

The role of high-mobility group box-1 (HMGB1) in the pathogenesis of asthma

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Clinical & Experimental Allergy

Summary

Background High-mobility group box 1 protein (HMGB1) belonging to endogenous danger signals prolongs eosinophil survival and acts as a chemoattractant.

Objective The authors evaluated the role of HMGB1 in the pathogenesis of asthma characterized by eosinophilic airway inflammation.

Methods Firstly, HMGB1 expressions in induced sputum obtained from human asthmatics were determined. This was followed by an evaluation of the role of HMGB1 in a murine model of asthma using anti-HMGB1 antibodies. Then the effect of HMGB1 on the receptor of advanced glycation end products (RAGE) expressions on CD11b-CD11c⁺ cells isolated from a murine model of asthma were measured to elucidate the mechanisms involved.

Results Sputum HMGB1 expressions were markedly higher in asthmatics than in normal controls, and were positively correlated with sputum eosinophilia and sputum TNF- α , IL-5 and IL-13 expressions. In a murine model of asthma, HMGB1 expressions in lung tissue and HMGB1 levels in bronchoalveolar lavage fluid were significantly elevated and eosinophilic airway inflammation, non-specific airway hyperresponsiveness, and pathological changes were attenuated by blocking HMGB1 activity. Furthermore, we found that enhanced RAGE expressions on CD11b-CD11c⁺ also significantly decreased when HMGB1 activity was blocked.

Conclusion and Clinical Relevance Our findings suggest that HMGB1 plays a key role in the pathogenesis of clinical and experimental asthma characterized by eosinophilic airway inflammation.

Keywords asthma, danger signal, eosinophil, high-mobility group box 1 protein (HMGB1)

Abbreviations AHR, Airway hyperresponsiveness; BAL, Bronchoalveolar lavage; ELISA, Enzyme-linked immune solvent assay; HMGB1, High-mobility group box 1 protein; OVA, Ovalbumin; RAGE, Receptor of advanced glycation end products; TLR, Toll-like receptor.

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Introduction

High-mobility group box 1 protein (HMGB1) is a highly conserved nuclear protein that belongs to the *Alarmins* family, members of which alert the immune system to tissue damage and trigger immediate response [1]. Since it was reported first that HMGB1 has cytokine activity that mediates endotoxin-related lethality [2], many investigators have observed that

HMGB1 is involved in pathological processes, such as, acetaminophen-induced liver injury [3], sepsis [4], and rheumatic diseases [5].

Recently, it was reported that airway epithelium damaged elicits the chemotactic movement in human eosinophils [6], and that HMGB1 enhances eosinophil survival and acts as a chemoattractant [7]. These reports are of special interest because eosinophils are believed to play important roles in the pathogenesis of allergic

airway diseases. Moreover, it was documented that HMGB1 is present in airway smooth muscle cells [8], and HMGB1 has been shown to support proliferation, chemotaxis, and the synthesis of metalloproteinases by stromal fibroblasts [9, 10]. Taken together, HMGB1 released in the airways during inflammatory process may contribute to important histopathological features of tissue remodelling.

Based on those findings, we tested our hypothesis that HMGB1 plays an important role in the pathogenesis of asthma characterized by eosinophilic airway inflammation. To the best of our knowledge, this is the first comprehensive report to show the potential contribution made by HMGB1 to asthma.

Methods

All subjects enrolled in this study provided written informed consent, and the study protocol was approved by the Seoul National University Hospital Institutional Review Board (H-1012-061-344). All experimental procedures were performed with the approval of the Seoul National University Institutional Animal Care and Use Committee. Methodological details are presented in supplementary materials.

HMGB1 expressions and levels in induced sputum obtained from asthmatics

To evaluate the clinical relevancy of HMGB1 in human asthma, we measured HMGB1 expressions in induced sputum samples from asthmatics. Induced sputum cell and mediator have been extensively studied and validated as a measure of airway inflammation [11]. Normal controls who showed negative methacholine airway hyperresponsiveness (AHR) without respiratory symptom complaint, and asthmatics were recruited at Seoul National University Hospital. Asthma was diagnosed in accordance with the guideline issued by the National Institutes of Health, US [12]. All asthmatics were treatment-naïve. Sputum induction, sample processing, RNA extraction, and semi-quantitative reverse transcription polymerase chain reaction for HMGB1 and inflammatory cytokine (*TNF- α* , *IL-5* and *IL-13*) were performed as previously described [13]. Levels of HMGB1 in sputum supernatants were measured using ELISA (enzyme-linked immune solvent assay) according to the manufacturer's instruction (SHINO-TEST Corporation, Tokyo, Japan).

