

HMGB1 regulates T helper 2 and T helper17 cell differentiation both directly and indirectly in asthmatic mice

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ABSTRACT

The Th (T helper) 2 response is characteristic of allergic asthma, and Th17 cells are involved in more severe asthma. Recent studies demonstrated that HMGB1 (High mobility group box 1 protein) regulates airway inflammation and the Th2, Th17 inflammatory response in asthma. HMGB1 can interact with Toll-like receptors (TLR) 2 and 4, and the receptor for advanced glycation end products (RAGE), activating the NF- κ B (nuclear factor kappa B) signaling pathway and inducing the release of downstream inflammatory mediators. Both Th cells and dendritic cells express TLR2, TLR4, and RAGE receptors. Therefore, we speculate that HMGB1 could regulate the differentiation of Th2, Th17 cells in asthma through direct and indirect mechanisms. An ovalbumin (OVA)-induced mouse asthmatic model was established. Anti-HMGB1 antibody or rHMGB1 was administered to OVA-sensitized mice 30 min prior to each challenge. For *in vitro* studies, magnetically separated CD4⁺ naive T cells were stimulated with or without rHMGB1 and/or anti-HMGB1 antibody. BMDCs (bone marrow-derived dendritic cells)-stimulated with or without rHMGB1 and/or anti-HMGB1 antibody were cocultured with CD4⁺ naive T cells. Our study showed that administration of rHMGB1 aggravated airway inflammation and mucus production, and induced Th2, Th17 polarization in asthmatic mice, and that anti-HMGB1 antibody weakened characteristic features of asthma and blocked the Th2, Th17 inflammatory responses. HMGB1 could directly act on naive T cells to induce differentiation of Th2, Th17 cells *in vitro* through activating the TLR2, TLR4, RAGE-NF- κ B signal pathway in CD4⁺ naive T cells. HMGB1 could also indirectly promote Th2, Th17 differentiation via activating the TLR2, TLR4, RAGE-NF- κ B signal pathway in DCs to mediate their maturation and antigen-presenting ability *in vitro*.

1. Introduction

Allergic asthma is one of the most common airway inflammatory diseases, characterized by recurrent and reversible airflow obstruction associated with airway hyperresponsiveness (AHR) and airway inflammation. Both clinical and experimental evidence support that the reciprocal regulation between Th (T helper) 1/Th2 and Th17/Treg balance plays an important role in the pathogenesis of asthma. The expression of both Th2 and Th17 cytokines, such as interleukin (IL)-4, IL-5, IL-13 and IL-17A, enhance the infiltration of inflammatory cells, allergen-specific IgE synthesis, airway mucus production, and

abundance of airway eosinophils, a primary feature of asthma (Vroman et al., 2015; Kim et al., 2010). A Th2 response is characteristic of allergic eosinophilic asthma, whereas Th17 cells contribute to severe and fatal asthma exacerbations and steroid therapy resistance (Choy et al., 2015; Zhao et al., 2013; Cosmi et al., 2011).

HMGB1, high mobility group box 1 protein, is a ubiquitous and highly conserved nucleoprotein that is released into the extracellular space upon stimulation by inflammation and infection, by monocytes, macrophages, and dendritic cells, and can also be released by dead cells (Lotze and Tracey, 2005). HMGB1, a pro-inflammatory mediator, belongs to the alarmin family and has a key role in different acute and

Abbreviations: Th cells, T helper cells; NF- κ B, nuclear factor kappa B; HMGB1, High mobility group box 1 protein; AHR, Airway hyperresponsiveness; IL, Interleukin; TLR, Toll-like receptors; RAGE, Receptors for advanced glycation end products; PBMCs, Peripheral blood mononuclear cells; DCs, Dendritic cells; rHMGB1, Recombinant mouse HMGB1; BALF, Bronchoalveolar lavage fluid; BMDCs, Bone marrow-derived DCs; MNCs, Mononuclear cells; cDNA, Complementary DNA; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IFN, Interferon; PMA, Phorbol 12-myristate 13-acetate; BFA, Brefeldin A; P-NF- κ B, Phosphorylation of NF- κ B; APCs, Antigen-presenting cells

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chronic immunity diseases (Cavone et al., 2015; Cuppari et al., 2015; Gangemi et al., 2015). It not only stimulates the immune system to produce an inflammatory response, caused by a variety of cytokines and pro-inflammatory cells, but is also involved in signal transduction. HMGB1 can interact with Toll-like receptors (TLR) 2 and 4, and receptors for advanced glycation end products (RAGE) by activating the MAPK and NF- κ B (nuclear factor kappa B) signal pathways, inducing the release of downstream inflammatory mediators (Lotze and Tracey, 2005; Yang et al., 2010; Manfredi et al., 2008). Recent studies have confirmed that HMGB1-mediated airway inflammation contributes to the pathogenesis of asthma, and the level of HMGB1 is related to the severity of asthma (Huang et al., 2012; Watanabe et al., 2011; Shim et al., 2012).

Both Th cells and other immune cells (dendritic cells and monocytes) express TLRs and RAGE. HMGB1 acts as an endogenous ligand for these receptors and regulates these cells' function. Multiple studies have shown that, *in vitro*, rHMGB1 stimulates splenic CD4⁺ T cells, leading to a significant increase of ROR γ t and IL-17 mRNA levels, augments the ratio of CD4⁺ IL-17⁺ T cells, and lowers the production and expression of FoxP3 mRNA and IL-10 from Treg cells (Shi et al., 2012; Su et al., 2011; He et al., 2012; Zhang et al., 2011; Zhu et al., 2011). TLRs or RAGE competitive antagonists can significantly reduce levels of IL-17 in the peripheral blood mononuclear cells (PBMCs) (Zhang et al., 2011; Zhu et al., 2011; Kim et al., 2013). rHMGB1 could both time- and dose-dependently increase the abundance of Th2 subsets, and thus reduce the Th1/Th2 ratio, stimulating the Th cells to shift from Th1 to Th2 (Huang et al., 2008). *In vivo*, exogenous HMGB1 induced a dominance of Th2 cells, a Th17-type response, and an attenuated Th1-type response (Ma et al., 2015; Wang et al., 2015; Lee et al., 2013). Thus, HMGB1 can directly affect TLRs and RAGE expressed by CD4⁺ T cells, thus regulating the polarization of Th2 and Th17. In a study of neutrophilic asthma in mice by Zhang et al. (2014, 2015), HMGB1 could promote the maturation of dendritic cells (DCs) and stimulate the secretion of IL-23. DCs activated by HMGB1 induced naive T cells to differentiate into Th17 cells, which secrete IL-17A. Anti-HMGB1 weakened airway inflammation and AHR, and reduced the number of Th17 cells and expression of Th17/DC-related cytokines in asthma. Therefore, it can be hypothesized that the effect of HMGB1 may also require the interaction between CD4⁺ T cells and other immune cells, by programming DCs to mount Th2, Th17-mediated immune responses.

Thus, we speculated that HMGB1 could regulate the differentiation of Th2 and Th17 cells through two mechanisms, both directly and indirectly, to participate in the pathogenesis of asthma; the first being: HMGB1 directly regulates Th0 cells to differentiate into Th2 and Th17 cells through the RAGE/TLR2 or 4-NF- κ B signal pathway, and the second being: HMGB1 mediates the maturation of DCs and their antigen-presenting ability via the RAGE/TLR2 or 4-NF- κ B signal pathway, indirectly promoting Th2, Th17 differentiation.

2. Materials and methods

2.1. Animals and model of allergic asthma

Female and male C57BL/6 mice aged 6–8 weeks were obtained from the Center for Animal Experiments of Wuhan University. Animal care and handling protocols were approved by the Animal Welfare Committee of Wuhan University. On day 0 and 14, female mice were intraperitoneally sensitized by a 20- μ g injection of OVA (Sigma Aldrich, St. Louis, MO, USA), emulsified in 200 μ l PBS, containing 2 mg aluminum hydroxide (Thermo Fisher Scientific Inc, Rockford, IL, USA). The OVA-sensitized mice were then given an intranasal challenge of 100 μ g OVA in 50 μ l PBS once a day, on days 21–23, and were intratracheally given recombinant mouse HMGB1 (rHMGB1, R&D Systems, Minneapolis, MN, USA) (Zhang et al., 2014, 2015) or purified anti-HMGB1 IgG2b (Biolegend Inc., San Diego, CA, USA) (Zhang et al.,

2014; Zhou et al., 2009) 30 min before each OVA challenge. Isometric PBS was administered as a control. The mice were euthanized 24 h after the final challenge, followed by collecting serum, bronchoalveolar lavage fluid (BALF), lungs, and spleen for subsequent analysis.

2.2. Bronchoalveolar lavage

BALF was collected with 0.5 ml PBS containing 1 mM EDTA, instilled by a syringe 3 times. BALF cells were harvested by centrifugation at 1500 rpm for 7 min at 4 °C. Slides for the cellular component were prepared by cytocentrifugation (TXD3 cytocentrifuge, Xiangyi, Changsha, China), then were stained with Wright-Giemsa (Jiangcheng Bioengineering Institute, Nanjing, China). In total, 400 cells were counted on each slide under a light microscope. BALF cell supernatant was collected for ELISA analysis.

2.3. Histological analysis

The left lung tissue were fixed in 4% paraformaldehyde for more than 24 h and then embedded in paraffin. Lung sections of 5 μ m were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) (Baso, Taiwan, China). Peribronchial inflammatory infiltration and goblet cells hyperplasia were assessed at 200 $\times$ magnification by microscope. The severity of peribronchial inflammation was graded semiquantitatively using lung inflammatory scores (Ma et al., 2015). The extent of goblet cell hyperplasia in each airway was estimated utilizing Image Pro Plus 6.0 (IPP 6.0) software, calculating Apas⁺/Pbm (Nie et al., 2015).

2.4. Generation of bone marrow-derived DCs (BMDCs)

BMDCs were prepared from femur bone marrow suspensions of male mice aged 5–8 weeks, then cultured at a density of 1–2 $\times$ 10⁶ cells/ml in RPMI1640, with 10% FBS (HyClone, Logan, UT, USA) and 0.1% 100 $\times$ Penicillin-Streptomycin solution (Solarbio, Beijing, China). GM-CSF and rmIL-4 (PeproTech Inc., Rocky Hill, NJ, USA) were added at 10 ng/mL. BMDCs were placed in a 37 °C, 5% CO₂ incubator, and the medium was refreshed every two days. After 7 days, the BMDCs were collected and co-cultured with naive T cells.

