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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-κB (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-κB signal pathway in $CD4^+$ naive T cells. HMGB1 could also indirectly promote Th2, Th17 differentiation via activating the TLR2, TLR4, RAGE-NF-κB 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-κB, 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-κB, Phosphorylation of NF-κB; 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-κB (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 RORγt and IL-17 mRNA levels, augments the ratio of $CD4^+$ $IL\text{-}17^+$ 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). rHMGBl could both time- and dose-dependently increase the abundance of Th2 subsets, and thus reduce the Thl/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-κB signal pathway, and the second being: HMGB1 mediates the maturation of DCs and their antigen-presenting ability via the RAGE/TLR2 or 4-NF-κB 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-μg injection of OVA (Sigma Aldrich, St. Louis, MO, USA), emulsified in 200 μl 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 μg OVA in 50 μl 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 μm 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× 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 $1–2 \times 10^6$ cells/ml in RPMI1640, with 10% FBS (HyClone, Logan, UT, USA) and 0.1% 100 × 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% CO_2 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 1×10^8 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 μg/mL) and soluble anti-CD28 (1 μg/mL) in complete RPMI 1640 medium for 3 days. OVA (10 μg/mL) was added, then cells were treated with medium in the absence or presence of rHMGB1 (50 μg/mL) or/and anti-HMGB1 antibody (10 μg/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 1×10^5 cells/well, then cocultured with 2.5×10^4 BMDCs/well, stimulated with or without rHMGB1(50 μg/mL) or/and anti-HMGB1(10 μg/mL) for 2 days (Zhang et al., 2014, 2015). OVA(10 μg/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.