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ORIGINAL ARTICLE



High mobility group box 1 inhibition by BoxA attenuates ovalbumin-induced allergic rhinitis in mice

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ABSTRACT

This study was designed to evaluate the effects of BoxA on allergic rhinitis (AR). Ovalbumin (OVA)-induced AR mice model was employed and BoxA was administered to AR mice. AR symptoms, levels of cytokines and chemokines, and the expression of high mobility group box 1 (HMGB1), TLR2, and TLR4 were measured. BoxA treatment significantly ameliorated AR symptoms, decreased level of histamine, OVA-specific antibodies, suppressed the infiltration of immune cells in nasal tissues, inhibited the expression of IL-4, IL-6, IL-5, TNF- α , IL-13, IL-17, IL-2 while promoting the expression of IL-10, suppressed the expression of HMGB1, TLR2, and TLR4 in AR mice. BoxA ameliorated allergic rhinitis in mice by inhibiting HMGB1.

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Introduction

Allergic rhinitis (AR) is a type of inflammation in the nose with symptoms including running nose, sneezing, watery and itchy eyes [1]. With increasing prevalence, AR strongly affected patients' life quality and generated great economic impact [2].

Multiple factors contribute to the pathogenesis of AR. IgE plays an essential role in AR by binding to the allergen and resulting in the release of inflammatory mediators. The level of circulating allergen-specific IgE is correlated with symptoms severity in AR patients and neutralization of IgE using monoclonal antibodies has been shown to ameliorate AR [3]. AR is also characterized by the infiltration of immune cells into nasal tissues. These cells include T cells, basophils, and eosinophils and produce cytokines and chemokines to promote inflammation. Immunotherapies targeting these cell responses have been recognized as important approaches to treat AR [4].

High mobility group box 1 (HMGB1) is a nuclear protein that could bind to DNA and regulate transcription of multiple genes [5]. HMGB1 has been known as a pro-inflammatory cytokine and is secreted by several immune cells. The secreted HMGB1 can signal through receptors for advanced glycation end products (RAGE), toll-like receptor (TLR)2, and TLR4 to trigger cytokine production [6]. HMGB1 has been implicated in the pathogenesis of several inflammatory diseases including AR [7]. Significantly increased nasal HMGB1 has been detected in children with AR and the HMGB1 level is positively related to symptom severity [8]. Targeting HMGB1 using its inhibitors has been shown to

ameliorate inflammatory diseases, such as AR [9], ischemia-reperfusion injury [10], and myocarditis [11].

HMGB1 is highly conserved and contains three functional domains including A box (BoxA), B box (BoxB), and C-terminal tail (C tail) [12]. BoxA is the DNA binding domain that has been shown to block the binding of HMGB1 to its receptors and suppress the pro-inflammatory effects of HMGB1. Therefore, BoxA is considered a specific HMGB1 antagonist [13]. The anti-inflammatory activity of BoxA has been described in several diseases [14–16]. However, the precise effects of BoxA on AR are not elucidated. Therefore, in the present study, we investigate the effects of BoxA on AR and explore the underlying mechanism using an ovalbumin (OVA)-induced AR mice model.

Materials and methods

AR mice model and treatment

6 weeks female BALB/c mice were obtained from Charles River (Beijing, China) and kept in an environment with a controlled light/dark circle (12/12 h), temperature (22–24 °C), and humidity (60 ± 5%). AR was induced as previously described [17]. Briefly, mice were intraperitoneally injected with 200 μ L phosphate buffer solution (PBS) containing 75 μ g OVA and 2 mg aluminium hydroxide (Sigma, St. Louis, MO, USA) on days 0, 7, and 14. Then mice were challenged with 500 μ g OVA in 20 μ L PBS into the nostrils on days 23, 25, and 27. The grouping and the treatment of the mice were shown in Table S1. The control group was challenged with PBS instead of OVA. For HMGB1 BoxA treatment, BoxA (HMGBiotech, Milan, Italy) was dissolved in PBS, and 5, 10, or 20 μ g BoxA

in 20 μ L PBS was administrated to nasal cavities of mice daily from day 28 to day 35. AR mice treated with 20 μ L PBS were used as control. On day 36, samples were harvested for analysis. The dosage of BoxA was chosen based on previous reports [18,19]. Twelve mice were included in each group. The study was approved by the Ethical Committee of the Second Hospital of Hebei Medical University.

Nasal symptoms measurement

On day 36, the numbers of sneezing and nose rubbing motions in 20 min were measured.

Nasal lavage fluid (NALF) preparation

NALF was collected on day 36 as described previously [20]. One millilitre of PBS was used for perfusion and collected. After centrifuge, the supernatant was stored at -80°C for analysis.

Histamine measurement

Histamine level in serum and NAFL was measured using a Histamine ELISA kit (Abcam, Beijing, China).

OVA-specific IgE, IgG1, and IgG2a measurement

OVA-specific IgE in serum and NALF, and OVA-specific IgG1 and IgG2a in serum were measured using commercial ELISA kits (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions.