2.5. Magnetic separation of CD4⁺ naive T cells and culture of CD4⁺ T cells

Mouse splenic mononuclear cells (MNCs) were prepared as a single-cell suspension at concentration of 1 $\times$ 10⁸ cells/ml. Based on the protocol of the MagniSort[®] mouse CD4 naive T cell enrichment kit (eBioscience Inc. an Affymetrix company, part of Thermo Fisher Scientific, Vienna, Austria), CD4⁺ naive T cells were collected by negative selection (Park et al., 2016). The purity was greater than 95%. The CD4⁺ naive T cells were then collected after incubation with anti-CD3 (2 μ g/mL) and soluble anti-CD28 (1 μ g/mL) in complete RPMI 1640 medium for 3 days. OVA (10 μ g/mL) was added, then cells were treated with medium in the absence or presence of rHMGB1 (50 μ g/mL) or/and anti-HMGB1 antibody (10 μ g/mL) for 24 h (Shi et al., 2012; Su et al., 2011; He et al., 2012; Zhang et al., 2011; Zhu et al., 2011; Kim et al., 2013; Huang et al., 2008; Zhang et al., 2014, 2015).

2.6. Coculture of CD4⁺ naive T cells with BMDCs

CD4⁺ T naive cells stimulated with anti-CD3 and anti-CD28 were plated at 1 $\times$ 10⁵ cells/well, then cocultured with 2.5 $\times$ 10⁴ BMDCs/well, stimulated with or without rHMGB1 (50 μ g/mL) or/and anti-HMGB1 (10 μ g/mL) for 2 days (Zhang et al., 2014, 2015). OVA (10 μ g/mL) was added. After incubation for 5 days, the culture supernatants were analyzed by ELISA, and the cells were used for analyses by western blotting, RT-PCR, and FACS.

2.7. Western blot analysis

Total protein (40 µg), extracted from the right lung using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA), then blocked with 5% non-fat milk in TBST (Tris-buffered saline containing Tween 20) for 2 h at room temperature. The PVDF membranes were incubated overnight with diluted primary antibodies (1:1000) at 4 °C and then treated with diluted HRP-conjugated secondary anti-rabbit antibody (1:50000, BOSTER Biological Technology Co., Wuhan, China) at 37 °C for 2 h. Chemiluminescent images were captured with Pierce enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA). The bands were analyzed using BandScan 5.0 (Glyko, Novato, CA, USA) gel imaging software. The relative protein expression level was calculated with GAPDH as a control.

2.8. Real-time PCR analysis

Total RNA was extracted from lung tissue, using Trizol reagent (Invitrogen/Thermo Fisher Scientific Inc., Carlsbad, CA, USA). RNA (1 µg) was reverse-transcribed to complementary DNA (cDNA), using rReverTra Ace qPCR RT kit (Toyobo, Tokyo, Japan). We obtained the primers from Sangon Biotech (Shanghai, China) and tested them for sequence specificity using Primer-BLAST from the National Center for Biotechnology Information (NCBI). Expression of target genes was quantified by RT-PCR with SYBR Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan). All primer sequences are presented in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. The relative expression of the target genes was calculated by the comparative $2^{-\Delta\Delta C_t}$ method.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-4, IL-5, IL-13, interferon gamma (IFN-γ), IL-10, IL-17A and IL-22 in BALF and cell culture supernatant was quantified using sandwich-type enzyme-linked immunosorbent assay kits (eBioscience, San Diego, CA, USA) according to the manufacturer's

Table 1
Primer sequences and product sizes.

Gene name	Primer sequences (5'–3')	Product size (bp)
TLR2	Forward: 5'-CAGTCCCAAGTCTAAAGTCG-3'	167
	Reversed: 5'-TCTACGGGCAGTGGTGAAA-3'	
TLR4	Forward: 5'-TGGGTCAAGGAACAGAAGCA-3'	151
	Reversed: 5'-TCACACTGACCACTGACACA-3'	
RAGE	Forward: 5'-CGGGACTCTTTACACTGCG-3'	187
	Reversed: 5'-CCTTCAGGCTCAACCAACA-3'	
NF-κB	Forward: 5'-GGACCTATGAGACCTTCAAGAG-3'	112
	Reversed: 5'-ACAGAAAGTTGAGTTTCGGGTAG-3'	
GATA3	Forward: 5'-CTGGAGGAGGAACGCTAATG-3'	239
	Reversed: 5'-AGATGTGGCTCAGGGATGAC-3'	
T-bet	Forward: 5'-CCATTCTCTGCTTTCACCG-3'	137
	Reversed: 5'-CTGCGCTTCTGCTTTCAC-3'	
RORγt	Forward: 5'-GCGGCTTTCAGGCTTCATGGAG-3'	221
	Reversed: 5'-GGGCGCTGAGGAAGTGGGAAAA-3'	
Foxp3	Forward: 5'-ACTCGCATGTTCGCCTACTT-3'	181
	Reversed: 5'-AGGGATTGGAGCACTTGTG-3'	
GAPDH	Forward: 5'-TGTGTCCGTCGTGGATCTGA-3'	150
	Reversed: 5'-TTGCTGTGAAGTCGAGGAG-3'	

a) TLR2, Toll-like receptor 2; b) TLR4, Toll-like receptor 4; c) RAGE, Advanced glycation end products receptor; d) NF-κB, Nuclear factor kappa-enhancer of activated B cells; e) GATA3, GATA binding protein 3; f) T-bet, T-box transcription factor; g) RORγt, Retinoic acid receptor-related orphan receptor γt; h) Foxp3, Forkhead box protein 3; i) GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

instructions. The levels of total IgE in serum were detected using specific ELISA kits (eBioscience, San Diego, CA, USA).

2.10. Flow cytometric analysis

For determination of DCs, lung MNCs were first suspended in FACS buffer at 2×10^6 cells/ml. PE-CD11c antibody (eBioscience, San Diego, CA, USA) and APC-Cy7-F4/80 antibody (Biolegend Inc., San Diego, CA, USA) staining was used to separate DCs from lung MNCs; DCs were measured as CD11c⁺/F4/80⁺ MNCs (Matsuoka et al., 2013). Next the cells were labeled with the following antibodies: FITC-MHCII, FITC-CD80, FITC-CD40 (eBioscience, San Diego, CA, USA), APC-CD86, APC-CCR5, APC-CCR7 (Biolegend Inc., San Diego, CA, USA). For analyzing the percentage of Th1, Th2, Th17, Treg in the CD4⁺ Th cell population, MNCs were first cultured for 2 h with ionomycin (Tocris Bioscience, Bristol, UK) at 500 ng/mL and phorbol 12-myristate 13-acetate (PMA, Tocris Bioscience, Bristol, UK) at 20 ng/mL, then $1 \times$ BFA (brefeldin A, Biolegend, San Diego, CA, USA) was used to block for 4 h. After staining with FITC-CD3e and PE-cy7-CD4 (eBioscience, San Diego, CA, USA), Th1, Th2 and Th17 cells were measured as CD3e and CD4 double-positive cells. They were stained with PE-IFN-γ, PE-IL-4, PE-IL-17A (eBioscience, San Diego, CA, USA) to distinguish them after fixation and permeabilization (Intracellular Fixation & Permeabilization Buffer Set, eBioscience, San Diego, CA, USA). For determination of Treg cells, MNCs were at first labeled double-positively with PE-cy7-CD4 and PE-CD25 (eBioscience, San Diego, CA, USA), then fixed and permeabilized (FoxP3 Fixation/Permeabilization Concentrate and Diluent, eBioscience, San Diego, CA, USA), and were finally stained for intranuclear region with FITC-FoxP3 (eBioscience, San Diego, CA, USA). For analyzing the percentage of CD44⁺ Th cells (differentiated Th cells) in the Th cell population, cultured T cells were stained with FITC-CD3e and PE-cy7-CD4, then were marked with APC-CD44 (eBioscience, San Diego, CA, USA). All Th cells and DCs were analyzed by flow cytometry (FACSARIA™ III, BD Biosciences, USA).

2.11. Statistical analysis

All data is reported as the mean $\pm$ SD, and analyzed using SPSS 22.0 software (IBM SPSS, Chicago, IL, USA). Comparison between groups was conducted via non-parametric tests and one-way analysis of variance. The presented data is one representative experiment from three. $P < 0.05$ was considered to be statistically significant. “*” denotes $P < 0.05$, “**” denotes $P < 0.01$, “***” denotes $P < 0.001$.

3. Results

3.1. HMGB1 aggravated airway inflammation, mucus production, and Th2 and Th17 responses in lung tissue of asthmatic mice

This study investigated the role of HMGB1-driven Th2 and Th17 responses in the OVA-induced asthmatic mice model. On day 21–23, mice were intratracheally given rHMGB1 and anti-HMGB1 antibody 30 min before each OVA challenge. In histological analysis, the OVA-sensitized and challenged mice showed cardinal pathological features, including numerous inflammatory cells assembling in the peribroncholar and perivascular zones, and goblet cell hyperplasia. Compared with that in the PBS-treated asthma group, rHMGB1 administration resulted in an increase of lung infiltration by inflammatory cells and the number of PAS-positive cells in the airways. On the contrary, anti-HMGB1 markedly weakened the asthmatic typical histological change (Fig. 1a–d). The number of eosinophils, lymphocytes, and neutrophils markedly increased in the BALF of mice exposed to OVA compared to that in control group. The intervention of rHMGB1 markedly increased eosinophil abundance in BALF. In addition, a reduction in the number of eosinophils and lymphocytes was observed in the anti-HMGB1 antibody-treated mice (Fig. 1e). The results of total IgE levels in serum