Role of HMGB1 in a murine model of asthma

To evaluate the role played by HMGB1 in the pathogenesis of asthma, we generated a murine model of asthma based on intraperitoneal sensitization with ovalbumin

(OVA) plus alumhydroxide (alum) and a subsequent intranasal challenge with OVA alone (see Fig. S1a). Female BALB/c mice (6 weeks old) were purchased from the Orient Bio (Sungnam, Korea). We measured non-specific airway hyperresponsiveness by Flexivent invasive measurement (SCIREQ, Montreal, Canada) of dynamic resistance as previously described [14]. Lung tissue and bronchoalveolar lavage (BAL) fluid were obtained and histological evaluations were performed as previously described [15]. Briefly, whole lungs were inflated and fixed under pressure fixation using Streck solution (Streck Laboratories, La Vista, NE, USA) and lung were H&E stained. The same magnification was used for all microscopic examinations of sample slides. HMGB1, IL-4, IL-5, IL-13, and GM-CSF levels in BAL fluid were measured by ELISA and HMGB1 expressions in lung tissues were measured using real time PCR. We also assessed the immunolocalization of HMGB1 on lung tissue sections and cells in cytospin preparations of BAL fluids expressing HMGB1 were visualized using confocal microscopy.

Then we specifically antagonized HMGB1 activity by injecting anti-HMGB1 antibodies intraperitoneally (200 μ g/mL; Abcam, Cambridge, UK) 6 hours after every challenge on days 14, 15, and 16 (see Fig. S1b). We observed that HMGB1 levels in BAL fluid significantly reduced by injection of blocking antibodies (see Fig. S2). After blocking the HMGB1 activity phenotype changes were evaluated and HMGB1, IL-4, IL-5, IL-13, and GM-CSF levels in BAL fluid were measured using ELISA. As it was reported that the receptor of advanced glycation end products (RAGE, one of the HMGB1 receptors) triggering by HMGB1 regulates several important functions that maintain lung homeostasis, including cell activation, differentiation, and migration [16–18], we also measured expressions of the HMGB1 receptors (RAGE, TLR2 and TLR4) on the CD11b-CD11c⁺ cells obtained from asthmatic mice before and after injecting anti-HMGB1 antibodies using flow cytometry (FACScalibur; Becton Dickinson, Palo Alto, CA, USA).

Statistical analysis

Results are expressed as means $\pm$ SEMs, and were assessed using unpaired Student *t*-test. Statistical significance was accepted for *P*-values of < 0.05 .

Results

HMGB1 expressions and levels in induced sputum obtained from asthmatics

A total of 50 asthmatics [men = 21 (42.0%); mean age ($\pm$ SD) = 48.7 $\pm$ 13.4] and 15 normal controls [men = 8

(53.3%); mean age ($\pm$ SD) = 28.6 ± 6.7] were enrolled (see Table S1). Mean age of asthmatics and normal controls was significantly different. So far, there has been no report suggesting an ageing effect on HMGB1 expressions (especially on lung tissue or inflammatory cells). However, it was reported that an age-dependent change of HMGB1 expressions on neurons was observed in mouse brain [19]. Therefore, age difference between asthmatics and normal controls enrolled in this study needs to be considered when our results are interpreted. Sputum HMGB1 expressions were significantly higher in asthmatics than in controls (Fig. 1a). In asthmatics, sputum HMGB1 expressions and HMGB1 levels in sputum supernatant were significantly higher in subjects with sputum eosinophilia (eosinophil proportion $\geq 3\%$ of sputum cells) than in subjects without sputum eosinophilia (Figs 1b and c). Sputum *TNF- α* , *IL-5* and *IL-13* expressions showed significant positive correlations with sputum HMGB1 expressions in asthmatics (Figs 1d–f).

Role of HMGB1 in the murine model of asthma

The HMGB1 expressions in lung tissue and HMGB1 levels in BAL fluid were significantly higher in asthmatic mice than those in control mice (Figs 2a and b). We found that HMGB1 was expressed on cells in cytospin preparations of BAL fluids (red dots in Fig. 2c). Mononuclear cells in BAL fluid obtained from asthmatic mice showed a wide range of HMGB1 distribution from nucleus to marginal cell membrane but HMGB1 distrib-

uted just around the nucleus in mononuclear cells obtained from control mice. HMGB1-positive cells were found in the interstitial and peri-vascular areas of mouse lung tissue and their numbers increased in asthmatic mice compared to control mice (Fig. 2d). However, a positive staining of epithelium was not prominent.