Inflammatory cell counting

Total cells in NALF were pelleted. After washing, cells were subjected to Wright-Giemsa staining using Wright-Giemsa Stain Kit (Abcam). The total number of different cells was counted under a microscope.

Cytokine measurement

Serum level of IL-4, IL-6, IL-5, TNF- α , IL-13, IL-10, IL-17, and IL-2 was measured using corresponding ELISA kits (Abcam).

Western blot

Total protein from mice nasal tissues was extracted using Protein Extraction Kit (Abcam). 25 μ g proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to transfer. After blocking, membranes were incubated with primary antibodies at 4°C for overnight. All primary antibodies were purchased from Abcam. The next day, after washing with phosphate-buffered saline with Tween 20 for three times, membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. The membranes were developed using

ECL Substrate Kit (Abcam, China). The bands intensity was analyzed using ImageJ.

Real-time PCR

TRizol reagent (Thermo Fisher, Waltham, MA, USA) was used to extract total RNA. The cDNA was synthesized using PrimeScript RT Reagent Kit (Takara, Beijing, China). The real-time PCR reactions were set up using GeneRead qPCR SYBR Green Mastermix (Qiagen, Germantown, MD, USA) and performed in 7500 Real-time PCR System (Applied Biosystems, USA). Primers used for real-time PCR included: TLR4 sense 5'-AGCTCCTGACCTTGGTCTTG-3', antisense 5'-CGCAGGGGAACCTCAATGAGG-3'; TLR2 sense 5'-CTC TTCAGCAAACGCTGTTCT-3', anti-sense 5'-GGCGTCTCC CTCTATTGTATTG-3'; HMGB1 sense 5'-GCTGACAAGGC TC GTTATGA-3', anti-sense 5'-CCTTTGATTTTGGGGC GGTA-3'; GAPDH sense 5'-AATGGATTGGAC GCATTG GT-3', anti-sense 5'-TTTGCCTGCTGCTGTTGAT-3'. The relative expression pattern was analyzed by the comparative threshold cycle ($2^{-\Delta\Delta\text{Ct}}$) method.

Histopathological examination

The heads of mice were fixed with 4% paraformaldehyde for 3 days and decalcified in the ethylenediaminetetraacetic acid solution for 6 days. Following this, the specimens were embedded in paraffin, cut into 4- μ m-thick sections, and stained with periodic acid-Schiff (PAS) for goblet cells and mucus. Goblet cells were counted in randomly selected high-power fields at 400 $\times$ magnification.

Statistics analysis

All data were presented as mean $\pm$ standard deviation (SD). One-way ANOVA followed Dunn's multiple comparisons test was used for different analysis. When $p < .05$, the difference was considered as significant.

Results

BoxA alleviated AR symptoms in mice

To investigate the effects of BoxA on AR, the OVA-induced AR mice model was used and BoxA was administrated to the mice (Figure 1(A)). The number of sneezing and nasal rubbing was recorded. As shown in Figure 1(B), AR mice had significantly increased sneezing numbers compared to normal control mice. In addition, significantly increased PAS-positive goblet cells were detected in the nasal mucosa of AR mice, indicating AR was successfully induced in these mice (Figure S1). AR mice treated with PBS had a similar number of sneezing and nasal rubbing to AR mice, indicating PBS treatment did not affect AR symptoms (Figure S2). There was no significant difference in sneezing number between AR mice and AR mice treated with 5 μ g BoxA. In contrast, AR mice treated with 10 and 20 μ g BoxA has remarkably reduced sneezing number when compared to AR mice. The sneezing number of AR mice treated with

20 μg BoxA was less than that of AR mice treated with 10 μg BoxA, suggesting BoxA suppressed sneezing in a dose-dependent manner in AR mice. Similarly, we observed a significantly increased number of nasal rubbing in AR mice when compared to control mice. BoxA reduced the number of nasal rubbing in AR mice in a dose-dependent manner (Figure 1(C)). These results demonstrated that BoxA alleviated the symptoms in AR mice.

BoxA decreased histamine level in AR mice

We continued to investigate the effects of BoxA on histamine levels in AR mice. We detected a significantly higher serum level of histamine in AR mice than in control mice

(Figure 2(A)). Although 5 μg BoxA treatment did not obviously affect serum histamine level in AR mice, the serum histamine level in AR mice treated with 10 and 20 μg BoxA was significantly decreased when compared to AR mice. Similarly, the histamine level in NALF was significantly upregulated in AR mice while BoxA treatment decreased histamine level in NALF of AR mice in a dose-dependent manner (Figure 2(B)). Collectively, these data showed BoxA decreased histamine level in AR mice.

