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Recombinant HMGB1 A box protein inhibits Th17 responses in mice with neutrophilic asthma by suppressing dendritic cell-mediated Th17 polarization

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ABSTRACT

High mobility group box chromosomal protein 1 (HMGB1) is a critical pro-inflammatory cytokine involved in diverse inflammatory diseases and has important immunomodulatory effects on allergic asthma. Our recent studies demonstrate that HMGB1 α ReloadFigure=Yes1 expression increases in the lung tissue and associates with interleukin-17⁺ (IL-17) helper T cell (Th17) responses in a murine model of neutrophilic asthma. In this study, to examine the immunomodulatory mechanisms of HMGB1, we evaluated the effects of recombinant HMGB1 A box (an antagonist of HMGB1) administration on allergic airway inflammation and lung antigen-presenting cell (APC) function in a murine model of neutrophilic asthma. In OVA-challenged mice, rHMGB1 A box attenuated HMGB1 expression, airway neutrophilic inflammation and hyper-responsiveness. In addition, the administration of rHMGB1 A box decreased the number of Th17 cells and IL-23⁺ CD11c⁺ APCs in lung cells. *In vivo*, rHMGB1 A box revealed an inhibitory effect of rHMGB1-activated dendritic cells (DCs) to produce IL-23 and induce a Th17 response. Finally, we showed that adoptive transfer of rHMGB1-activated DCs was sufficient to restore the characteristics of neutrophilic asthma in a DCs-driven model of asthma, whereas the transfer of rHMGB1 A box plus rHMGB1-activated DCs significantly reduced these inflammation phenotypes. These data demonstrate that rHMGB1 A box may have therapeutic effects on controlling Th17 polarization and airway inflammation in neutrophilic asthma by blocking the HMGB1 pathway on DCs.

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1. Introduction

Allergic asthma is commonly thought to be an idiopathic disease characterized by airway obstruction to inhaled environmental antigens and an airway inflammation associated with dysregulated immune responses mediated by CD4⁺ T cells polarized to a Th2 or Th17 cell [1, 2]. Th2 cells have been described as the pathogenesis of eosinophil-predominant asthma; however, the mechanisms involved in the development of Th17 responses remain unknown [3].

Th17 cells are a distinct subset of T cells that have been found to produce interleukin 17 (IL-17), and play an important role in the development of non-eosinophilic asthma (NEA), resulting in more severe

phenotypes and steroid-resistant neutrophilic airway inflammation [4–6]. For example, passive Th17 transfer in mice induces neutrophilic airway inflammation and airway hyper-responsiveness (AHR), which are not attenuated by steroids, indicating a potential role for Th17 cells in steroid-resistant asthma [7]; IL-17 is a pro-inflammatory cytokine mainly secreted from helper T (Th) 17 cells, and it is important for the induction of neutrophil recruitment and migration at sites of inflammation [8]; Th17-related cytokines in sputum are significantly elevated in severe asthmatics and are correlated with sputum neutrophil counts [9]. The evidence suggests that Th17 cells are important in promoting and sustaining neutrophilic inflammation as observed in severe asthma.

Dendritic cells (DCs) are considered the most important antigen-presenting cells (APCs) in allergen sensing and in the consecutive activation of Th cells and their differentiation toward Th2 and Th17 effector or regulatory T cells (Treg) [10]; on the other hand, IL-23 and IL-6, the cytokines that preferentially expand Th17 cells, play a significant role in the development of chronic inflammatory diseases, including neutrophilic asthma [11]. High mobility group box 1 (HMGB1) is a primarily nuclear protein involved in the induction of airway inflammation in patients with asthma [12,13]. HMGB1, composed of the A box, B box and C tail domains, is a critical pro-inflammatory cytokine involved in diverse

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inflammatory diseases [14]. The B box mediates pro-inflammatory activity, while the A box alone acts as a specific antagonist of HMGB1 by competing with its receptors [15]. Some evidences demonstrated that HMGB1 could be secreted into the extracellular milieu, acts as a pro-inflammatory cytokine and mediates the downstream inflammatory responses in diverse inflammatory diseases. These findings inferred that increased HMGB1 expression could enhance Th17 cell polarization in acute allograft rejection or chronic asthma [16,17]; however, the molecular dialogue between HMGB1 and Th17 cell polarization in the neutrophilic asthma remains to be explored. Our recent study showed that HMGB1 expression in lung tissue was increased in a murine model of neutrophilic asthma induced by ovalbumin (OVA) plus lipopolysaccharide (LPS) and was positively correlated with neutrophil numbers and the levels of IL-17A in bronchoalveolar lavage fluid (BALF) [18]. We hypothesize that HMGB1 directs Th17 cell polarization by regulating IL-23 or IL-6 cytokine production by DCs, and HMGB1 A box inhibits the Th17 response and Th17-mediated neutrophilic airway inflammation in asthma.

To test this hypothesis, a murine model of neutrophilic asthma was established by the intranasal administration of lipopolysaccharide (LPS) plus ovalbumin (OVA) during sensitization and was challenged with OVA. First, we analyzed the role of recombinant HMGB1 A box (rHMGB1 A box) in regulating HMGB1 expression, Th17 responses and airway inflammation in this murine model of neutrophilic asthma; we also evaluated the effect of rHMGB1 A box on the Th17 polarization and the induction of IL-23⁺ CD11c⁺ antigen-presenting cells (APCs) in vivo. To demonstrate the key role of blocking HMGB1 pathway in DC-primed allergic disease, we further investigated whether the adoptive transfer of rHMGB1 A box plus rHMGB1-activated DCs was sufficient to attenuate the neutrophilic inflammation and the Th17 response in a DC-driven model of asthma. This study will uncover the immunopathological mechanisms for HMGB1-induced Th17 responses that are mediated by DCs.