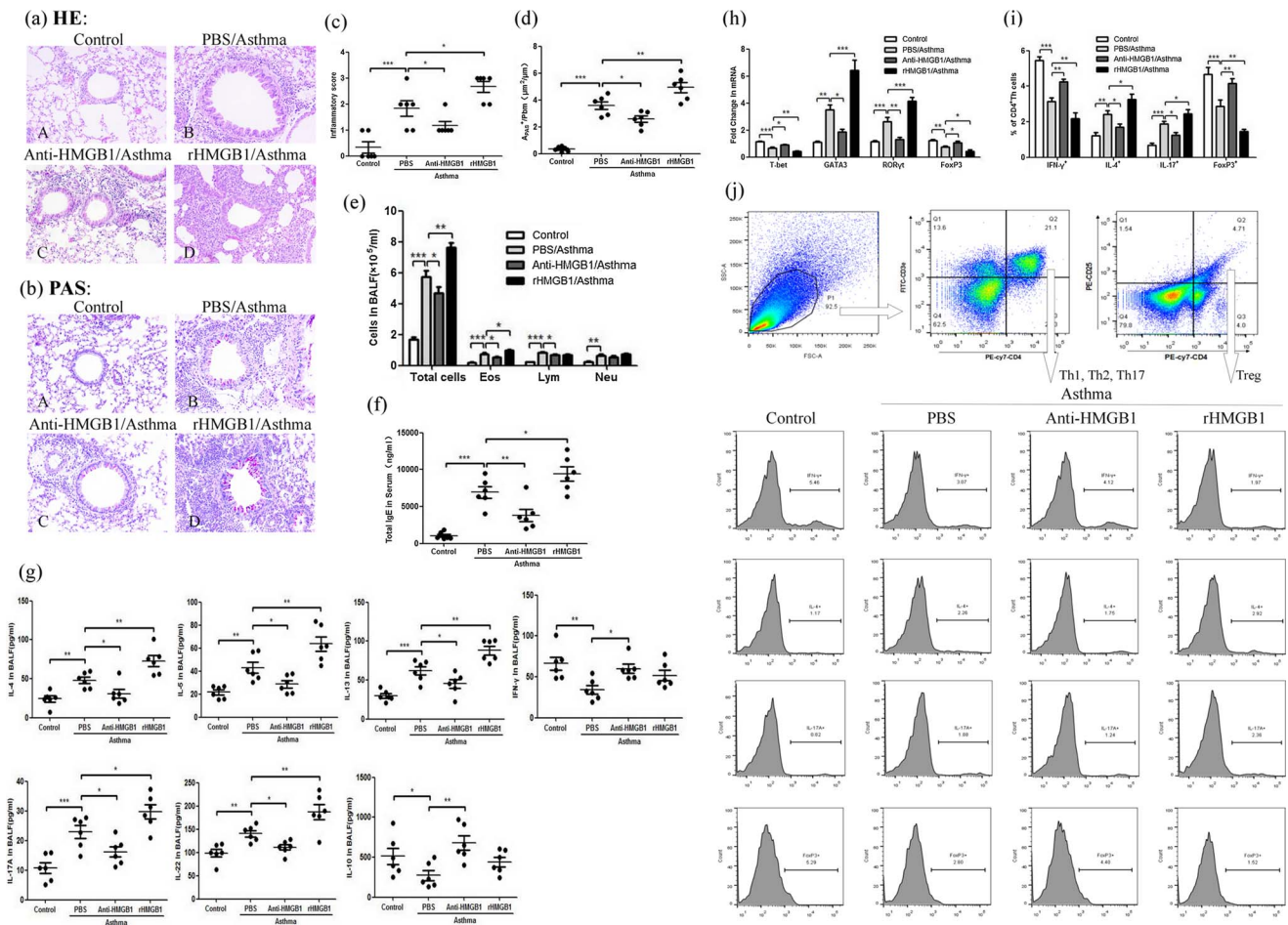


Fig. 1. Increased allergic airway inflammation and Th2, Th17 polarization by administration of rHMGB1, Anti-HMGB1 antibody had the opposite effect.

(a, c) Histological analysis of lung sections and inflammatory score. (b, d) PAS-positive areas per unit length were determined. (a: A–D) Representative results for inflammatory infiltration (HE, $\times 200$). (b: A–D) representative results for mucus secretion (PAS, $\times 200$). (e) The number of inflammatory cells (total cells, eosinophils, lymphocytes, neutrophils) in BALF. (f) The total IgE level was measured in serum. (g) ELISA results of inflammatory cytokines (IL-4, 5, 13, 17A, 22, 10, IFN- γ) secretion in the BALF. (h) T-bet, GATA3, ROR γ t, FoxP3 mRNA expression of lung tissue were measured. (i) Percentage of IFN- γ $^+$, IL-4 $^+$, IL-17A $^+$, FoxP3 $^+$ -expressing CD4 $^+$ Th cells in lung. (j) Lung CD3e and CD4 double-positive Th cells were examined for IFN- γ (Th1), IL-4 (Th2), IL-17A (Th17) secretion, and lung CD4 and CD25 double-positive Th cells were examined for FoxP3 (Treg) expression. The numbers below the parentheses indicate the percentage of positive cells. All data are represented as mean $\pm$ SEM ($n = 4-6$ mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

were in line with the above evidence (Fig. 1f). The levels of IL-4, IL-5, IL-13, IL-22 and IL-17A in the BALF were markedly increased in the PBS-treated asthma group. These increased levels of cytokines in BALF were further increased by rHMGB1 administration, but were significantly reduced by anti-HMGB1 antibody treatment. On the other hand, the levels of IFN- γ and IL-10 were augmented by anti-HMGB1 antibody treatment (Fig. 1g). In addition, a rise in the percentage of CD4 $^+$ Th cells expressing IL-4 $^+$ and IL-17A $^+$, and a decrease in the percentage of CD4 $^+$ Th cells expressing IFN- γ $^+$ and FoxP3 $^+$, in lung MNCs was observed in the asthmatic groups as compared with that in the control group. These changes were further enhanced in the rHMGB1-intervened asthmatic groups in comparison with that in the PBS-treated asthma group. Anti-HMGB1 antibody treatment lowered these effects (Fig. 1i and j). We further determined whether HMGB1 regulated the mRNA expression of Th cell-related transcription factors in the lungs obtained from asthmatic mice. The administration of rHMGB1 effectively augmented the expression of GATA3 and ROR γ t, and lowered the expression of T-bet and FoxP3, in the lung tissues compared with that in the PBS-treated asthmatic mice. Anti-HMGB1 antibody had the opposite effect (Fig. 1h). Thus, HMGB1 aggravated airway inflammation, mucus production, and the Th2 and Th17 responses in the lung tissue of asthmatic mice.

3.2. HMGB1 activated TLR2, TLR4, and RAGE-NF- κ B signal pathway of CD4 $^+$ naive T cells *in vitro*

Next, we confirmed whether HMGB1 could directly act on naive T cells, and explored its downstream signaling pathway. CD4 $^+$ naive T cells were separated from splenic MNCs, using a magnetic negative selection kit, and then were stimulated with or without rHMGB1 and/or anti-HMGB1 antibody. As shown in Fig. 2a and b, the levels of TLR2, TLR4, RAGE, NF- κ B p65, and phosphorylation of NF- κ B p65 (P-NF- κ B p65) proteins showed significant increases in the rHMGB1 group. However, this increase was apparently attenuated by anti-HMGB1 antibody treatment in the rHMGB1 + anti-HMGB1 group. We also observed a similar change in levels of TLR2, TLR4, RAGE, and NF- κ B mRNA expression (Fig. 2c). These results show that HMGB1 could act on CD4 $^+$ naive T cells *in vitro* via the TLR2, TLR4, and RAGE-NF- κ B signal pathway.

3.3. HMGB1 directly induced differentiation of Th2 and Th17 cells *in vitro*

Next, we verified whether HMGB1 could directly activate CD4 $^+$ naive T cells via the TLR2, TLR4, and RAGE-NF- κ B signal pathways, and induce differentiation of CD4 $^+$ naive T cells. Flow cytometry analysis were used to analyze CD4 $^+$ naive T cells stimulated with or without rHMGB1 and/or anti-HMGB1 antibody. CD44 expression is usually

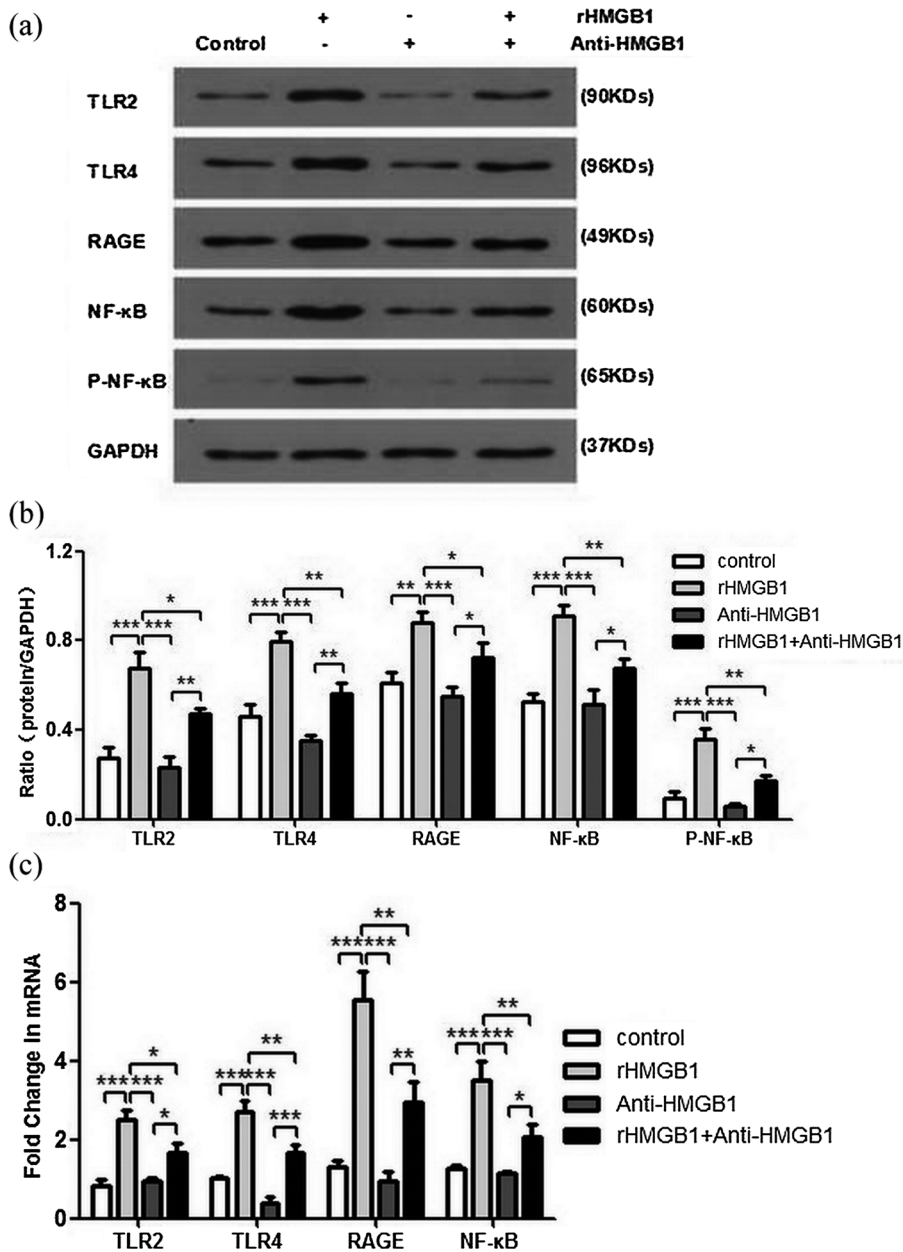


Fig. 2. rHMGB1 upregulated the expression of TLR2, TLR4, RAGE, and NF-κB in CD4⁺ T cells.