BoxA decreased OVA antibody level in AR mice

Next, we evaluated the effects of BoxA on OVA-specific IgE and IgG levels in AR mice. The level of OVA-specific IgE in

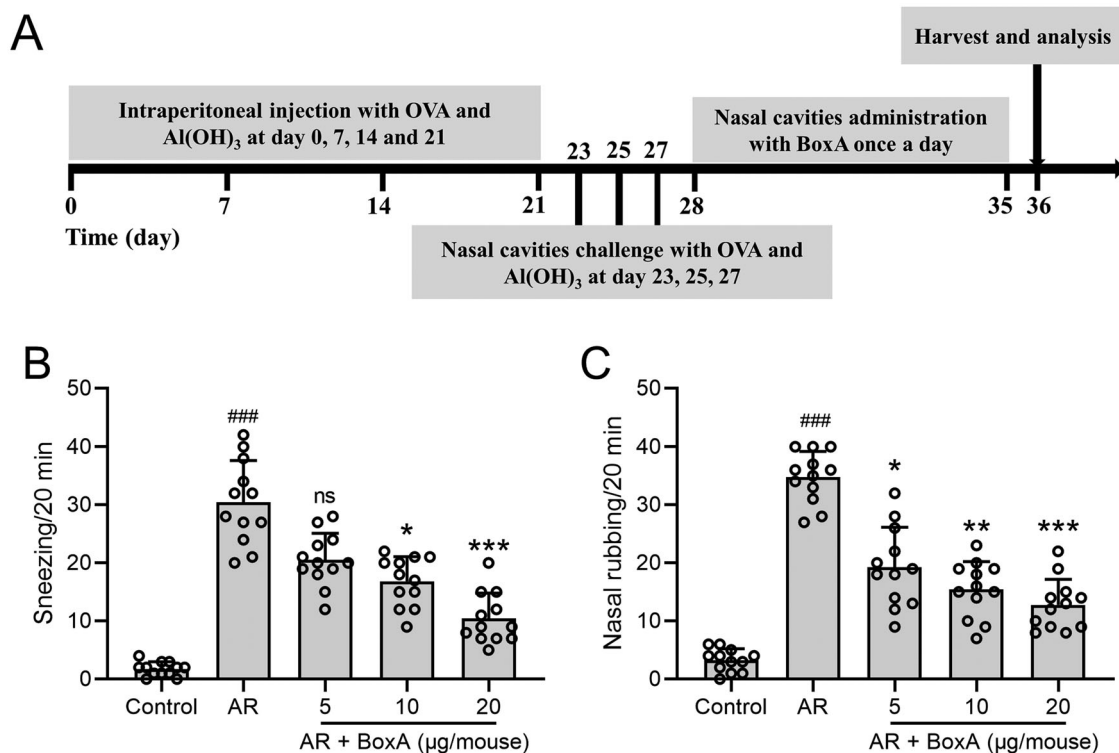


Figure 1. BoxA ameliorates allergic symptoms in AR mice. (A) Schematic diagram of the experimental protocol for mouse ovalbumin-induced allergic rhinitis mouse model. Number of sneezing (B) and nasal rubbing events (C) in 20 min duration were compared. Data are presented as mean $\pm$ SD from 12 mice in each group. ### $p < .001$ compared to control. * $p < .05$, ** $p < .01$, *** $p < .001$, ns means no significance compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

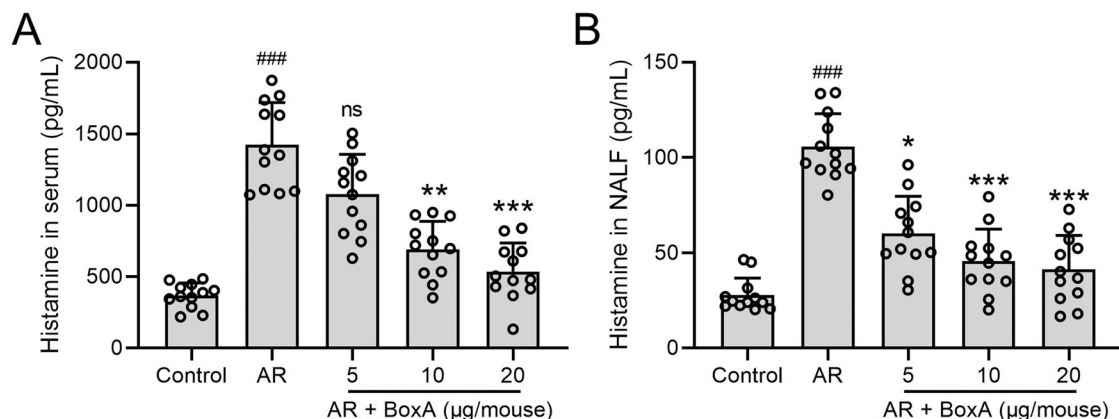


Figure 2. BoxA decreases histamine level in serum (A) and nasal lavage fluid (NALF) (B). Data are presented as mean $\pm$ SD from 12 mice in each group. ### $p < .001$ compared to control. * $p < .05$, ** $p < .01$, *** $p < .001$, ns means no significance compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

serum (Figure 3(A)) and NALF (Figure 3(B)) of AR mice was significantly higher than that in control mice. Five micrograms BoxA treatment slightly decreased both serum and NALF levels of OVA-specific IgE while 10 and 20 μ g BoxA treatment significantly decreased IgE level (Figures 3(A,B)). We also detected remarkably elevated OVA-specific IgG1 (Figure 3(C)) and IgG2a (Figure 3(D)) in AR mice while BoxA treatment decreased the serum level of both OVA-specific IgG1 and IgG2a. These data showed that BoxA decreased OVA-specific antibodies in AR mice.