2. Materials and methods

2.1. Mice

Pathogen-free female C57BL/6 mice were used, aged between 6 and 8 weeks and maintained in specific pathogen-free conditions, with the temperature at 20–22 °C and the humidity at 50–60%. The dark/light cycle was 12/12 h. The Institutional Animal Care and Use Committee approved all animal experiments.

2.2. Protocols and pharmacologic treatments

A murine model of neutrophilic asthma characterized by Th17 responses was generated as described previously [19]. As shown in Fig. 1A, mice received intranasal sensitization with 75 µg OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) plus 10 µg LPS (*Escherichia coli* serotype 026:B6; Sigma-Aldrich) on days 0, 1, 2 and 7, and were then challenged by intranasal instillation of 50 µg OVA alone on days 14, 15, 21 and 22. All mice were analyzed 24 h after the last OVA challenge.

In this study, the mice were divided randomly into four groups ($n = 6$ per group) as follows: (i) mice sensitized with phosphate-buffered saline (PBS) and challenged with OVA (control group); (ii) mice sensitized with OVA plus LPS and challenged with OVA (asthma group); (iii) mice treated with mouse serum albumin (MSA) (Sigma-Aldrich) half an hour before sensitization to OVA plus LPS and the same challenge with OVA later (MSA group); and (iv) mice treated with rHMGB1 A box (R&D Systems) half an hour before sensitization to OVA plus LPS and the same challenge with OVA later (A box group). rHMGB1 A box or MSA was administered intra-nasally (150 µg/kg) on days 0, 1, 2 and 7 before sensitization (Fig. 1A). The dose of rHMGB1 A box was predetermined using staining analysis of airway inflammation in mice receiving 100–300 µg/kg of rHMGB1 A box.

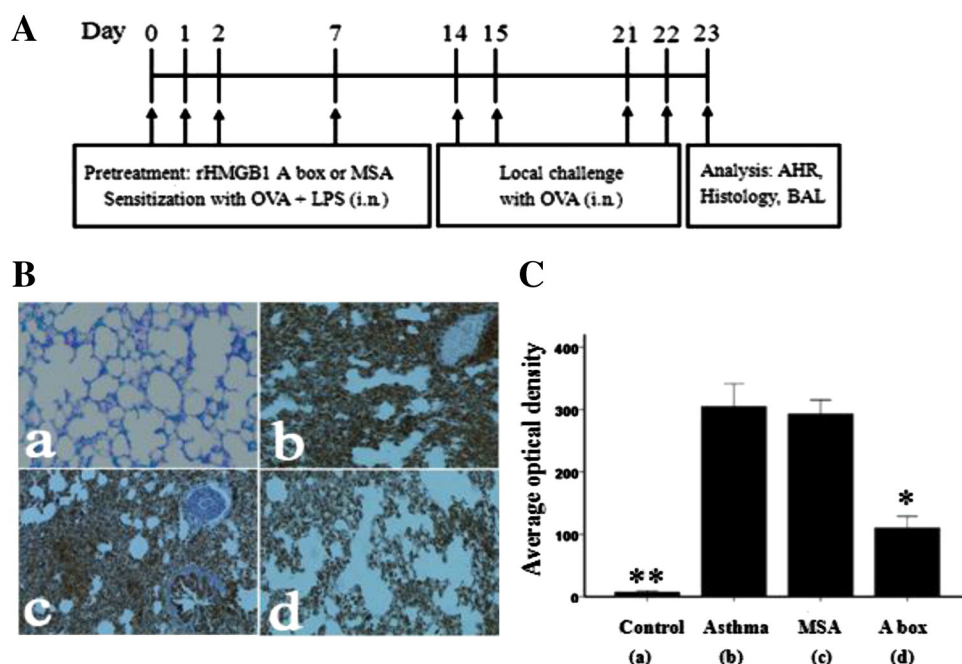


Fig. 1. Intranasal treatment of HMGB1 A box inhibited HMGB1 expression in the lung. (A) Protocol of a murine model of neutrophilic asthma and experimental intervention. Mice were intranasally sensitized with OVA plus LPS on days 0, 1, 2 and 7, and then challenged with OVA on days 14, 15, 21 and 22. rHMGB1-A box or MSA was intranasally treated a half an hour before each OVA challenge. One day after the final challenge, mice were sacrificed for further analysis. (B) Representative images of immunohistochemical (IHC) staining for HMGB1 in lung tissue from mice in the control (a), asthma (b), MSA-treated (c) and rHMGB1 A box-treated groups (d). (C) Histogram of average optical density represents HMGB1 expression. The values represent the means $\pm$ standard error of the means (SEM) ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

2.3. Bronchoalveolar lavage fluid (BALF)

To assess differential BALF cell counts, lungs were lavaged twice by instillation and withdrawal of 1 ml PBS through a tracheal cannula as described previously [20]. The cells in the BALF samples were centrifuged and loaded onto slides. For differential cell counts, cyto-centrifuged preparations were fixed and stained with Diff-Quick (Kokusaishiyaku, Kobe, Japan) and differentiated morphologically by counting 300 cells/slide. The levels of IL-23, IL-17A, IL-4 and interferon-gamma (IFN- γ) in the BALF were determined using ELISA according to the manufacturer's instructions (eBioscience).

2.4. Histological examination

For histopathological assessment, lungs were fixed and embedded in paraffin. Sections (5 mm) from all lobes were stained with haematoxylin and eosin (H&E). The sections were observed using a microscope at 200 $\times$ magnification.

2.5. Airway hyper-reactivity (AHR)

AHR was assessed in conscious, unrestrained mice by whole-body plethysmography (model PLY 3211, Buxco Electronics, Troy, NY, USA), as described previously [21]. Briefly, the mice were placed in the plethysmograph chamber and exposed to aerosolized, normal saline solution (baseline readings) and subsequently to cumulative concentrations of β -methacholine that ranged from 3 to 24 mg/ml. The aerosol was generated using an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed, and enhanced pause (Penh) readings were taken for 3 min and averaged. The values were then reported as the Penh index.