After blocking HMGB1 activity with an intraperitoneal injection of neutralizing antibodies, methacholine AHR and total cells, macrophage and eosinophils counts in BAL fluid significantly decreased (Figs 3a and b). GM-CSF and IL-5 levels in BAL fluid showed significant reductions after injection of neutralizing antibodies (Figs 3c and d), although IL-4 and IL-13 levels in BAL fluid showed no differences (data not shown). In addition, histological improvements were also observed (Fig. 4). We observed that RAGE and TLR2 expression on the CD11b-CD11c⁺ cells (we defined these cells as dendritic cells) significantly increased in asthmatic mice compared with control mice and these changes were significantly attenuated by injection of HMGB1 neutralizing antibodies (Figs 5a and b). However, these changes were not observed in TLR4 expressions (Fig. 5c).

Discussion

In the present study, we observed that HMGB1 expressions were significantly elevated in the induced sputum samples of asthmatics as compared with normal controls. Furthermore, we found that HMGB1 levels in

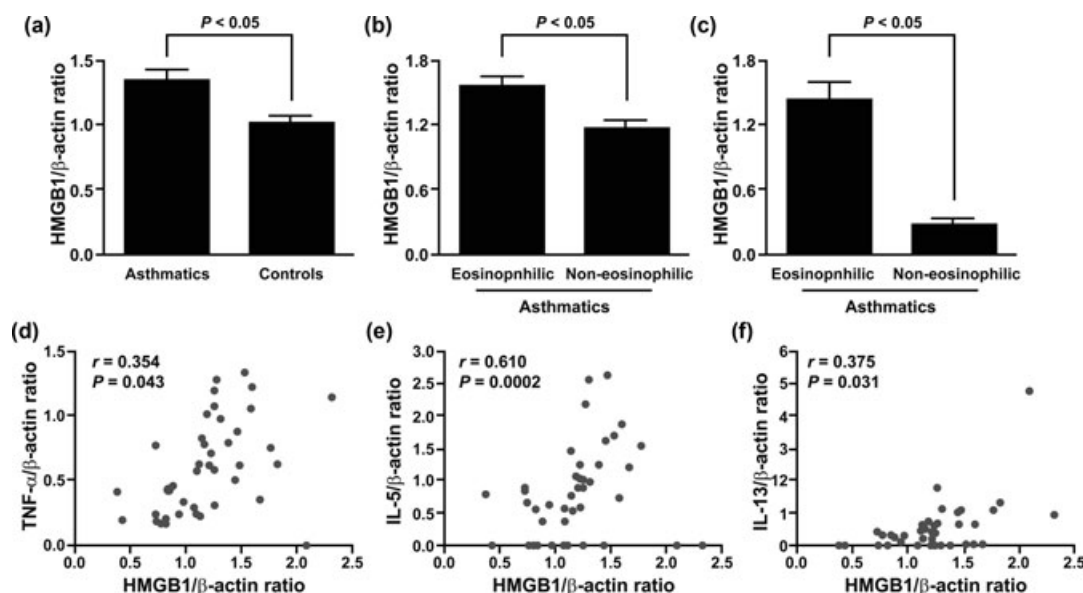


Fig. 1. HMGB1 levels in sputum supernatant, sputum HMGB1 expressions and correlation with sputum *TNF- α* , *IL-5* and *IL-13* expressions (human data). (a) Sputum HMGB1 expressions of asthmatics and controls. (b) Sputum HMGB1 expressions of asthmatics with or without sputum eosinophilia. (c) HMGB1 levels in sputum supernatant of asthmatics with or without sputum eosinophilia. (d) Correlation between sputum HMGB1 and *TNF- α* expressions. (e) Correlation between sputum HMGB1 and *IL-5* expressions. (f) Correlation between sputum HMGB1 and *IL-13* expressions. (Correlations were measured by Pearson correlation, *r*; correlation coefficient).