BoxA suppressed inflammatory cells infiltration in NALF of AR mice

Next, we evaluated the effects of BoxA on inflammatory cells infiltration in AR mice. As presented in Figure 4(A), the total number of leucocytes in NALF of AR mice was significantly greater than that in NALF of control mice. BoxA treatment decreased the number of leucocytes in NALF and significantly decreased leucocytes number in NALF was detected in AR mice treated with 10 and 20 μ g BoxA. Respectively, AR mice had significantly increased total numbers of eosinophils (Figure 4(B)), lymphocytes (Figure 4(C)), and neutrophils (Figure 4(D)) in NALF. In contrast, AR mice treated with BoxA had decreased eosinophils, lymphocytes, and neutrophils and the total numbers in NALF were significantly decreased in AR mice treated with 10 and 20 μ g BoxA. Collectively, these results showed BoxA

decreased leucocytes numbers in NALF of AR mice. We further compared the histopathology of mice from different groups. As shown in Figure S1, significantly increased PAS-positive goblet cells in nasal mucosa were detected in AR mice when compared with the mice. In contrast, the number of PAS-positive goblet cells was significantly decreased in BoxA-treated AR mice.

BoxA suppressed inflammatory cytokine production in AR mice

We continued to evaluate whether BoxA treatment affected cytokine production in AR mice. We measured the serum level of multiple cytokines in AR mice treated with 20 μ g BoxA. We detected significantly elevated serum level of inflammatory cytokines including IL-4 (Figure 5(A)), IL-6 (Figure 5(B)), IL-5 (Figure 5(C)), TNF- α (Figure 5(D)), IL-13 (Figure 5(E)), IL-17 (Figure 5(G)), and IL-2 (Figure 5(H)) in AR mice. In contrast, the serum level of the anti-inflammatory cytokine IL-10 was significantly decreased in AR mice (Figure 5(F)). Twenty micrograms BoxA treatment attenuated the upregulation of IL-4 (Figure 5(A)), IL-6 (Figure 5(B)), IL-5 (Figure 5(C)), TNF- α (Figure 5(D)), IL-13 (Figure 5(E)), IL-17 (Figure 5(G)), and IL-2 (Figure 5(H)) while promoted the serum level of IL-10 in serum of AR mice. Collectively, these results demonstrated that BoxA suppressed inflammatory cytokine production in AR mice.

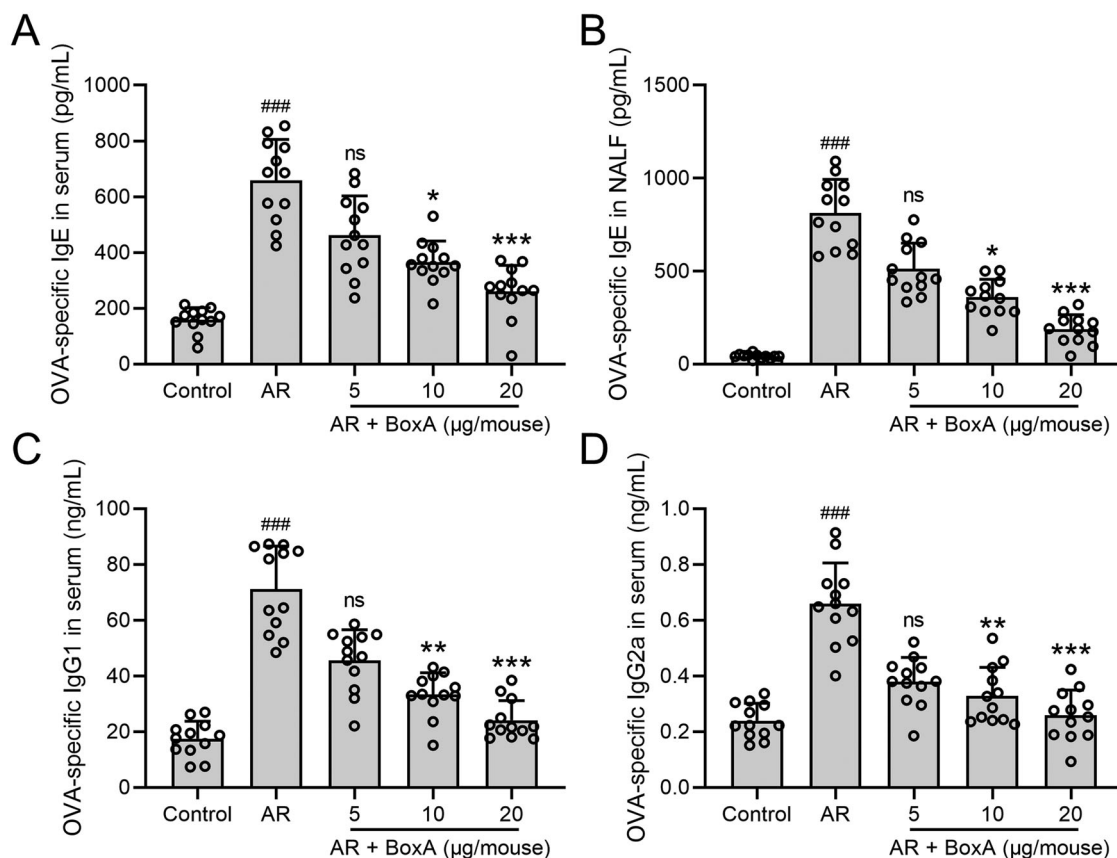


Figure 3. BoxA decreases the OVA-specific IgE in serum (A) and nasal lavage fluid (NALF) (B). Box A attenuates the OVA-specific IgG1 (C) and IgG2a (D) in serum. Data are presented as mean $\pm$ SD from 12 mice in each group. ### p < .001 compared to control. * p < .05, ** p < .01, *** p < .001, ns means no significance compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