2.6. Measurement of HMGB1 expression

The paraffin-embedded lung tissues were stained to detect the expression of HMGB1 as described previously [22]. Briefly, Paraffin-embedded tissue sections were de-waxed, rehydrated and stained with primary antibodies for HMGB1 (Santa Cruz Biotechnology Inc.) that was diluted at 1:50 and biotinylated secondary antibody that was diluted at 1:500. The bound peroxidase was visualized using the DAB method. We counted 10 randomly chosen fields per section using a microscope at 200 $\times$ magnification, and the intensity of HMGB1 protein staining was determined as the average optical density using IPP software (Image-Pro Plus 6.0, Media, Cybernetics). A non-stained region was selected and set as the background.

2.7. Culture and treatment of DCs

Bone marrow-derived DCs (BMDCs) were generated as previously described [20]. In brief, bone marrow was flushed from femurs and tibia of mice and plated at 2×10^5 cells/ml in complete RPMI 1640 plus 5 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) and 1 ng/ml IL-4 (PeproTech). After 8 days of culture, DCs were purified using CD11c purification kits (Miltenyi-Biotec, Auburn, CA, USA) and treated with medium in the absence or presence of rHMGB1 (500 ng/ml) (R&D Systems, Minneapolis, MN) with or without various concentrations of rHMGB1 A box (10, 50, 100 or 200 ng/ml) (R&D Systems) for 2 days. Cytokine concentrations in the cell culture supernatants were measured using ELISA kits (IL-23 and IL-6; R&D Systems). rHMGB1 and rHMGB1 A box was endotoxin tested with Limulus amoebocyte lysate (Zhanjiang A&C Biological, China), and both products were considered to be endotoxin free.

2.8. Co-culture of DCs with CD4⁺ T Cells

CD4⁺ T cells were purified from the spleens of OVA-sensitized mice using a 'Mouse CD4⁺ T cell isolation kit' (Stem Cell Technologies, Vancouver, BC, Canada), following the manufacturer's instructions. OVA-sensitized murine model was constructed as described previously [20]. Briefly, On days 0, 7 and 14, 0.1 mL of an OVA/Al(OH)₃ mixture containing 50 μ g OVA (grade V; Sigma-Aldrich, St Louis, MO, USA) and 1 mg Al(OH)₃ (Pierce, Rockford, Illinois, USA) was injected intraperitoneally. From days 21 to 25, the mice inhaled nebulized OVA in normal saline (10 g/L) for 30 min each day. The purity of isolated CD4⁺ T cells was greater than 98%. CD4⁺ T cells (1×10^5 cells/well) were mixed in a 96-well plate with BMDCs (2.5×10^4 cells/well) stimulated with rHMGB1 (500 ng/ml) in the absence or presence of rHMGB1 A box (200 ng/ml). OVA (10 μ g/mL) was added, and the mixed cells were cultured at 37 °C in 5% CO₂. After 5 days of incubation, the supernatants were analyzed for IL-17 using ELISA, and the expression of intracellular IL-17 in CD4⁺ T cells were analyzed using FACS analysis.

2.9. Flow cytometry analysis

To detect the intracellular IL-17 expression by CD4⁺ T cells in the DC-T cell co-culture system of lung cells, the cells were incubated with 10 μ g/ml brefeldin A (eBioscience) for 2 h and stained with FITC-anti-CD4 mAb (eBioscience) for 30 min at 4 °C. After incubation with the fixation/permeabilization solution (eBioscience), the cells were stained with PE-anti-IL-17 mAb (eBioscience) for 30 min. Data were acquired by FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). Lung cells in this study were obtained according to previously reported methods [23]. Briefly, the lungs were minced into small pieces and digested with RPMI 1640 solution containing collagenase XI and DNase I (both from Roche Applied Science, Indianapolis, USA), followed by the lysis of erythrocytes using ACK buffer (eBioscience).

To detect IL-23⁺ CD11c⁺ APCs in lung tissue, low-density lung cells were prepared as described previously [24]. Briefly, digested lung cells (10×10^6 /ml) were resuspended in high-density Percoll ($\rho = 1.075$ g/ml), overlaid with an equal volume of lower density Percoll ($\rho = 1.030$ g/ml), and centrifuged at 400 $\times$ g for 20 min. Low-density lung cells, which were enriched for mononuclear cells, were recovered from the 1.075/1.030 Percoll interface and washed with Hanks' balanced salt solution (HBSS). Low-density lung cells were then stained with FITC-anti-CD11c (eBioscience) and PE-anti-IL-23 mAb (eBioscience) and analyzed using a FACSCalibur flow cytometer.

2.10. Adoptive transfer of DCs

The establishment of a murine model of asthma using adoptive transfer of bone marrow-derived DCs (BMDCs) has been described previously [20]. Briefly, BMDCs were pulsed with OVA and injected into anaesthetized, naïve mice via the intra-tracheal route. Ten days after intra-tracheal injection, the mice were challenged with OVA (1% w/v in PBS; grade V; Sigma-Aldrich) aerosol during a daily, 30-min challenge on three consecutive days. In this experiment, the mice were divided randomly into three groups ($n = 6$ per group) as follows: (i) mice received an i.t. injection of 2×10^6 PBS-treated and non-pulsed DCs (PBS/DCs); (ii) mice received an i.t. injection of 2×10^6 rHMGB1-treated OVA-DCs (rHMGB1/OVA-DCs); and (iii) mice received an i.t. injection of 2×10^6 rHMGB1 A box plus rHMGB1-treated OVA-DCs (rHMGB1 + A box/OVA-DCs). The OVA-DCs were treated with rHMGB1 or rHMGB1 A box at concentrations of 500 ng/ml or 200 ng/ml, respectively. All mice were sacrificed 24 h after the final challenge for further analyses.