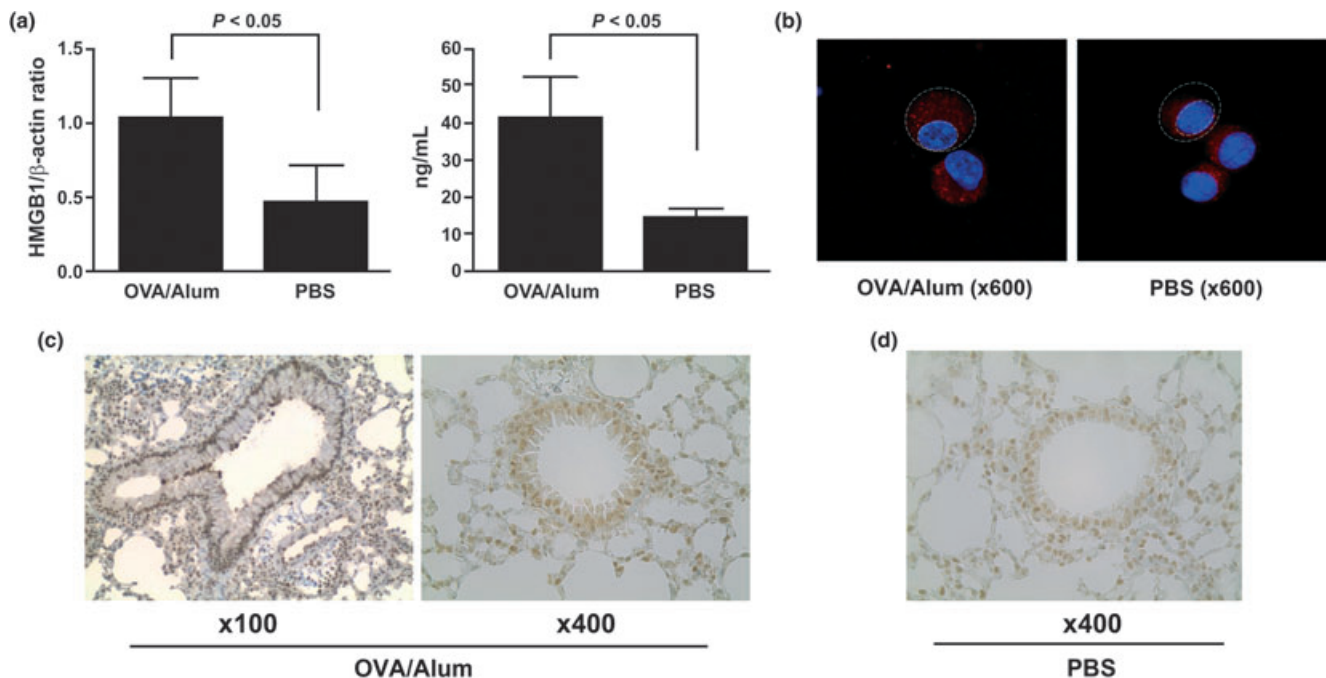


Fig. 2. HMGB1 levels in BAL fluid, HMGB1 expressions in lung tissues and immunolocalization of HMGB1 (murine model of asthma data). (a) HMGB1 expressions in lung tissues [asthmatic mice (OVA/Alum) vs. control mice (PBS)]. (b) HMGB1 levels in BAL fluid [asthmatic mice (OVA/Alum) vs. control mice (PBS)]. (c) Immunofluorescence confocal microscopic images of mononuclear cells in cytospin preparations of BAL fluids [anti-HMGB1 (red) and anti-DAPI (blue), asthmatic mice (OVA/Alum) vs. control mice (PBS)]. One representative experiment of three replicates is shown ($\times 600$). (d) Immunohistochemistry staining of lung tissue sections obtained from asthmatic mice (OVA/Alum) [HMGB1 (dark brown), $\times 100$ & $\times 400$]. E. Immunohistochemistry staining of lung tissue sections obtained from control mice (PBS) [HMGB1 (dark brown), $\times 400$].