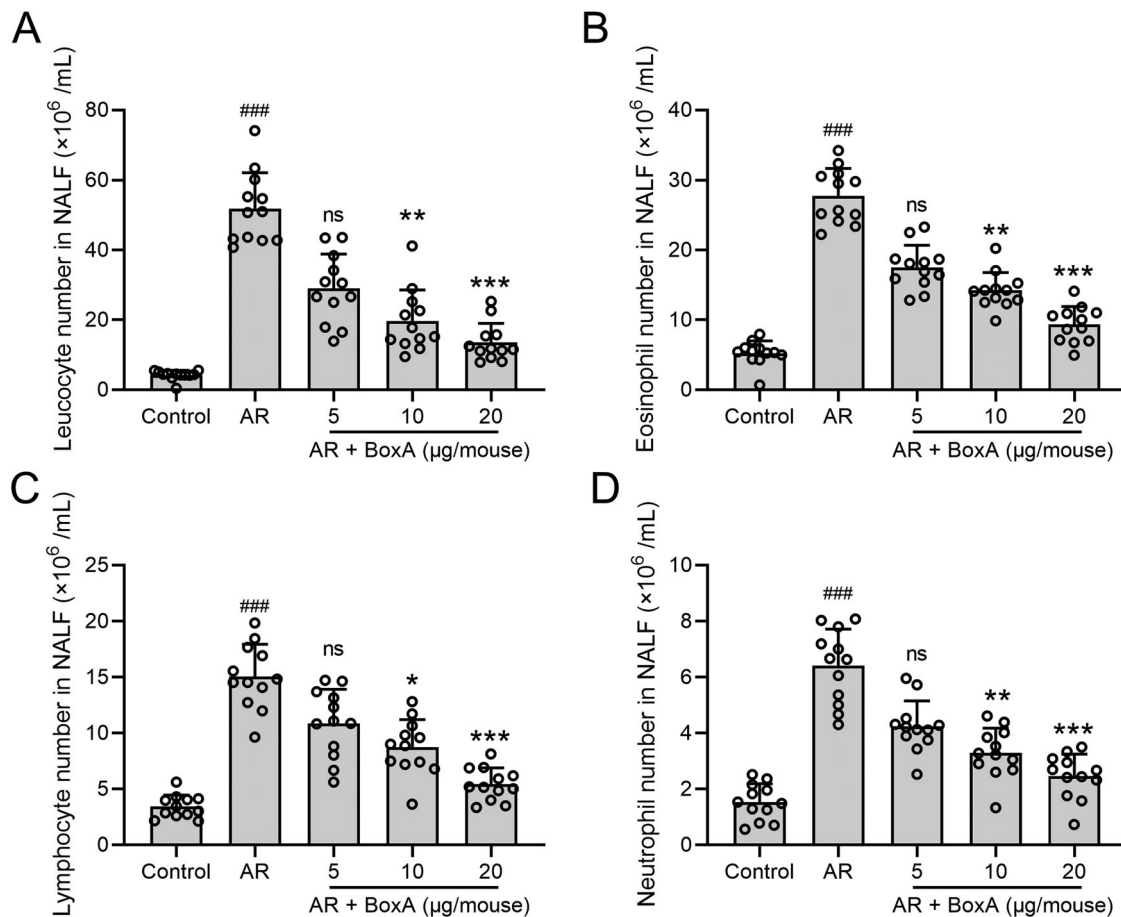


Figure 4. BoxA decreases the number of leucocytes (A), eosinophils (B), lymphocytes (C), and neutrophils (D) in the nasal lavage fluid (NALF) of AR mice. Data are presented as mean $\pm$ SD from 12 mice in each group. ### $p < .001$ compared to control. * $p < .05$, ** $p < .01$, *** $p < .001$, ns means no significance compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

BoxA suppressed HMGB1, TLR2, and TLR4 expression in AR mice

Finally, we evaluated the effects of BoxA on the expression of HMGB1, TLR2, and TLR4 in AR mice. We detected significantly increased protein levels of HMGB1 (Figures 6(A,B)), TLR2 (Figures 6(A,C)), and TLR4 (Figures 6(A,D)) in nasal tissues of AR mice when compared to control mice. Correspondingly, the mRNA expression of TLR2 (Figure 6(E)) and TLR4 (Figure 6(F)) and HMGB1 (Figure S3) was significantly elevated in nasal tissues of AR mice. In contrast, BoxA treatment decreased the protein level of HMGB1, TLR2, and TLR4 and suppressed the mRNA expression of TLR2, TLR4, and HMGB1 in nasal tissues of AR mice. These results showed that BoxA downregulated the expression of HMGB1, TLR2, and TLR4 in AR mice.