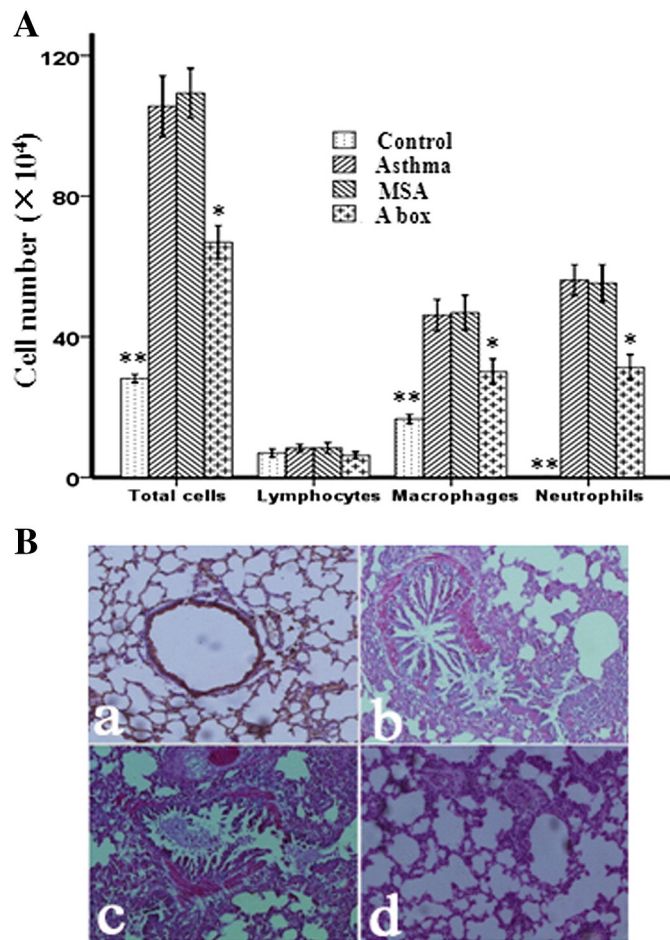


Fig. 2. Intranasal treatment of rHMGB1 A box inhibited neutrophilic airway inflammation. (A) Treatment of HMGB1 A box inhibited the infiltration of neutrophils and macrophages into BALF. BALF was collected 24 h after the last OVA challenge, and the number of total and differential cells counts in the BALF of mice in the control, asthma, MSA-treated and rHMGB1 A box-treated groups was analyzed by counting. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group. (B) Treatment of rHMGB1 A box inhibited the neutrophilic inflammation in lung tissue. Representative results of lung pathology (original magnification, 200 $\times$) in mice from the Control (a), Asthma (b), MSA-treated (c) and A box-treated groups (d) were analyzed using H&E staining. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

2.11. Statistical analysis

Data were expressed as the means $\pm$ standard error of the means (SEM). Differences between groups were analyzed using SPSS for windows (version 16.0) and unpaired, two-tailed, parametric Student's *t*-test or ANOVA tests. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. rHMGB1 A box inhibits HMGB1 expression in lung tissue

Because HMGB1 has been identified as a crucial cytokine involved in many inflammatory diseases, we supposed that rHMGB1 A box could ameliorate airway inflammation via blocking HMGB1 signaling. It was found that HMGB1 expression was elevated significantly in the asthma group mice compared with the control group mice; however, this

effect was suppressed by the administration of rHMGB1 A box, suggesting that rHMGB1 A box has an inhibitory effect on HMGB1 expression (Fig. 1B).

3.2. rHMGB1 A box prevents the neutrophilic airway inflammation

To estimate the effects of rHMGB1 A box on airway inflammation, rHMGB1 A box was administered intranasally in the murine neutrophilic asthma model. OVA sensitization and challenge induced a substantial increase in total cells and neutrophils in BALF. This was due largely to increased neutrophils numbers, but almost no eosinophils (Fig. 2A), which is one of the characteristics of neutrophilic bronchial asthma. The administration of rHMGB1 A box before airway sensitization markedly decreased the number of total cells and neutrophils in BALF (Fig. 2A). The treatment, the same amount of MSA, did not influence the BALF cell counts.

H&E-stained lung tissue sections showed increased numbers of inflammatory cells, including neutrophils, in the peribronchial and perivascular regions of the asthma group mice compared to the control group mice (Fig. 2B). The administration of rHMGB1 A box decreased the infiltration of inflammatory cells, including primary neutrophils (Fig. 2B). These data suggest that rHMGB1 A box ameliorates neutrophilic airway inflammatory in this asthma model.

3.3. rHMGB1 A box inhibits the development of AHR

Next, to assess the effect of rHMGB1 A box on AHR, the relative increase of Penh in response to methacholine inhalation was evaluated. After OVA challenge, AHR was increased in the asthma group mice, while the administration of rHMGB1 A box before airway sensitization inhibited the development of AHR significantly (Fig. 3).

3.4. rHMGB1 A box suppresses local Th17 response in vivo

To analyze the Th1/Th2/Th17 response in airway, cytokine levels in BALF were measured. Th17-associated cytokines, IL-17 and IL-23, were increased in the asthma group mice, and the increase of IL-17 and IL-23 was attenuated significantly by rHMGB1 A box (Fig. 4A). In contrast, no significant differences in IL-4 (Th2-associated

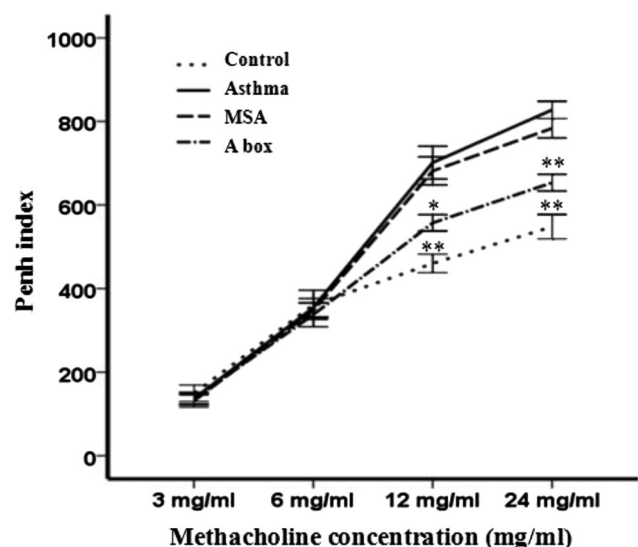


Fig. 3. Intranasal treatment of HMGB1 A box decreased AHR. The Penh values represent the OVA-induced AHR to methacholine stimuli and were measured in mice from the control, asthma, MSA-treated, and rHMGB1 A box-treated groups using a body plethysmograph. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group.

cytokine) levels were seen among the asthma group and A box group mice. The level of IFN- γ (Th1-associated cytokine) decreased in rHMGB1 A box administered mice, but this did not reach statistical significance.

