.
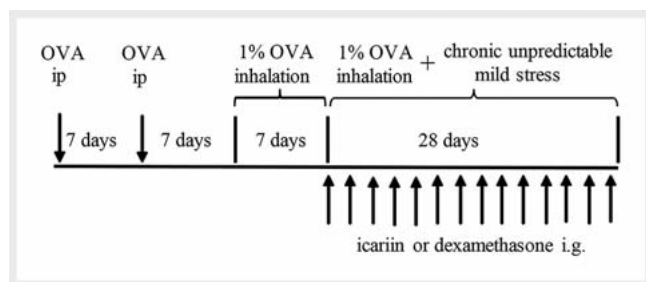
▶ **Fig. 8** Experimental protocols. Mice were subjected to OVA exposure combined with continuous chronic unpredictable mild stress (CUMS). Briefly, mice were sensitized twice with OVA followed with 1% OVA inhalation each day for 5 consecutive weeks. From days 21 to 48, mice were subjected to CUMS, and, simultaneously, mice in the treatment groups received oral icariin at 25, 50, and 100 mg/kg or 0.5 mg/kg of dexamethasone (Dex) for 4 weeks.

and time in the center zone, was quantified using the automatic video tracking system (SMART 3.0; Panlab S. I.). The center field was defined as the central $25 \times 25\,cm^2$ area of the open field, which was one-fourth of the total area.

Forced swim test

A modified version of the forced swim test was performed to assess the effects of icariin treatment on depression-like behavior. Briefly, subjects were gently placed in a glass cylinder filled with 15 cm of water at room temperature ($25 \pm 1\,°C$). The mice were placed in the cylinders for 6 min and the immobility time of the last 4 min was determined by the automatic video tracking system (SMART V3.0; Panlab S. I.). Immobility, in which the mice remained immobile or made only small limb movements necessary to float, was regarded as depression-like behavior.

Tail suspension test

The tail suspension test was conducted to assess the behavioral despair of mice, as previously described [27]. The mice were suspended by their tails using an elastic band attached to the tail (approximately 1 cm from the tip of the tail). The distance between the tip of the nose of the mouse and the laboratory bench was approximately 20 cm. Mice were suspended for a period of 6 min and the time spent immobile during the last 4 min of 6 min was scored for each mouse by an observer blinded to the test.

Measurement of airway hyperresponsiveness

Buxco's unrestrained whole-body plethysmography systems were performed to evaluate AHR to methacholine [28]. Briefly, mice were kept in a closed chamber and the pressure fluctuations were recorded during the breathing cycle. After mice reached a stable baseline, aerosolized PBS or various concentrations of methacholine (6.25, 12.5, or 25 mg/mL) were administered to the mice via a jet nebulizer in the chamber. The "enhanced pause" (Penh), as a dimensionless parameter, was used to indicate changes in airway resistance [29].

Serum collection and analysis

Blood samples were collected from all experimental animals following full anesthesia by an intraperitoneal injection of pentobarbital sodium. Serum was collected after centrifugation at $1500 \times g$ for 10 min and then stored at $-80\,°C$. OVA-specific IgE and corticosterone levels were then measured by ELISA according to the manufacturer's instructions.

Cytokine analysis in bronchoalveolar lavage fluid

To obtain BALF, a tracheal tube was inserted in the mice and lung lavage was performed twice with 0.8 mL of sterilized normal saline. The collected BALF was centrifuged at $500 \times g$ at $4\,°C$ for 10 min. The supernatants were then collected and stored at $-80\,°C$ for subsequent cytokine measurements. Subsequently, interleukin levels (IL-4, IL-5, IL-13, INF-γ, IL-6, TNF-α) in the BALF supernatant were detected by ELISA according to the instructions of the manufacturer.