Discussion

In this study, we utilized the OVA-induced AR murine model to evaluate the potential effects of BoxA, an HMGB1 antagonist, on AR. Our results demonstrated that administration of BoxA to AR mice significantly attenuated AR symptoms, decreased IgE level, suppressed leucocytes infiltration to nasal tissue, and inhibited the inflammatory cytokine production. We further determined that BoxA

suppressed the expression of HMGB1, TLR2, and TLR4. Therefore, our study suggested BoxA could be used as a therapeutic agent to treat AR.

In AR, the allergen stimulates the early and late responses, which are mediated by various inflammatory cells. In early-phase response, the inhaled allergens bind to IgE and the IgE further bind to Fc receptor (FcR) on mast cells and basophils, which leads to degranulation of mast cells and basophils and release of histamine. We detected elevated serum levels of histamine and OVA-specific IgE in AR mice. Previous studies have shown that anti-IgE and anti-histamine treatments have been shown to reduce AR-specific symptoms [3,21]. Similarly, we demonstrated that in BoxA-treated AR mice, the serum level of histamine and OVA-specific IgE were significantly decreased. These effects contributed to the protective role of BoxA on AR. In late-phase response, inflammatory cells including T cells, basophils, and eosinophils accumulate in local tissue. These leucocytes secrete a variety of cytokines and chemokines, resulting in promoting the infiltration of eosinophils, T cells, and basophils into the nasal mucosa and inducing the release of pro-inflammatory mediators [22]. In the present study, we detected obvious infiltration of leucocytes into nasal tissue in AR mice, together with enhanced secretion of inflammatory cytokines. In contrast, BoxA treatment suppressed the infiltration of leucocytes and inflammatory