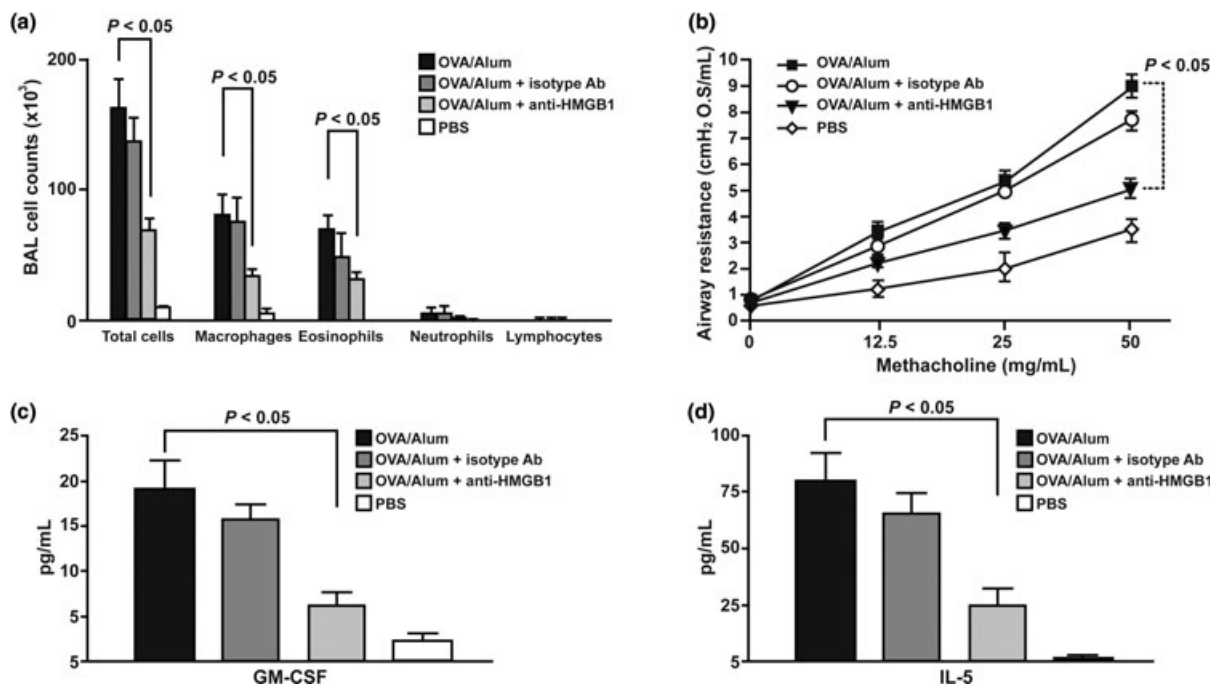


Fig. 3. Phenotypic changes in asthmatic mice after blocking HMGB1 activity. (a) Inflammatory cells in BAL fluid. (b) Airway resistance. (c) GM-CSF levels in BAL fluid. (d) IL-5 levels in BAL fluid. (OVA/Alum; asthmatic mice, PBS; control mice, isotype Ab; treated by isotype antibody, anti-HMGB1; treated by anti-HMGB1 antibody).

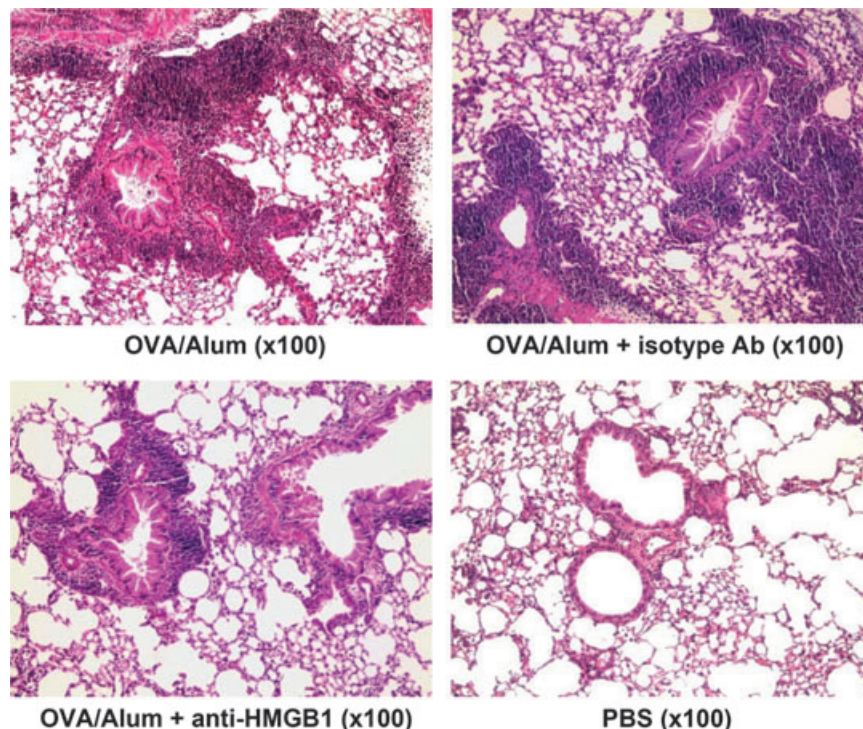


Fig. 4. Pathology changes in asthmatic mice after blocking HMGB1 activity. (OVA/Alum; asthmatic mice, PBS; control mice, isotype Ab; treated by isotype antibody, anti-HMGB1; treated by anti-HMGB1 antibody, $\times 100$).