Because the administration of rHMGB1 A box suppressed the level of IL-17A in BALF, we investigated the possibility that this suppression was mediated through the decreased production of Th17 cells in the lung. As expected, the asthma group mice exhibited dramatically increased Th17 cells production in the lung tissue compared with the control group mice, while the administration of rHMGB1 A box significantly down-regulated the production of Th17 cells in lung tissue (Fig. 5B). These results suggested that rHMGB1 A box could suppress the production of Th17 cells *in vivo*.

3.5. rHMGB1 A box decreases the number of IL-23⁺ CD11c⁺ APCs in lung tissue

Because lung antigen-presenting cells (APCs) have been documented to possess the potential to induce T helper cell polarization, we investigated whether rHMGB1 A box treatment has a regulatory effect on CD11c⁺ APCs function. Consistent with the reduced level of IL-23 in the BALF, the administration of rHMGB1 A box significantly decreased IL-23 production, which is an important Th17 polarized-cytokine that promotes the differentiation and proliferation of Th17 by lung CD11c⁺ APCs (Fig. 5). These results demonstrate that rHMGB1 A box could decrease the number of IL-23⁺ CD11c⁺ APCs, thus inhibiting the development of Th17-mediated immune responses in the lung.

3.6. rHMGB1 A box inhibits HMGB1-stimulated BMDCs by inducing Th17 responses *in vitro*

To ascertain whether rHMGB1 A box could inhibit the production of Th17 polarization-related factors by HMGB1-stimulated APCs *in vitro*, we initially exposed BMDCs to HMGB1 and various concentrations of rHMGB1 A box and then measured their capacity to express IL-23 and IL-6 in culture supernatants. Results showed that rHMGB1 (500 ng/ml) significantly increased the IL-23 levels in a 2-day culture, while HMGB1 plus rHMGB1 A box markedly decreased the IL-23 production by BMDCs when rHMGB1 A box concentrations reached 100 ng/ml or 200 ng/ml (Fig. 6A). A slight increase in IL-6 production was observed after rHMGB1 treatment, but rHMGB1 A box plus rHMGB1 did not have significant effects on IL-6 production (data not shown). The results suggest that rHMGB1 A box have an inhibitory effect on rHMGB1-stimulated DCs to secrete Th17-polarized cytokines, such as IL-23, *in vitro*.

To examine the Th17 development and contribution of rHMGB1 A box to HMGB1-stimulated BMDCs, *in vitro* antigen presentation was analyzed by the co-culture of HMGB1-stimulated BMDCs with OVA peptide and CD4⁺ T cells from OVA-sensitized mice in the absence or presence of rHMGB1 A box. Results showed that rHMGB1-stimulated BMDCs significantly enhanced IL-17A secretion in the culture supernatants (Fig. 6B) and increased the percentage of IL-17⁺ CD4⁺ T cells (Fig. 6C). In contrast, rHMGB1 A box significantly down-regulated the expression of IL-17 induced by rHMGB1-stimulated BMDCs, while addition of MSA had no effects on IL-17 expression, suggesting that the rHMGB1 A box suppressed Th17 responses induced by HMGB1-stimulated BMDCs.