CD4⁺ naive T cells separated from splenic MNCs were treated with or without rHMGB1 or/and anti-HMGB1 antibody after incubation with anti-CD3 (2 μg/mL) and anti-CD28 (1 μg/mL) in complete medium for 3 days. (a) Levels of TLR2, TLR4, RAGE, NF-κB p65, and P-NF-κB p65 in CD4⁺ T cells were measured by western blotting. GAPDH served as a loading control. (b) Densitometric ratio analyses (TLR2, TLR4, RAGE, NF-κB p65 and P-NF-κB p65/GAPDH) using BandScan 5.0 gel image software. (c) Levels of TLR2, TLR4, RAGE, and NF-κB in CD4⁺ T cells were measured by real-time PCR. GAPDH served as loading control. All data are represented as mean ± SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001

used to distinguish murine naive from differentiated T cells. As shown in Fig. 3a and b, rHMGB1 administration significantly increased CD44 expression in Th cells, as compared to the control group. However, the increases were weakened upon anti-HMGB1 stimulation. In comparison with the control group, rHMGB1 administration showed a significant increase in the mRNA expression of transcription factors GATA3 and RORγt. These changes were decreased in rHMGB1 + anti-HMGB1 administration group (Fig. 3c). Flow cytometry analysis also revealed that rHMGB1 stimulation increased the percentage of CD4⁺ Th cells expressing IL-4⁺, IL-17A⁺, IFN-γ⁺ and FoxP3⁺, as compared with that in the control group. Stimulation with anti-HMGB1 antibody weakened this increase of IL-4⁺ and IL-17A⁺ expression, and further enhanced the increase in IFN-γ⁺ and FoxP3⁺ expression in the rHMGB1 plus anti-HMGB1 antibody stimulation group (Fig. 3d and e). We also measured the Th2- and Th17-related cytokines in the cell culture supernatant. The results showed that in the rHMGB1 group, the levels of IL-4, IL-5, IL13, IL17A, and IL-22 were significantly increased compared with that in the control. However, anti-HMGB1 antibody clearly inhibited this increase (Fig. 3f). These results suggest that HMGB1 could directly activate

CD4⁺ naive T cells, and induce the differentiation of Th2 and Th17 cells *in vitro*.

3.4. HMGB1 activated TLR2, TLR4, and RAGE-NF-κB signal pathway in DCs *in vitro*

Next, we further evaluated whether HMGB1 could indirectly active naive T cells and induce their differentiation by activating DCs. We first examined whether HMGB1 could activate the TLR2, TLR4, and RAGE-NF-κB signal pathway in DCs. Western blot analysis showed that rHMGB1 augmented TLR2, TLR4, RAGE, NF-κB p65, and P-NF-κB p65 protein expression in DCs (Fig. 4a and b) with an accompanied rise in the expression of TLR2, TLR4, RAGE and NF-κB mRNA (Fig. 4c). In comparison with the rHMGB1 group, the rHMGB1 + anti-HMGB1 group showed a significant reduction in the expression of TLR2, TLR4, RAGE, NF-κB p65, and P-NF-κB p65 proteins (Fig. 4a and b) and a decrease in the mRNA expression of TLR2, TLR4, RAGE and NF-κB mRNA (Fig. 4c). Therefore, HMGB1 could act on DCs via TLR2, TLR4, and RAGE-NF-κB signal pathway *in vitro*.

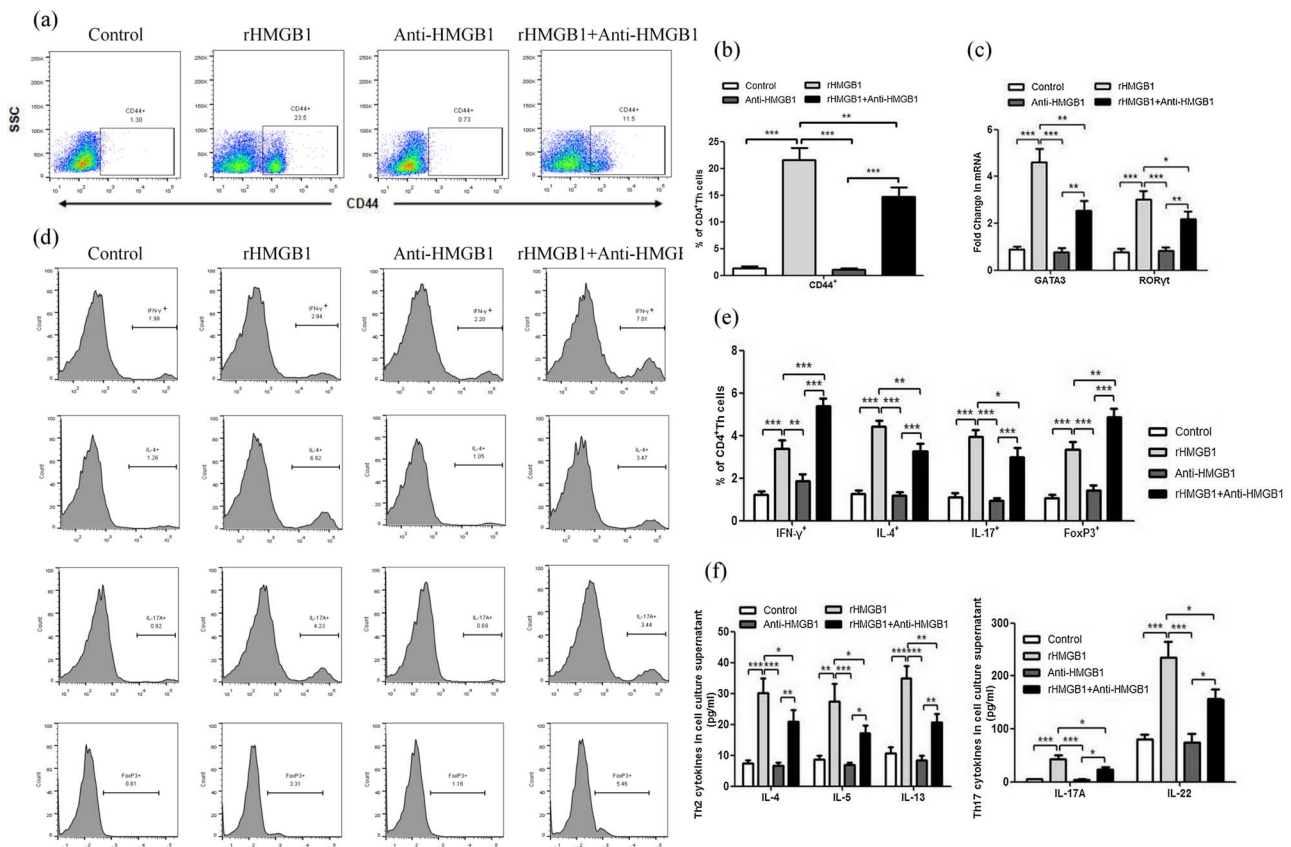


Fig. 3. rHMGB1 activated CD4⁺ naive T cells, and induce the differentiation towards Th2, Th17 cells.

CD4⁺ naive T cells separated from splenic MNCs were treated with or without rHMGB1 or/and anti-HMGB1 antibody after incubation with anti-CD3 (2 µg/mL) and anti-CD28 (1 µg/mL) antibodies in complete medium for 3 days. (a) CD44⁺ Th cells (differentiated Th cells) were examined from CD3e⁺CD4⁺ Th cells by flow cytometric analysis. (b) The percentage of CD3e⁺CD4⁺ Th cells expressing CD44⁺ in different groups. (c) Real-time PCR analyses of GATA3, RORγt and GAPDH expression in different groups. (d) CD3e⁺CD4⁺ Th cells were examined for IFN-γ (Th1), IL-4 (Th2) and IL-17A (Th17) secretion from CD4⁺ naive T cells, and CD4, CD25 double-positive Th cells were examined for FoxP3 (Treg) expression. The numbers below the parentheses indicate the percentage of positive cells. (e) Percentage of IFN-γ⁺, IL-4⁺, IL-17A⁺, and FoxP3⁺-expressing CD4⁺ Th cells in CD4⁺ naive T cells. (f) ELISA results of Th2 cytokines (IL-4, IL-5, IL-13), Th17 cytokines (IL-17A, IL-22) secretion in cell culture supernatant. All data are represented as mean ± SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

3.5. HMGB1 induced the maturation of DCs in asthmatic mice

Subsequently, we observed the effect of HMGB1 on DCs of lung tissue *in vivo*. Whilst detecting surface antigen presenting molecule (MHCII), costimulating factors (CD80, CD86, CD40), and chemotactic factors (CCR5, CCR7) of lung CD11c⁺/F4/80[−] MNCs (DCs), we found that the percentage of MHCII, CD80, CD86, CD40, CCR7-positive expression was significantly elevated, and the percentage of CCR5-positive expression was lowered in PBS-treated asthma group in contrast to that in the control. The increase of MHCII, CD80, CD86, CD40 and CCR7 levels was obviously higher in the asthma group administrated with rHMGB1 relative to that in the PBS-treated asthma group. It was also observed that the expression of CCR5 was reduced after rHMGB1 administration. The opposite results were observed in the presence of anti-HMGB1 antibody treatment (Fig. 5a and b). Combined with the experimental results of the previous step, this data demonstrated that HMGB1 could induce the maturation of DCs and enhance their antigen-presenting ability via TLR2, TLR4, and RAGE-NF-κB signal pathway *in vivo*.