Histopathological evaluation of the lung

After BALF collection, lung tissue slices were fixed with 4% neutral-buffered formalin. The fixed tissue blocks were then cut into thin sections (3–4 µm) and stained with H&E. The histopathological evaluation was performed blindly using randomized sections with Image-Pro Plus software at a magnification of 100×. A five-point scoring system was adopted to analyze the severity of inflammatory cell infiltration in the airway: 0, no cells; 1, a few cells; 2, a ring of cells, 1 cell layer deep; 3, a ring of cells, 2 to 4 cells deep; and 4, a ring of cells which was > 4 cells deep [30].

Splenocyte isolation and corticosterone sensitivity assay

The corticosterone sensitivity assay was evaluated as previously described [31]. A single-cell splenocyte suspension was prepared and then cultured in triplicate in 96-well plates (2.5×10^5 cells/well) to perform cell proliferation assays and cytokine evaluation at a volume of 100 and 300 µL/well, respectively. Cells were treated with corticosterone (dose range 0, 0.05, 0.5, 5 µM) and 1 µg/mL LPS at $37\,°C$ with 5% CO_2. Cell supernatants were collected after 18 h of incubation and frozen at $-20\,°C$ for cytokines analysis of IL-6 and TNF-α by ELISA. At the end of the 48-h culture, a CCK-8 assay was performed to evaluate cell viability, and absorbance was read at 490 nm by an ELISA plate reader. Cell viability is expressed as percentage of OD of the saline-treated control.

Glucocorticoid receptor DNA-binding activity

To determine GR DNA-binding activity in nuclear extracts, splenocytes were treated with 1×10^{-7} M Dex for 2 h. Then nuclear extraction was performed as previously described [32]. GR DNA-binding activity was assessed by adding 20 µg of nuclear extract to TransAM GR kits, according to the manufacturer's instructions. Briefly, nuclear extracts were incubated in wells of the 96-well plates coated with a GR-binding consensus oligonucleotide sequence for 1 h, then incubated with the supplied primary anti-GR antibody (1 : 1000) for 1 h, and finally with a peroxidase-conjugated secondary antibody (1 : 1000) for 1 h. The color development was read at 450 nm following addition of the substrate, and then the OD of the GR was recorded.

Western blotting analysis

Splenocytes and lung tissues were homogenized to extract total protein using RIPA protein extraction reagent. An amount of 50 μg proteins was subjected to SDS-PAGE and electroblotted to a PVDF membrane (Bio-Rad). Immunoblotting was then performed by incubating the membranes with the following antibodies: phosphor-GR ser226 (1 : 1000 dilution), GRα (1 : 1000 dilution), phosphophorylated-p38 MAPK Thr180/Tyr182 (1 : 1000 dilution), p38 MAPK (1 : 1000 dilution), and GAPDH (1 : 2000 dilution), followed with secondary HRP-conjugated antibodies (1 : 1000 dilution). After the incubation, the membrane was then developed by enhanced chemiluminescence according to the manufacturer's instructions. The protein bands were then quantified and expressed as the band intensity of the phosphorylated proteins normalized to the relevant total proteins.

Statistical analysis

Results were analyzed by GraphPad Prism v.9.0 (GraphPad software). Data are expressed as the mean ± standard error (SEM). The significance of differences between groups was determined by one-way ANOVA followed by post hoc Dunnett tests. A value of $p < 0.05$ was accepted as statistically significant.

Contributors' Statement

Data collection: H. L. Jin, Y. Zhou, J. Ye, C. H. Qiu, W. Z. Jin; design of the study: L. M. Wang, H. L. Jin, Y. Zhou; statistical analysis: H. L. Jin, J. Ye, C. H. Qiu, W. Z. Jin; analysis and interpretation of the data: H. L. Jin, Y. Zhou, L. M. Wang, J. Ye, C. H. Qiu, W. Z. Jin; drafting the manuscript: H. L. Jin, Y. Zhou, L. M. Wang, C. H. Qiu; critical revision of the manuscript: L. M. Wang, J. Ye, W. Z. Jin.

Conflict of Interest

The authors declare that they have no conflict of interest.

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