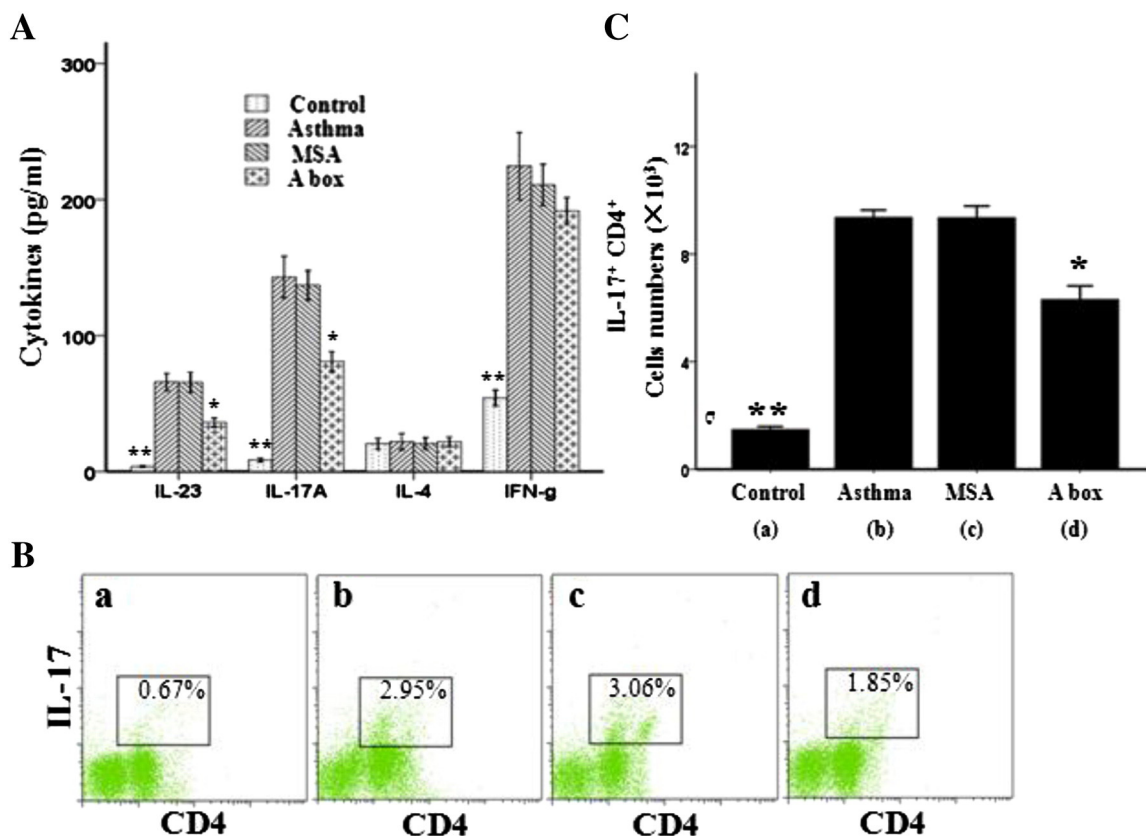


Fig. 4. Intranasal treatment of rHMGB1 A box suppressed the Th17 responses in the lung. (A) Treatment of rHMGB1 A box suppressed Th17 cytokine production after OVA challenge. BALF were collected 24 h after the last OVA challenge, the production of IL-23, IL-17A, IL-4 and IFN- γ in the BALF of mice from control, asthma, MSA-treated and rHMGB1 A box-treated groups were assessed using ELISA. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group. (B) Treatment of rHMGB1 A box down-regulated the count of Th17 cells in the lung. Representative results of the percentage of Th17 cells in CD3⁺ gated lung cells from mice in the control (a), asthma (b), MSA-treated (c) and rHMGB1 A box-treated groups (d) were analyzed using FACS. (C) Histogram shows the number of Th17 cells in mice from the different groups. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

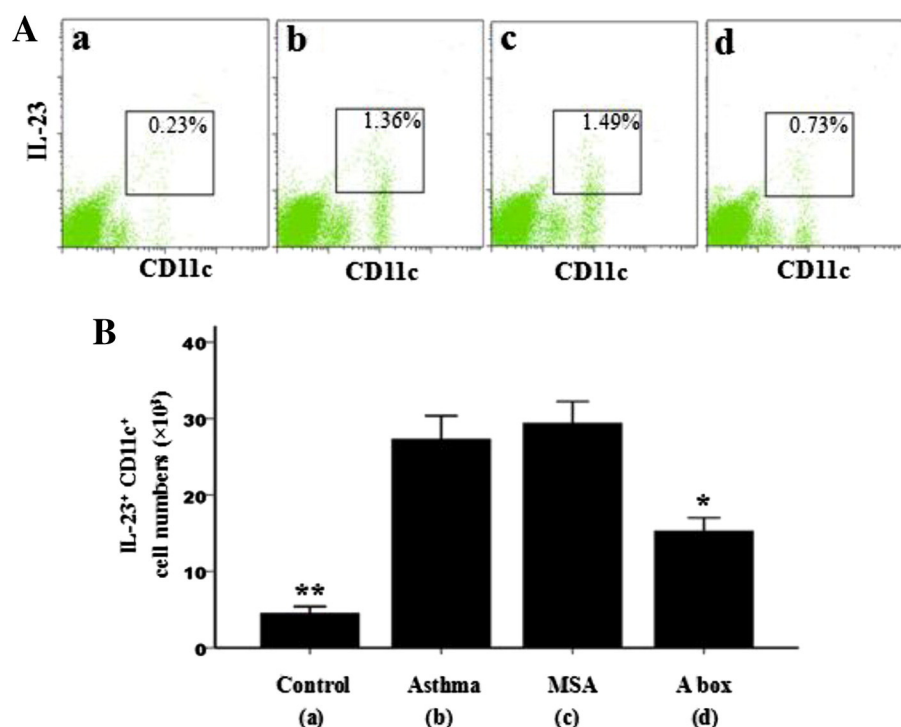


Fig. 5. Intranasal treatment of HMGB1 A box decreased the IL-23 expression by lung CD11c⁺ APCs. (A) Treatment of anti-HMGB1 decreased the percentage of IL-23⁺ CD11c⁺ APCs in low-density lung cells. Representative results of the percentage of IL-23⁺ CD11c⁺ APCs in lung from mice in the Control (a), Asthma (b), MSA-treated (c) and HMGB1 A box-treated groups (d) were analyzed using FACS. (B) Histogram shows the number of IL-23⁺ CD11c⁺ APCs in mice from the different groups. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3.7. rHMGB1 A box inhibits the Th17 priming induced by rHMGB1-stimulated BMDCs in vivo

To evaluate the role of rHMGB1 A box on HMGB1-stimulated BMDCs in Th17 polarization in vivo, we adoptively transferred rHMGB1 or rHMGB1 plus rHMGB1 A box-treated OVA-DCs into the airways of mice. As expected, adoptive transfer of rHMGB1-stimulated DCs resulted in a marked neutrophilic inflammation that was characterized by infiltration of neutrophils into the BALF (Fig. 7B) and Th17/Th1 priming, as shown by the increase in IL-23, IL-17A and IFN- γ levels in the BALF (Fig. 7C). However, adoptive transfer of rHMGB1 plus rHMGB1 A box-stimulated DCs significantly decreased the neutrophils in the BALF and the Th17 response, as shown by the decrease in IL-23 and IL-17A levels (Figs. 7B and 7C). These data confirmed that rHMGB1 A box inhibits the capability of rHMGB1-stimulated DCs to induce neutrophilic inflammation and Th17 skewing in asthmatic mice.