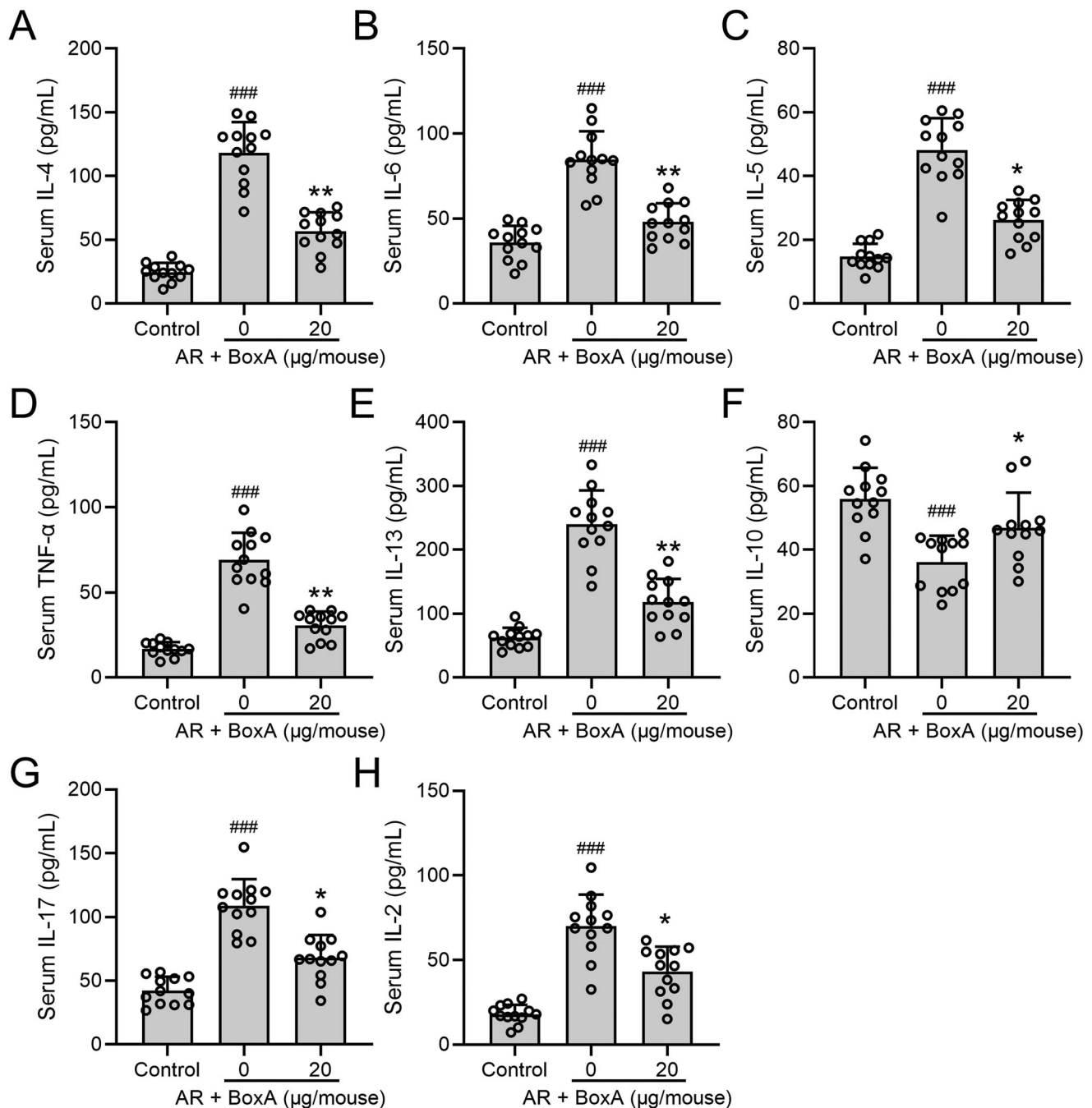


Figure 5. BoxA suppressed the inflammatory cytokines secretion in the serum of AR mice. IL-4 (A), IL-6 (B), IL-5 (C), TNF- α (D), IL-13 (E), IL-10 (E), IL-17 (F), IL-2 (H). Data are presented as mean $\pm$ SD from 12 mice in each group. ### p < .001 compared to control. * p < .05, ** p < .01 compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

cytokine production in AR mice. These effects also contributed to the protective role of BoxA on AR.

HMGB1 is a DNA binding protein that not only binds to and bends DNA but also functions to regulate gene expression by interacting with several transcriptional factors, such as p53 [23], NF- κ B [24], and modulating their activities. HMGB1 can also be secreted by macrophages, monocytes, dendritic cells [25,26]. Once secreted outside, HMGB1 can bind to cell surface receptors including TLR2, TLR4, and RAGE [27–29]. The binding of HMGB1 to these receptors results in cell infiltration and production of inflammatory cytokines, such as TNF- α , IL-1 α , IL-1, IL- β , and IL-6 [26].

The contribution of HMGB1 to the pathogenesis of AR has been described and inhibiting HMGB1 has been shown to protect against AR. Chen and colleagues demonstrated that Ethyl pyruvate prevented the release of HMGB1 and attenuated the AR in mice [9]. Yuan et al. demonstrated that silent information regulator 1 (SIRT1) suppressed the expressions of HMGB1 and attenuates AR in mice [17]. BoxA is the conversed domain of HMGB1 and is known as an HMGB1 antagonist which inhibits the binding of HMGB1 to its receptors. HMGB1 played important role in ischemia-reperfusion injury by binding to RAGE and activating inflammatory signalling pathways [30]. In the same