3.6. HMGB1 indirectly induced differentiation of Th2 and Th17 cells *in vitro*

The above studies have indicated that HMGB1 activated TLR2, TLR4, and the RAGE-NF-κB signal pathways of DCs to induce their maturation and elevate their antigen presenting ability. Next, we evaluated whether HMGB1 indirectly induced the differentiation of Th2

and Th17 cells by mediating the function of DCs. Through coculturing CD4⁺ naive T cells with BMDCs-stimulated with or without rHMGB1 (50 µg/mL) and/or anti-HMGB1 antibody (10 µg/mL) for 5 days, we carried out flow cytometry analysis to detect the percentage of different Th cells. As illustrated in Fig. 6a and b, the expressions of CD44 in Th cells were significantly increased in coculturing CD4⁺ naive T cells with BMDCs-stimulated by rHMGB1 groups, in comparison with only T cells and T cells-cocultured with BMDCs without any intervention groups. In addition, rHMGB1 + anti-HMGB1-stimulated BMDCs down-regulated the increases. In real-time PCR detection of transcription factors GATA3 and RORγt, we observed similar changes (Fig. 6c). As shown in Fig. 6d and e, compared with only T cells and T cells-cocultured with BMDCs without any intervention, DCs stimulated by rHMGB1 markedly increased the percentage of IFN-γ⁺ CD3e⁺CD4⁺ T cells, IL-4⁺ CD3e⁺CD4⁺ T cells, IL-17⁺ CD3e⁺CD4⁺ T cells and FoxP3⁺ CD25⁺CD4⁺ T cells. The rHMGB1 + anti-HMGB1-stimulated BMDCs significantly further upregulated the percentage of IFN-γ⁺ CD3e⁺CD4⁺ T cells and FoxP3⁺ CD25⁺CD4⁺ T cells, and down-regulated the percentage of IL-4⁺ CD3e⁺CD4⁺ T cells and IL-17⁺ CD3e⁺CD4⁺ T cells, in comparison with the that in the rHMGB1-stimulated BMDCs group. IL-4, IL-5, IL-13, IL-17A, and IL-22 secretion in the coculture cell supernatants was enhanced in the rHMGB1-stimulated BMDCs cocultured with T cells group. However, no statistically significant differences were observed between the anti-HMGB1-stimulated BMDCs group and two control groups (Fig. 6f). In conclusion, HMGB1 could indirectly induce the differentiation of Th2 and Th17 cells *in vitro* through the activation DCs via TLR2, TLR4, and RAGE-NF-

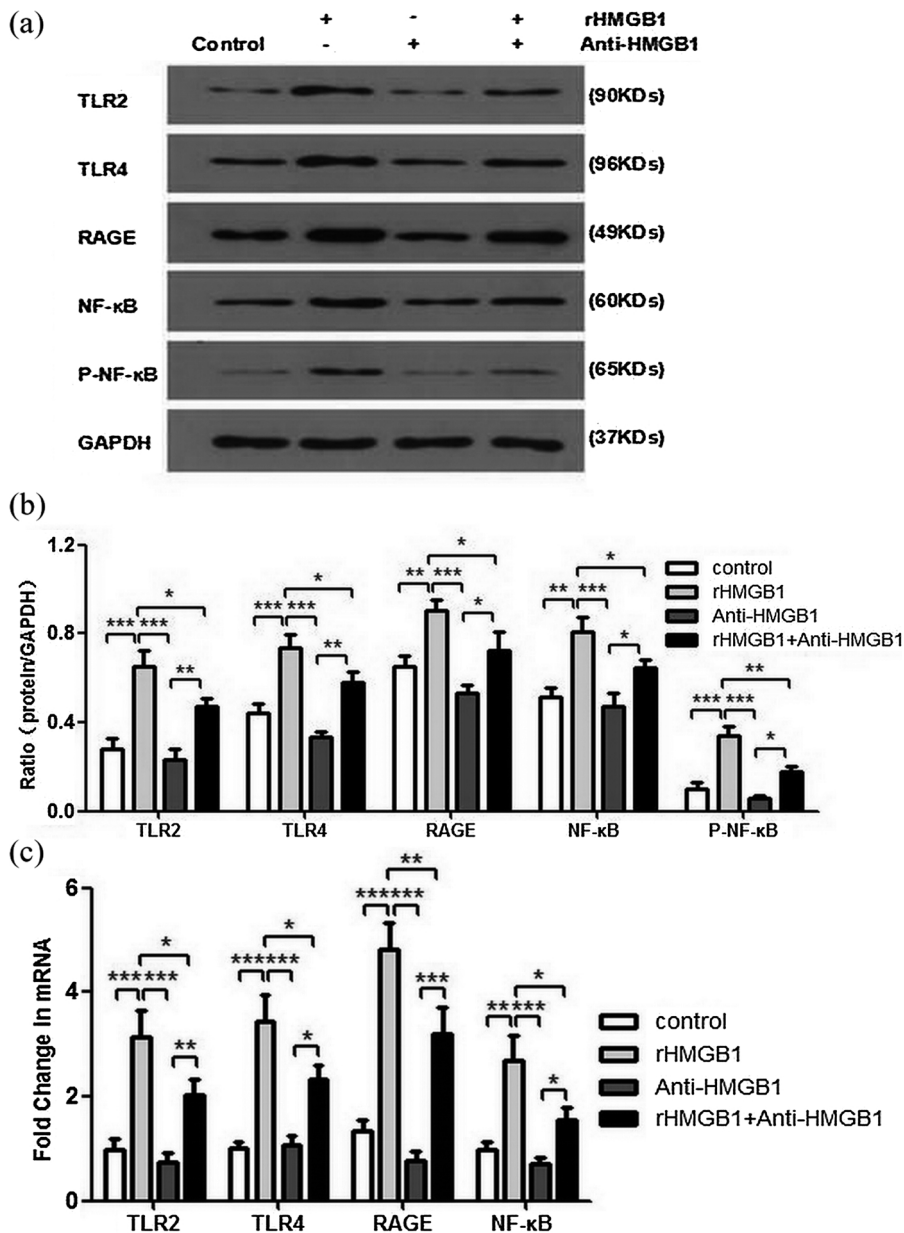


Fig. 4. rHMGB1 upregulated the expression of TLR2, TLR4, RAGE, and NF-κB in BMDCs.

BMDCs were prepared from femur bone marrow suspensions and treated with or without rHMGB1 (50 μg/mL) or/and anti-HMGB1 antibody (10 μg/mL) for 2 days. (a) Levels of TLR2, 4, RAGE, NF-κB p65, and P-NF-κB p65 in BMDCs were measured by western blotting. GAPDH served as loading control. (b) Densitometric ratio analyses (TLR2, TLR4, RAGE, NF-κB p65, and P-NF-κB p65/GAPDH) using BandScan 5.0 gel image software. (c) Levels of TLR2, TLR4, RAGE, and NF-κB in BMDCs were measured by real-time PCR. GAPDH served as loading control. All data are represented as mean ± SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

κB signal pathway.

4. Discussion

HMGB1 is a DNA-binding nuclear protein that functions to alert the innate immune system (Lotze and Tracey, 2005; Klune et al., 2008). Recently, some studies demonstrated that HMGB1, as a proinflammatory mediator, has a key role in allergic asthma, and regulates the Th2, Th17 inflammatory responses that are characteristic of allergic eosinophilic asthma (Shim et al., 2012; Ma et al., 2015; Lee et al., 2013; Zhang et al., 2014, 2015). Extracellular HMGB1 signals through multiple receptors, including TLR2, TLR4, and RAGE, triggering intracellular signal transduction and orchestrating migration and activation (Chen et al., 2015; Kang et al., 2014; Watanabe et al., 2015; Kang et al., 2015). However, little is known about how HMGB1 induces the polarization of Th2, Th17 cells in asthma.

In the present study, we found that HMGB1 regulated airway inflammation, mucus production, and Th2 and Th17 responses in an asthmatic mice model. HMGB1 induced Th2, Th17 inflammatory responses via two mechanisms, both direct and indirect, to participate in

the pathogenesis of asthma. Through the TLR2, TLR4, and RAGE-NF-κB signal pathway, HMGB1 could directly act on naive T cells to induce Th2, Th17 polarization. HMGB1 also could mediate the maturation and antigen-presenting ability of DCs, to indirectly promote Th2, Th17 differentiation.

The association between HMGB1 and asthma has been identified by several studies. Watanabe et al. (2011) were the first to test the level of HMGB1 in sputum of asthmatic patients. They found that the level of HMGB1 in sputum was significantly higher in asthmatic patients than in controls, and the HMGB1 level was relative to the severity of asthma. In 2012, Shim et al. 2012 first created an asthmatic mice model and treated the mice with anti-HMGB1 antibody to analyze lung tissue and BALF. They observed that expression of RAGE and TLR2 of DCs was augmented in the asthmatic group compared with that in the control and these changes were obviously attenuated by anti-HMGB1. Recently, Hou et al. (2015) found that anti-HMGB1 diminished inflammatory cytokine levels, inflammatory cell accumulation, mucus production in the airway, and hyperresponsiveness and airway remodeling in a chronic asthmatic mice model. These studies are in accordance with our present study, which proved that HMGB1 intervened in airway

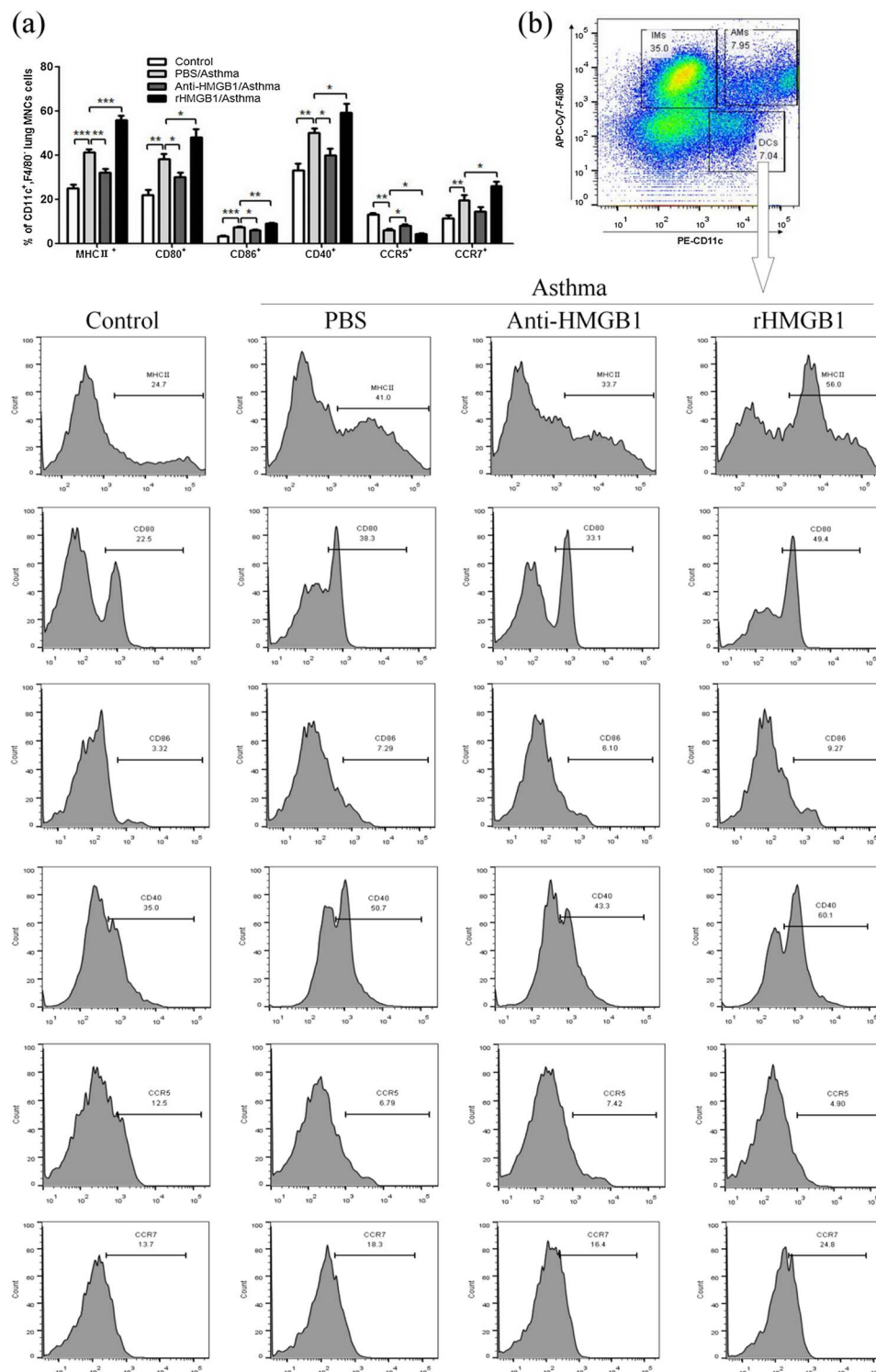


Fig. 5. rHMGB1 upregulated the surface maturation markers of lung DCs in OVA-induced asthmatic mice.