4. Discussion

Previous research in animal models indicates that the development of neutrophilic asthma might be caused by the enhancement of the Th17 immune response, but the pathogenesis of the abnormal Th17 polarization are still elusive. Recent studies indicate that abnormal Th17 polarization in neutrophilic asthma are well associated with the steroid-insensitivity and severity of asthmatics, a better understanding of its pathogenesis and the development of Th17 polarization in neutrophilic asthma will be helpful to currently available therapies for steroid-insensitive or severe asthma [25]. Here we show for the first time that rHMGB1 A box, which is an antagonist for HMGB1, represses HMGB1 expression and acts on DCs to attenuate the local Th17 response following allergic airway inflammation.

In the present study, rHMGB1 A box alleviated neutrophilic airway inflammation in a mouse asthmatic model by reducing HMGB1 expression, the influx of neutrophils and AHR at least in part by the regulation of Th17 polarization via blocking the HMGB1 pathway, indicating that the development of neutrophilic airway inflammation is dependent upon the activation of the HMGB1 pathway. Shim et al. [26] reported that sputum HMGB1 expressions were markedly higher in asthmatics than in normal controls and were positively correlated with sputum eosinophilia. Zhou et al. [13] reported that asthmatics exhibited a higher percentage of neutrophils and higher HMGB1 in induced sputum samples, and positive correlations were observed between HMGB1 and percentage of neutrophils. Lee et al. [17] reported increases in HMGB1 expression in the lungs from a mouse model of chronic asthma, and decreases in Th2 and Th17 cells after anti-HMGB1 antibody (Ab) treatment. These studies indicated that HMGB1 will be an important pathological mechanism underlying allergic asthma, especially in neutrophilic asthma, and HMGB1 might be a potential therapeutic target for allergic asthma.

In fact, HMGB1 signaling exists on different kinds of immune cells and regulates cellular behavior, such as the maturation and migration of DCs [27,28]. In the present study, we focused upon the function of CD11c⁺ APCs and showed that HMGB1 A box treatment modulated immune responses by inhibiting the number of the IL-23⁺ CD11c⁺ APCs population and Th17 polarization in lung tissue. In the pathogenesis of asthma, CD11c⁺ APCs are especially potent to induce Th2 polarization during ongoing airway inflammation, and result in AHR and airway eosinophilia. Also, CD11c⁺ APCs are reported to produce Th17-polarizing cytokines, such as IL-23 or IL-6, stimulating Th17 responses [29]. Tanaka et al. [30] stated that CD11c⁺ DCs activated by a combination of TSLP and TLR3 ligands promote naive CD4⁺ T cells to differentiate into Th17 cells under Th2-polarizing conditions; Kao et al. reported that *H. pylori* alters the DC-polarized Th17/Treg balance toward a Treg-biased response, which suppresses the effective induction of *H. pylori*-specific