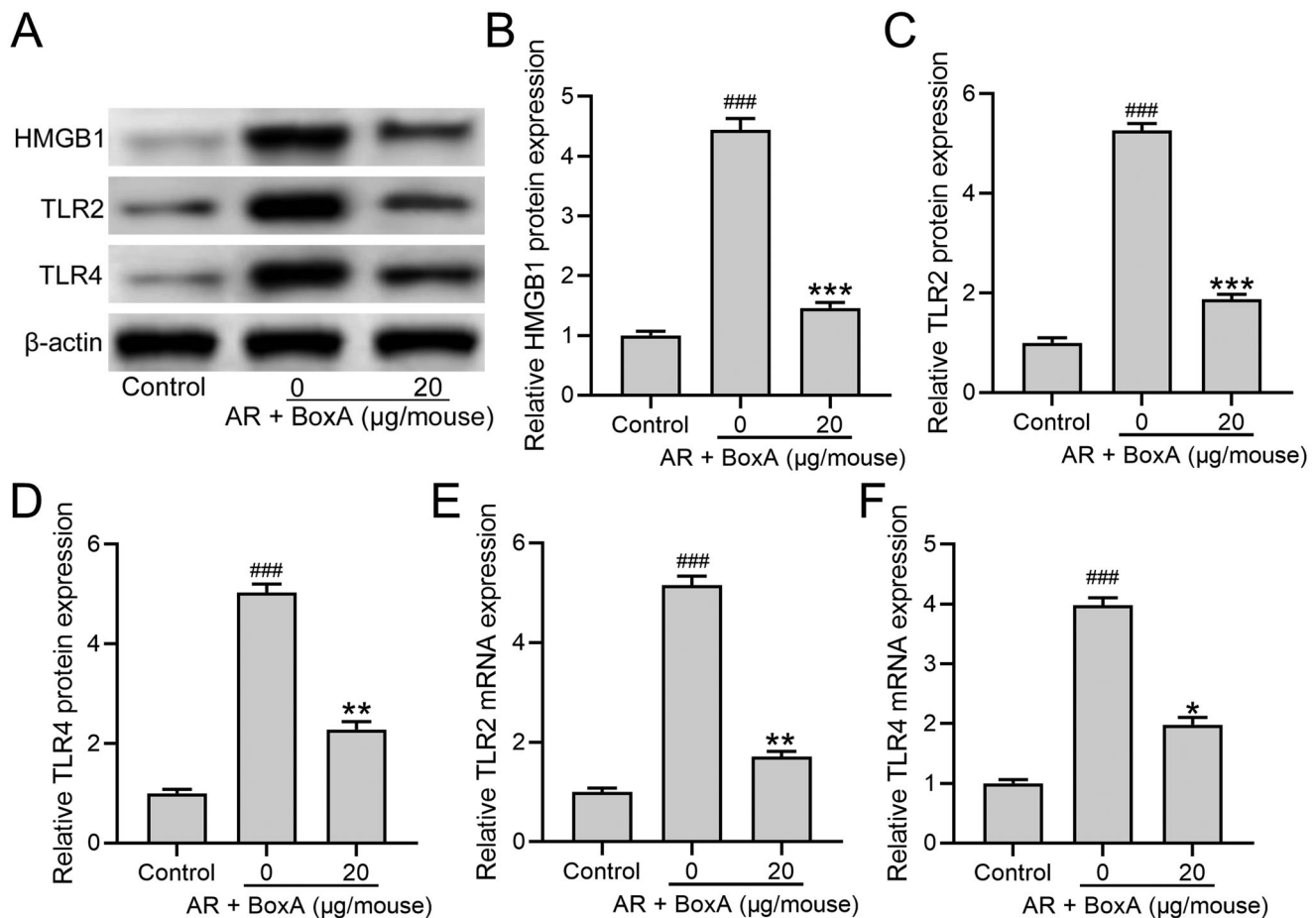


Figure 6. BoxA inhibits HMGB1, TLR2, TLR4 expressions in nasal mucosa from AR mice. (A) The protein expressions of HMGB1, TLR2, and TLR4 in nasal mucosa from indicated groups were measured by western blot. β -actin was used as a loading control (B–D). qRT-PCR was used to measure the mRNA expressions of TLR2 (E) and TLR4 (F) in nasal mucosa from indicated groups. $n = 3$ for each group from 12 mice in each group. Data are presented as mean $\pm$ SD. ### $p < .001$ compared to control. * $p < .05$, ** $p < .01$, *** $p < .001$ compared to AR group. One-way ANOVA followed Dunn's multiple comparisons test.

study, BoxA treatment significantly reduced the damage reduced the inflammation in the reperfused heart. Our present study demonstrated that BoxA treatment significantly reduced the expression of HMGB1, TLR2, and TLR4 in AR mice, suggesting BoxA inhibited HMGB1/TLR2 and HMGB1/TLR4 signalling pathway. Up-regulated expression of TLR2 and TLR4 is associated with RA [31]. Knocking down TLR4 by siRNA repressed NF- κ B activation and significantly relieved OVA-induced AR symptoms [32]. The binding of HMGB1 to TLR2, TLR4, and RAGE results in the activation of NF- κ B signalling pathways. Inhibition of NF- κ B also suppressed the allergic response in AR mice [33]. Although the activation of NF- κ B was not tested in the present study, it is very likely that BoxA treatment inhibited NF- κ B activation in AR mice. RAGE is another receptor for HMGB1 while its precise role in AR is not well-elucidated. It should be interesting to test the RAGE expression in AR and evaluate the effects of BoxA on RAGE expression and its downstream signalling pathway.

Goblet cell hyperplasia is one important feature of AR [34]. In the present study, we detected significantly increased PAS-positive goblet cells in AR mice. In contrast, the number of PAS-positive goblet cells in BoxA-treated AR mice was significantly decreased, indicating blockage of HMGB1 suppressed goblet cell hyperplasia. The relation

between HMGB1 and goblet cell hyperplasia has been described previously. Using a murine model of chronic asthma, Hou and colleagues found anti-HMGB1 antibody treatment led to a reduction in the number of PAS-stained goblet cells in the airway and they speculated HMGB1 induced goblet cell hyperplasia indirectly through cytokines [35]. In the study by Zhu et al., they found IL-13-transgenic mice had goblet cell hyperplasia [36], suggesting IL-13 promoted goblet cell hyperplasia. In the present study, we demonstrated that box A treatment inhibited the expression of multiple cytokines including IL-13, suggesting HMGB1 promotes goblet cell hyperplasia through regulating cytokine production.

In the present study, we demonstrated the protective effects of BoxA on AR. However, several issues need to be addressed in future studies. For example, to better understand the effect of BoxA, it is useful to compare the effects of BoxA to antihistaminic or nasal corticosteroid, which have efficient clinical effects against AR. In addition, determining the best route for BoxA administration is also useful.

Conclusion

We demonstrated that the administration of BoxA alleviated the symptoms in AR mice. BoxA targeted HMGB1 and

suppressed HMGB1, TLR2, and TLR4 expression in AR mice. Our results suggested BoxA should be a therapeutic agent to treat AR.

Author contributions

Liyan Yue, Jinhui Dong, Jianxing Wang, Xiaoyan Yin, Xiumin Ren, Ou Xu, and Chunguang Shan: data collection and manuscript writing. Liyan Yue, Jinhui Dong, Jianxing Wang, Xiaoyan Yin, Xiumin Ren, Ou Xu, and Chunguang Shan: data collection and analysis. Liyan Yue and Jinhui Dong: project development and manuscript writing.

Disclosure statement

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the ethics committee of the Second Hospital of Hebei Medical University.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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