Lung DCs were measured as CD11c⁺/F4/80⁻ MNCs, IMs = interstitial macrophages (CD11c⁺/F4/80⁺ MNCs), AMs = alveolar macrophages (CD11c⁺/F4/80⁺ MNCs). (a) Percentage of DCs (CD11c⁺/F4/80⁻) expressing CD80⁺, CD86⁺, CD40⁺, CCR5⁺, and CCR7⁺. (b) CD80⁺, CD86⁺, CD40⁺, CCR5⁺, and CCR7⁺ expressing DCs (CD11c⁺/F4/80⁻) were examined from the left lung by flow cytometric analysis. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

inflammation, mucus production, and Th2, Th17 inflammatory responses in an acute asthmatic model upon rHMGB1 and anti-HMGB1 antibody administration.

TLR2, TLR4, and RAGE act as the major receptors for HMGB1. NF- κ B and MAPK are also involved in the intracellular signaling of HMGB1 (Chen et al., 2015; Kang et al., 2014; Watanabe et al., 2015; Kang et al., 2015). *In our vitro* experiments suggested that HMGB1 directly acts on

TLR2, TLR4, and RAGE-NF- κ B signal pathway of naive T cells and induces them to differentiate into Th2, Th17 cells. In recent research of Di Candia et al. (2017), it was observed that HMGB1 could contribute to airway smooth muscle cells dysfunction and AHR in asthma, in a TLR4-dependent manner. Shi et al. (2012), in researching rheumatoid arthritis, found that CD4⁺ T cells from C57BL/6 the mice spleen directly stimulated by rHMGB1 *in vitro* could increase the abundance of

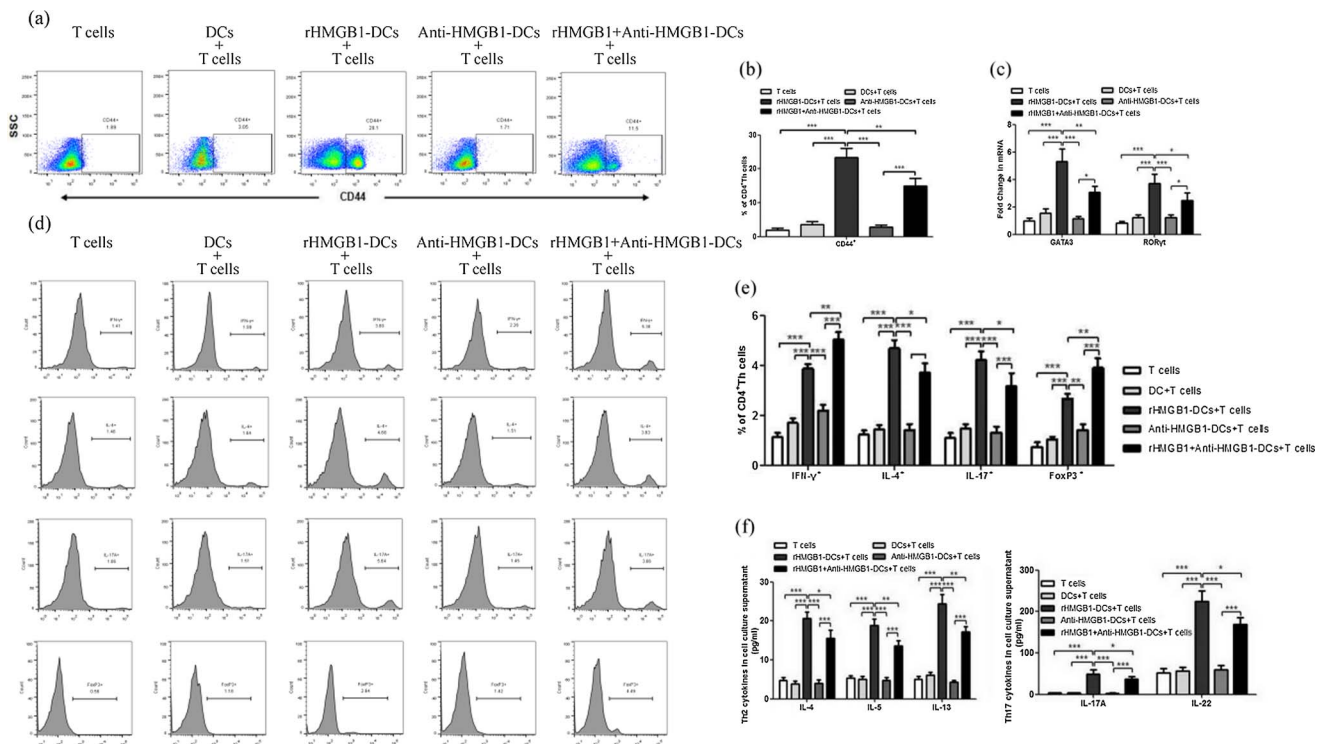


Fig. 6. rHMGB1 induce the differentiation of Th2 and Th17 cells through activating DCs. CD4⁺ naive T cells separated from splenic MNCs cocultured with BMDCs-stimulated with or without rHMGB1 (50 µg/mL) or/and anti-HMGB1 (10 µg/mL) for 5 days. (a) CD44⁺ Th cells (differentiated Th cells) were examined from CD3e⁺CD4⁺ Th cells by flow cytometric analysis. (b) The percentage of CD3e⁺CD4⁺ Th cells expressing CD44⁺ in different groups. (c) Real-time PCR analyses of GATA3, RORγt and GAPDH expression in different groups. (d) CD3e, CD4 double-positive Th cells were examined for IFN-γ (Th1), IL-4 (Th2), and IL-17A (Th17) secretion from CD4⁺ naive T cells, and CD4, CD25 double-positive Th cells were examined for FoxP3 (Treg) expression. The numbers below the parentheses indicate the percentage of positive cells. (e) Percentage of IFN-γ⁺, IL-4⁺, IL-17A⁺, and FoxP3⁺-expressing CD4⁺ Th cells in CD4⁺ naive T cells. (f) ELISA results of Th2 cytokines (IL-4, IL-5, IL-13), Th17 cytokines (IL-17A, IL-22) in cell culture supernatant. All data are represented as mean ± SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

Th17 subset and mRNA levels of Th17 cell-associated factors. Li et al. (2014) confirmed that HMGB1 regulated the Treg/Th17 balance via TLR4-IL-6 pathway *in vitro*. Huang et al. (2008) and Wang et al. (2015) considered that HMGB1 provoked an increase in the abundance of Th2 subset and lowered the ratio of Th1 to Th2 cells, blocking HMGB1-inhibited expression of inflammatory cytokines secreted by Th2, Th17 cells, and promoting Th1 cell-related cytokine expression. These findings are in line with our results.

DCs are pivotal for the initial immune response, and as APCs (antigen-presenting cells) in allergic asthma. Studies have confirmed that the activation and maturity of DCs, represented by the surface expression of MHCII and diverse costimulatory molecules, as well as inflammatory cytokines, play major roles during the induction of the T cell immune responses in asthma (Zhang et al., 2014, 2015; Lutz, 2016). In murine lung tissue, DCs have three subsets: Conventional dendritic cells (cDCs), Plasmacytoid DCs (pDCs), inflammatory DCs (infDCs) (Froidure et al., 2016). Among them cDCs play a critical role in allergen sensitization and Th2 recall responses (Plantinga et al., 2013; Lambrecht and Hammad, 2012), infDCs expresses the high-affinity receptor for IgE (FcεRI) in mice play crucial role in inducing effector T-cell responses murine asthma (Froidure et al., 2016; Lambrecht and Hammad, 2012; Hammad et al., 2010). Human lung tissue include distinct subsets of DCs: myeloid DCs1 (mDC1), mDC2, pDCs, and infDCs. mDC1 are strong inducers of T-cell activation, infDCs could present antigens and to migrate to regional lymph nodes, induce Th17 differentiation in CD4 T cells (Froidure et al., 2016). HMGB1 has been reported to activate DCs, up-regulate DC surface markers (CD80, CD83 and CD86), enhance DC production of cytokines (IL-6, IL-12, and TNF-α), and induce the migration and maturity of DCs. The effect of HMGB1 on DCs could be inhibited by suppressing TLRs or RAGE (Yang et al., 2007; Marsh et al., 2017; Gougeon et al., 2012; Zhu et al., 2017; Perrin-

Cocon et al., 2017; Liu et al., 2016; Saenz et al., 2014). Zhang et al. (2017) recently reported that blocking HMGB1/RAGE signalling inhibited Th17 polarisation induced by rHMGB1-activated airway DCs *in vitro* using sRAGE, a decoy to inhibit interactions of RAGE, in neutrophilic asthma. In our study, HMGB1 could act on DCs via TLR2, TLR4, and RAGE-NF-κB signal pathway, and induce the maturation and antigen-presenting ability of DCs. We also found that the activated DCs next induced the polarization of Th2, Th17 cells upon coculturing with CD4⁺ naive T cells. That is, HMGB1 could induce the activation and maturity of DCs via TLR2, TLR4, and RAGE-NF-κB signal pathway, to indirectly enhance the Th2, Th17 inflammatory response in asthma. Increasing evidence demonstrates that HMGB1, as the alarmin uric acid, programs DCs to shift Th1 to Th2 inflammatory response, and to mount Th2 production of cytokines, with enhancement of T lymphocyte immune function (Hammad and Lambrecht, 2015; Pang et al., 2014; Zhao et al., 2012; Liu et al., 2011; Zhang et al., 2010). Zhang et al., in research into neutrophilic asthma, also confirmed that rHMGB1 activated DCs to produce IL-23, the activated DCs induced naive lymphocytes to produce IL-17A, and anti-HMGB1 antibody attenuated IL-17A secretion (Zhang et al., 2014, 2015). Some experiments demonstrated that HMGB1 significantly decreased Treg cell frequency, modulating the Treg/Th17 ratio *in vivo* (Li et al., 2016; Ding et al., 2016). However, several findings showed that HMGB1 was positively related with FoxP3 or IL-10 expression, therefore, HMGB1 expression may activate Tregs. This might be in connection with immune evasion (Gougeon et al., 2012; Cheng et al., 2017; Wild et al., 2012).