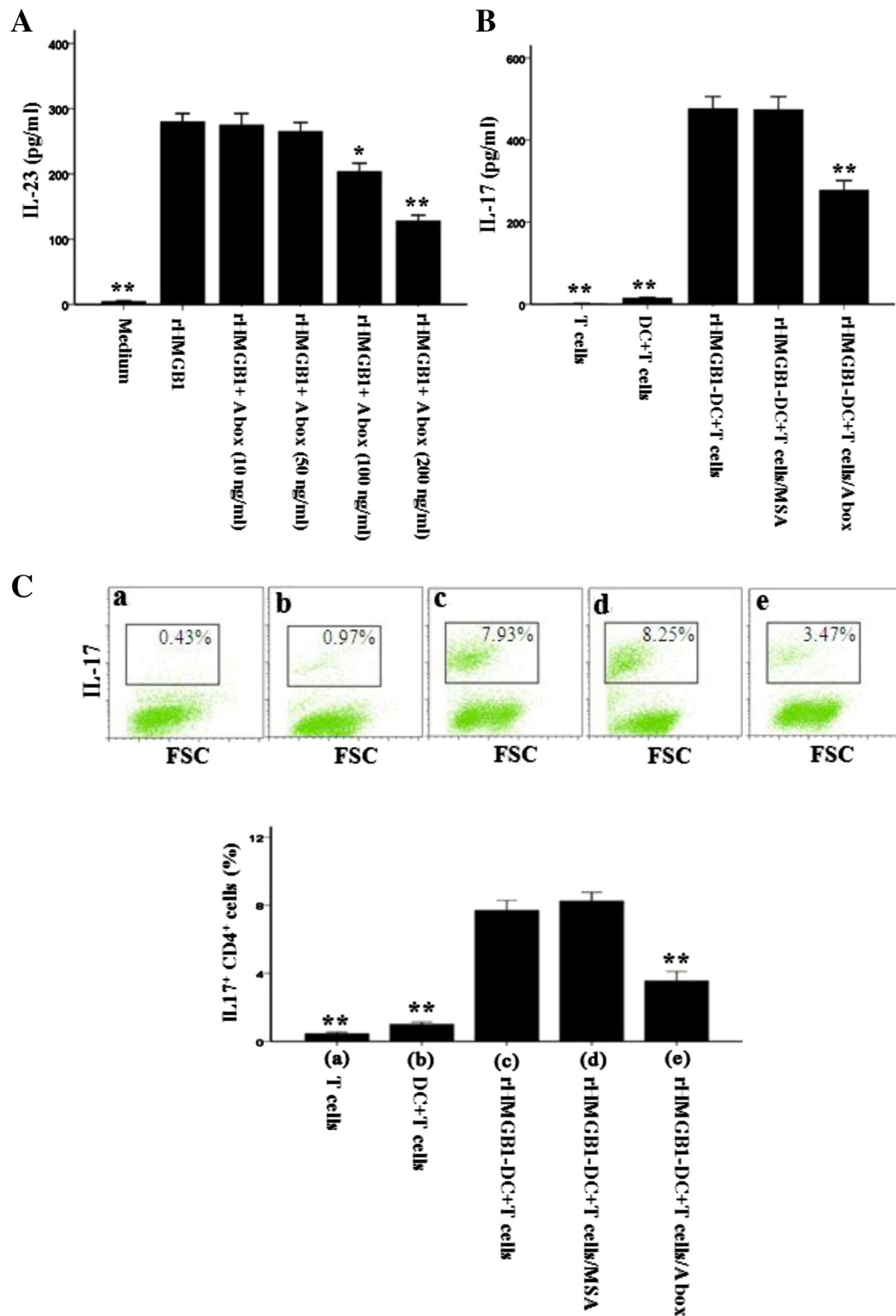


Fig. 6. rHMGB1 A box down-regulated HMGB1-treated BMDCs inducing Th17 responses *in vitro*. (A) rHMGB1 A box inhibited HMGB1-treated BMDCs to produce IL-23. BMDCs were cultured in medium or treated with 500 ng/ml HMGB1 alone, or co-cultured with graded concentrations of rHMGB1 A box for 2 days. The IL-23 levels in the culture supernatants were measured using ELISA. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the rHMGB1-treated BMDCs group. (B and C) rHMGB1 A box inhibited Th17 polarization induced by the HMGB1-treated BMDCs. CD4⁺ T cells from the spleens of OVA-sensitized mice (1×10^5) were cultured alone or co-cultured with BMDCs (2.5×10^4) that were treated with or without 500 ng/ml HMGB1 in the presence or absence of 200 ng/ml HMGB1 A box or MSA, and the supernatants were analyzed for IL-17A using ELISA (B). The expression of intracellular IL-17 by CD4⁺ T cells was analyzed using FACS (C). The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the rHMGB1-treated BMDCs group. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Th17 immunity [31]. In this study, HMGB1-activated DCs co-cultured with CD4⁺ T cells from OVA-sensitized mice induced Th17 responses. Moreover, transfer of HMGB1-activated DCs leads to the induction

of neutrophilic airway inflammation, while transfer of HMGB1 plus HMGB1 A box-activated DCs results into the amelioration of neutrophilic airway inflammation, suggesting that HMGB1 A box inhibits HMGB1-

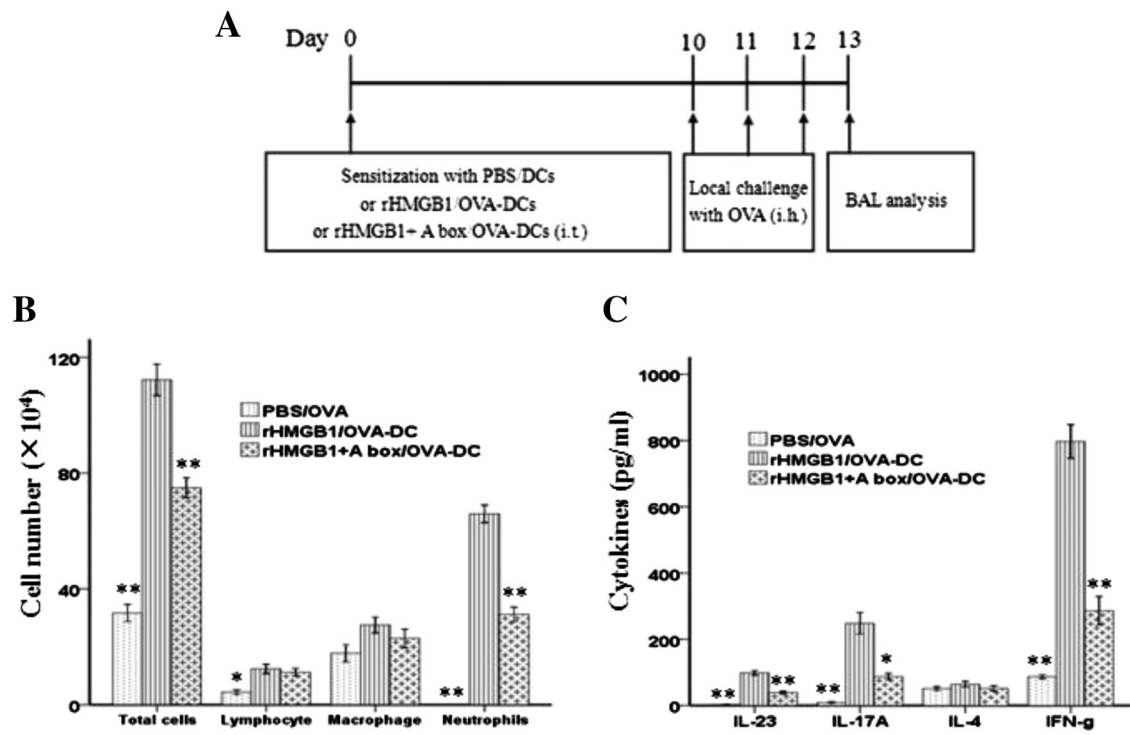


Fig. 7. rHMGB1 A box suppressed the ability of rHMGB1-treated BMDCs to prime Th17 responses *in vivo*. (A) Protocol of a murine model of asthma by the transfer of BMDCs and experimental intervention. Mice received an intra-tracheal (i.t.) injection of 2×10^6 PBS-treated, non-pulsed BMDCs (PBS/DCs), rHMGB1-treated, OVA-pulsed BMDCs (rHMGB1/OVA-DCs) or rHMGB1 plus rHMGB1 A box-treated, OVA-pulsed BMDCs (rHMGB1 + A box/OVA-DCs). The mice were then exposed to OVA challenge on days 10–12. One day after the final challenge, the mice were sacrificed for further analysis. (B) The total and differential cell counts and (C) cytokine profiles in the BALF were assessed 24 h after the final OVA exposure. The values represent the means $\pm$ SEM ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the asthma group.

activated DCs function to induce Th17 responses. These results confirm the evidence that the overexpression of HMGB1 is an important character in neutrophilic asthma, and HMGB1-activated DCs have the potential to develop Th17 polarization.

In conclusion, the HMGB1 pathway is important in the regulation of allergic airway inflammation, and HMGB1 A box is probably an effective, preventative or therapeutic strategy for neutrophilic asthma by blocking the HMGB1 pathway on DCs.

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Disclosure

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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