In conclusion, our present study provides evidence of the role of HMGB1 in an OVA-induced murine asthma model, and further confirmed how HMGB1 induces the polarization of Th2, Th17 cells to participate in the pathogenesis of asthma. Through the RAGE/TLR2, TLR4-NF-κB signal pathway, HMGB1 could directly and indirectly

induce Th2, Th17 immune responses. The first mechanism was: HMGB1 directly acted on naive T cells to induce Th2, Th17 polarization. The second mechanism was: HMGB1 first activated and induced maturation of DCs, then indirectly promoted Th2, Th17 differentiation through DCs.

Disclosures

The authors declare that they have no conflict of interests.

Authors' contributions

RTL and JY designed the research; RTL, JW, FFZ, WJX, GZH, HC and YMW performed the experiments; RTL, JW, RFL and BL analysed the data; RTL and JW produced the figures; RTL and JY wrote the manuscript.

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References

- Cavone, L., Cuppari, C., Manti, S., Grasso, L., Arrigo, T., 2015. Increase in the level of proinflammatory cytokine HMGB1 in nasal fluids of patients with rhinitis and its sequestration by glycyrrhizin induces eosinophil cell death. *Clin Exp Otorhinolaryngol.* 8, 123–128. <http://dx.doi.org/10.3342/ceo.2015.8.2.123>.
- Chen, Y., Huang, X.J., Yu, N., Xie, Y., Zhang, K., Wen, F., Liu, H., Di, Q., 2015. HMGB1 contributes to the expression of P-Glycoprotein in mouse epileptic brain through toll-like receptor 4 and receptor for advanced glycation end products. *PLoS One* 10 (10), e0140918. <http://dx.doi.org/10.1371/journal.pone.0140918>.
- Cheng, L.S., Li, J., Liu, Y., Wang, F.P., Wang, S.Q., She, W.M., Wu, S.D., Qi, X.L., et al., 2017. HMGB1-induced autophagy: a new pathway to maintain Treg function during chronic hepatitis B virus infection. *Clin. Sci. (Lond.)* 131 (5), 381–394. <http://dx.doi.org/10.1042/CS20160704>.
- Choy, D.F., Hart, K.M., Borthwick, L.A., Shikotra, A., Nagarkar, D.R., Siddiqui, S., Jia, G., Ohri, C.M., et al., 2015. Th2 and Th17 inflammatory pathways are reciprocally regulated in asthma. *Sci. Transl. Med.* 7 (301), 301ra129. <http://dx.doi.org/10.1126/scitranslmed.aab3142>.
- Cosmi, L., Liotta, F., Maggi, E., Romagnani, S., Annunziato, F., 2011. Th17 cells: new players in asthma pathogenesis. *Allergy* 989–998. <http://dx.doi.org/10.1111/j.1398-9995.2011.02576.x>.
- Cuppari, C., Manti, S., Chirico, V., Caruso, R., Salpietro, V., Giacchi, V., Laganà, F., Arrigo, T., et al., 2015. Sputum high mobility group box-1 in asthmatic children: a non-invasive sensitive biomarker reflecting disease status. *Ann. Allergy Asthma Immunol.* 115, 103–107. <http://dx.doi.org/10.1016/j.anai.2015.06.008>.
- Di Candia, L., Gomez, E., Venereau, E., Chachi, L., Kaur, D., Bianchi, M.E., Challiss, R.A.J., Brightling, C.E., et al., 2017. HMGB1 is upregulated in the airways in asthma and potentiates airway smooth muscle contraction via TLR4. *J. Allergy Clin. Immunol.* 140 (2), 584–587. <http://dx.doi.org/10.1016/j.jaci.2016.11.049>.
- Ding, J.W., Zheng, X.X., Zhou, T., Tong, X.H., Luo, C.Y., Wang, X.A., 2016. HMGB1 modulates the Treg/Th17 ratio in atherosclerotic patients. *J. Atheroscler. Thromb.* 23 (6), 737–745. <http://dx.doi.org/10.5551/jat.31088>.
- Froidure, A., Shen, C., Pillette, C., 2016. Dendritic cells revisited in human allergic rhinitis and asthma. *Allergy* 71, 137–148. <http://dx.doi.org/10.1111/all.12770>.
- Gangemi, S., Casciaro, M., Trapani, G., Quartuccio, S., Navarra, M., Picoqia, G., Imbalzano, E., 2015. Association between HMGB1 and COPD: a systematic review. *Mediat. Inflamm.* 2015, 164913. <http://dx.doi.org/10.1155/2015/164913>.
- Gougeon, M.L., Melki, M.T., Saidi, H., 2012. HMGB1, an alarmin promoting HIV dissemination and latency in dendritic cells. *Cell Death Differ.* 19 (1), 96–106. <http://dx.doi.org/10.1038/cdd.2011.134>.
- Hammad, H., Lambrecht, B.N., 2015. Barrier epithelial cells and the control of type 2 immunity. *Immunity* 43 (1), 29–40. <http://dx.doi.org/10.1016/j.immuni.2015.07.007>.
- Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M.A., Kool, M., et al., 2010. Inflammatory dendritic cells not basophils are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 207, 2097–2111. <http://dx.doi.org/10.1084/jem.20101563>.
- He, Z., Shotorbani, S.S., Jiao, Z., Su, Z., Tong, J., Liu, Y., Shen, P., Ma, J., et al., 2012. HMGB1 promotes the differentiation of Th17 via up-regulating TLR2 and IL-23 of CD14+ monocytes from patients with rheumatoid arthritis. *Scand. J. Immunol.* 76 (5), 483–490. <http://dx.doi.org/10.1111/j.1365-3083.2012.02759.x>.
- Hou, C., Kong, J., Liang, Y., Huang, H., Wen, H., Zheng, X., Wu, L., Chen, Y., 2015. HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts. *Cell Mol. Immunol.* 12, 409–423. <http://dx.doi.org/10.1038/cmi.2014.60>.
- Huang, L.F., Yao, Y.M., Meng, H.D., Zhao, X.D., Dong, N., Yu, Y., Sheng, Z.Y., 2008. The effect of high mobility group box-1 protein on immune function of human T lymphocytes in vitro. *Chin. Crit. Care Med.* 20 (1), 7–13.
- Huang, L., Yao, Y., Sheng, Z., 2012. Novel insights for high mobility group box 1 protein-mediated cellular immune response in sepsis: a systemic review. *World J. Emerg. Med.* 3, 165–171. <http://dx.doi.org/10.5847/wjem.j.issn.1920-8642.2012.03.001>.
- Kang, R., Chen, R., Zhang, Q., Hou, W., Wu, S., Cao, L., Huang, J., Yu, Y., et al., 2014. HMGB1 in health and disease. *Mol. Aspects Med.* 0, 1–116. <http://dx.doi.org/10.1016/j.mam.2014.05.001>.
- Kang, N., Hai, Y., Yang, J., Liang, F., Gao, C.J., 2015. Hyperbaric oxygen intervention reduces secondary spinal cord injury in rats via regulation of HMGB1/TLR4/NF- κ B signaling pathway. *Int. J. Clin. Exp. Pathol.* 8 (2), 1141–1153.
- Kim, H.Y., DeKruyff, R.H., Umetsu, D.T., 2010. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat. Immunol.* 11, 577–584. <http://dx.doi.org/10.1038/ni.1892>.
- Kim, H.Y., Choi, J.Y., Kim, C.W., Lee, C.D., Yoon, S.K., Bae, S.H., Han, N.I., Jhun, J.Y., et al., 2013. Basic Research: the pathogenic role of the interaction of the receptor for advanced glycation end products ligand and T helper 17 cells in chronic hepatitis B. *KASL* 2013 (1), 140 (210.101.116.28).
- Klune, J.R., Dhupar, R., Cardinal, J., Billiar, T.R., Tsung, A., 2008. HMGB1: endogenous danger signaling. *Mol. Med.* 14, 476–484. <http://dx.doi.org/10.2119/2008-00034>.
- Lambrecht, B.N., Hammad, H., 2012. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu. Rev. Immunol.* 30, 243–270. <http://dx.doi.org/10.1146/annurev-immunol-020711-075021>.
- Lee, C.C., Lai, Y.T., Chang, H.T., Liao, J.W., Shyn, W.C., Li, C.Y., Wang, C.N., 2013. Inhibition of high-mobility group box 1 in lung reduced airway inflammation and remodeling in a mouse model of chronic asthma. *Biochem. Pharmacol.* 86 (7), 940–949. <http://dx.doi.org/10.1016/j.bcp.2013.08.003>.
- Li, J., Wang, F.P., She, W.M., Yang, C.Q., Li, L., Tu, C.T., Wang, J.Y., Jiang, W., 2014. Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. *J. Viral Hepatitis* 21, 129–140. <http://dx.doi.org/10.1111/jvh.12152>.
- Li, J., Huang, L., Wang, S., Yao, Y., Zhang, Z., 2016. Astragaloside IV attenuates inflammatory reaction via activating immune function of regulatory T-cells inhibited by HMGB1 in mice. *Pharm. Biol.* 54 (12), 3217–3225. <http://dx.doi.org/10.1080/13880209.2016.1216133>.
- Liu, Q.Y., Yao, Y.M., Yan, Y.H., Dong, N., Sheng, Z.Y., 2011. High mobility group box 1 protein suppresses T cell-mediated immunity via CD11c (low) CD45RB (high) dendritic cell differentiation. *Cytokine* 54 (2), 205–211. <http://dx.doi.org/10.1016/j.cyt.2011.01.008>.
- Liu, H., Innamarato, P.P., Kodumudi, K., Weber, A., Nemoto, S., Robinson, J.L., Crago, G., McCordle, T., et al., 2016. Intrasternal rose bengal in melanoma elicits tumor immunity via activation of dendritic cells by the release of high mobility group box 1. *Oncotarget* 7 (25), 37893–37905. <http://dx.doi.org/10.18632/oncotarget.9247>.
- Lotze, M.T., Tracey, K.J., 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* 5, 331–342. <http://dx.doi.org/10.1046/j.1365-2842.2003.01141.x>.
- Lutz, M.B., 2016. Induction of CD4+ regulatory and polarized effector/helper T cells by dendritic cells. *Immune Netw.* 16 (1), 13–25. <http://dx.doi.org/10.4110/in.2016.16.1.13>.
- Ma, L., Zeng, J., Mo, B., Wang, C., Huang, J., Sun, Y., Yu, Y., Liu, S., 2015. High mobility group box 1: a novel mediator of Th2-type response-induced airway inflammation of acute allergic asthma. *J. Thorac. Dis.* 7 (10), 1732–1741. <http://dx.doi.org/10.3978/j.issn.2072-1439.2015.10.18>.
- Manfredi, A.A., Capobianco, A., Esposito, A., De Cobelli, F., Canu, T., Monno, A., Raucci, A., Sanvito, F., et al., 2008. Maturing dendritic cells depend on RAGE for in vivo homing to lymph nodes. *J. Immunol.* 180 (4), 2270–2275. <http://dx.doi.org/10.4049/jimmunol.180.4.2270>.
- Marsh, A.M., Nguyen, A.H., Parker, T.M., Agrawal, D.K., 2017. Clinical use of high mobility group box 1 and the receptor for advanced glycation end products in the prognosis and risk stratification of heart failure: a literature review. *Can. J. Physiol. Pharmacol.* 95, 253–259. <http://dx.doi.org/10.1139/cjpp-2016-0299>.
- Matsuoka, T., Chun, F.W., Stott, B., Durham, S.R., Shamji, M.H., 2013. Immunomodulatory effects of IL-27 on allergen-induced Th2 responses. *J. Allergy Clin. Immunol.* 131 (2), AB203. <http://dx.doi.org/10.1016/j.jaci.2012.12.1393>.
- Nie, H., Yang, Q., Zhang, G., Wang, A., He, Q., Liu, M., Li, P., Yang, J., et al., 2015. Invariant NKT cells act as an adjuvant to enhance Th2 inflammatory response in an OVA-induced mouse model of asthma. *PLoS One* 10 (4), e0119901. <http://dx.doi.org/10.1371/journal.pone.0119901>.
- Pang, X., Zhang, Y., Wei, H., Zhang, J., Luo, Q., Huang, C., Zhang, S., 2014. Expression and effects of high-mobility group box 1 in cervical cancer. *Int. J. Mol. Sci.* 15 (5), 8699–86712. <http://dx.doi.org/10.3390/ijms15058699>.
- Park, J.H., Choi, Y., Song, M.J., Park, K., Lee, J.J., Kim, H.P., 2016. Dynamic long-range chromatin interaction controls expression of IL-21 in CD4+ T cells. *J. Immunol.* 196 (10), 4378–4389. <http://dx.doi.org/10.4049/jimmunol.1500636>.
- Perrin-Cocon, L., Aublin-Gex, A., Sestito, S.E., Shirey, K.A., Patel, M.C., André, P., Blanco, J.C., Vogel, S.N., et al., 2017. TLR4 antagonist PF7 inhibits LPS-induced cytokine production and glycolytic reprogramming in dendritic cells, and protects mice from lethal influenza infection. *Sci. Rep.* 7, 40791. <http://dx.doi.org/10.1038/srep40791>.
- Plantinga, M., Guillems, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., et al., 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite

- allergen. *Immunity* 38, 322–335. <http://dx.doi.org/10.1016/j.immuni.2012.10.016>.
- Saenz, R., Futalan, D., Leutenetz, L., Eekhout, F., Fecteau, J.F., Sundelius, S., Sundqvist, S., Larsson, M., et al., 2014. TLR4-dependent activation of dendritic cells by an HMGB1-derived peptide adjuvant. *J. Transl. Med.* 12, 211. <http://dx.doi.org/10.1186/1479-5876-12-211>.
- Shi, Y., Sandoghchian Shotorbani, S., Su, Z., Liu, Y., Tong, J., Zheng, D., Chen, J., Liu, Y., et al., 2012. Enhanced HMGB1 expression may contribute to Th17 cells activation in rheumatoid arthritis. *Clin. Dev. Immunol.* 2012, 295081. <http://dx.doi.org/10.1155/2012/295081>.
- Shim, E.J., Chun, E., Lee, H.S., Bang, B.R., Kim, T.W., Cho, S.H., Min, K.U., Park, H.W., 2012. The role of high-mobility group box-1 (HMGB1) in the pathogenesis of asthma. *Clin. Exp. Allergy* 42, 958–965. <http://dx.doi.org/10.1111/j.1365-2222.2012.03998.x>.
- Su, Z., Sun, C., Zhou, C., Liu, Y., Zhu, H., Sandoghchian, S., Zheng, D., Peng, T., et al., 2011. HMGB1 blocked attenuates experimental autoimmune myocarditis and suppresses Th17-cell expansion. *Eur. J. Immunol.* 41 (12), 3586–3595. <http://dx.doi.org/10.1002/eji.201141879>.
- Vroman, H., van den Blink, B., Kool, M., 2015. Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? *Immunobiology* 220, 254–261. <http://dx.doi.org/10.1155/2015/879783>.
- Wang, L., Zhang, J., Wang, B., Wang, G., Xu, J., 2015. Blocking HMGB1 signal pathway protects early radiation-induced lung injury. *Int. J. Clin. Exp. Pathol.* 8 (5), 4815–4822.
- Watanabe, T., Asai, K., Fujimoto, H., Tanaka, H., Kanazawa, H., Hirata, K., 2011. Increased levels of HMGB-1 and endogenous secretory RAGE in induced sputum from asthmatic patients. *J. Respir. Med.* 105, 519–525. <http://dx.doi.org/10.1016/j.rmed.2010.10.016>.
- Watanabe, K., Karuppagounder, V., Arumugam, S., Thandavarayan, R.A., Pitchaimani, V., Sreedhar, R., Afrin, R., Harima, M., et al., 2015. Pruni cortex ameliorates skin inflammation possibly through HMGB1-NF κ B pathway in house dust mite induced atopic dermatitis NC/Nga transgenic mice. *J. Clin. Biochem. Nutr.* 14–75.
- Wild, C.A., Brandau, S., Lotfi, R., Mattheis, S., Gu, X., Lang, S., Bergmann, C., 2012. HMGB1 is overexpressed in tumor cells and promotes activity of regulatory T cells in patients with head and neck cancer. *Oral Oncol.* 48 (5), 409–416. <http://dx.doi.org/10.1016/j.oraloncology.2011.12.009>.
- Yang, D., Chen, Q., Yang, H., Tracey, K.J., Bustin, M., Oppenheim, J.J., 2007. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *J. Leukoc. Biol.* 81 (1), 59–66. <http://dx.doi.org/10.1189/jlb.0306180>.
- Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., et al., 2010. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc. Natl. Acad. Sci. U. S. A.* 107 (26), 11942–11947. <http://dx.doi.org/10.1073/pnas.1003893107>.
- Zhang, L.T., Yao, Y.M., Yao, F.H., Huang, L.F., Dong, N., Yu, Y., Sheng, Z.Y., 2010. Association between high-mobility group box-1 protein release and immune function of dendritic cells in thermal injury. *J. Interferon. Cytokine Res.* 30 (7), 487–495. <http://dx.doi.org/10.1089/jir.2009.0055>.
- Zhang, Y., Yao, Y.M., Huang, L.F., Dong, N., Yu, Y., Sheng, Z.Y., 2011. The potential effect and mechanism of high-mobility group box 1 protein on regulatory T cell mediated immunosuppression. *J. Interferon Cytokine Res.* 31 (2), 249–257. <http://dx.doi.org/10.1089/jir.2010.0019>.
- Zhang, F., Huang, G., Hu, B., Fang, L.P., Cao, E.H., Xin, X.F., Song, Y., Shi, Y., 2014. Anti-HMGB1 neutralizing antibody ameliorates neutrophilic airway inflammation by suppressing dendritic cell-mediated Th17 polarization. *Mediators Inflamm.* 2014, 257930. <http://dx.doi.org/10.1155/2014/257930>.
- Zhang, F., Huang, G., Hu, B., Qian, G.S., Song, Y., 2015. Recombinant HMGB1 A box protein inhibits Th17 responses in mice with neutrophilic asthma by suppressing dendritic cell-mediated Th17 polarization. *Int. Immunopharmacol.* 24 (1), 110–118. <http://dx.doi.org/10.1016/j.intimp.2014.11.005>.
- Zhang, F., Su, X., Huang, G., Xin, X.F., Cao, E.H., Shi, Y., Song, Y., 2017. sRAGE alleviates neutrophilic asthma by blocking HMGB1/RAGE signalling in airway dendritic cells. *Sci. Rep.* 7, 14268. <http://dx.doi.org/10.1038/s41598-017-14667-4>.
- Zhao, G.J., Yao, Y.M., Lu, Z.Q., Hong, G.L., Zhu, X.M., Wu, Y., Wang, D.W., Dong, N., et al., 2012. Up-regulation of mitofusin-2 protects CD4+ T cells from HMGB1-mediated immune dysfunction partly through Ca(2+)-NFAT signaling pathway. *Cytokine* 59 (1), 79–85. <http://dx.doi.org/10.1016/j.cyto.2012.03.026>.
- Zhao, J., Lloyd, C.M., Noble, A., 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol.* 6 (2), 335–346. <http://dx.doi.org/10.1038/mi.2012.7>.
- Zhou, H., Wang, Y., Wang, W., Jia, J., Li, Y., Wang, Q., Wu, Y., Tang, J., 2009. Generation of monoclonal antibodies against highly conserved antigens. *PLoS One* 4 (6), e6087. <http://dx.doi.org/10.1371/journal.pone.006087>.
- Zhu, X.M., Yao, Y.M., Liang, H.P., Xu, C.T., Dong, N., Yu, Y., Sheng, Z.Y., 2011. High-mobility group box 1 protein regulate immuno-suppression of regulatory T cells through toll like receptors 4. *Cytokine* 54 (3), 296–304. <http://dx.doi.org/10.1016/j.cyto.2011.02.017>.
- Zhu, J., Wang, F.L., Wang, H.B., Dong, N., Zhu, X.M., Wu, Y., Wang, Y.T., Yao, Y.M., 2017. TNF- α mRNA is negatively regulated by microRNA-181a-5p in maturation of dendritic cells induced by high mobility group box-1 protein. *Sci. Rep.* 7 (1), 12239. <http://dx.doi.org/10.1038/s41598-017-12492-3>.