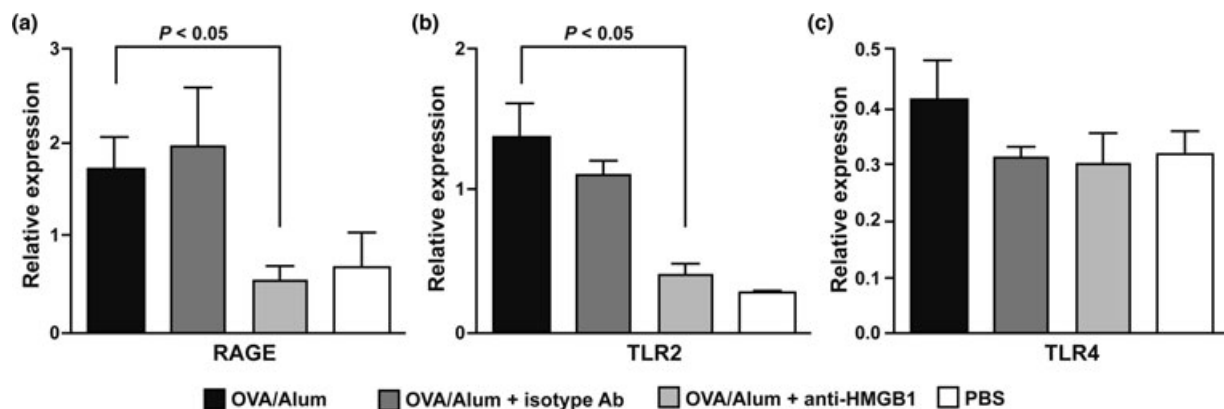


Fig. 5. Changes in HMGB1 receptor expressions on the CD11b-CD11c⁺ cells obtained from asthmatic mice after blocking HMGB1 activity. (a) RAGE. (b) *TLR2*. (c) *TLR4*. [The relative RAGE expression was calculated as follows: mean fluorescence intensity (MFI) of RAGE/MFI of isotype-matched control of all the CD11b-CD11c⁺ cells analysed.]. (OVA/Alum; asthmatic mice, PBS; control mice, isotype Ab; treated by isotype antibody, anti-HMGB1; treated by anti-HMGB1 antibody).

sputum supernatant were significantly elevated in asthmatics with sputum eosinophilia and showed positive correlations with sputum *TNF- α* , *IL-5* and *IL-13* expressions. Based on these observations, we tested our hypothesis that HMGB1 plays an important role in the pathogenesis of asthma characterized by eosinophilic airway inflammation using a murine model of asthma. We confirmed that HMGB1 expressions in lung tissue and HMGB1 levels in BAL fluid are significantly higher in asthmatic mice and that eosinophilic airway

inflammation, methacholine AHR, GM-CSF, and IL-5 levels in BAL fluid and histological changes were attenuated by blocking HMGB1 activity. Moreover, we found that enhanced RAGE and TLR2 expressions on the CD11b-CD11c⁺ cells also significantly decreased by blocking HMGB1 activity. A recent report suggested the existence of an autocrine/paracrine loop in which binding of HMGB1 to RAGE and potentially other HMGB1 receptors, amplifies inflammatory and remodelling signals [8].

The HMGB1 is passively released not only from necrotic or damaged cells [3], but also actively secreted by innate immune cells and acts as a pro-inflammatory cytokine [20, 21]. Examination of HMGB1 immunostaining in airway structural cells showed bronchial epithelial cells and airway smooth muscle cells represent a potential source of the secreted soluble form of HMGB1 in the human airways [8]. In addition, alveolar macrophages were identified as an important source of this protein in the human lungs [8]. Along these observations, we found that HMGB1-positive cells located in the interstitial and peri-vascular areas of mouse lung tissue and their numbers increased in asthmatic mice compared with control mice. And we also found that mononuclear cells in BAL fluid obtained from asthmatic mice might be the source of HMGB1. Those observations suggested that HMGB1 is secreted actively and may play an important role in the pathogenesis of airway diseases involving many types of cells in the lung.

Eosinophils are found preferentially in necrotic areas [6, 22]. It has reported that necrotic airway epithelial cells activate human eosinophils [6] and that HMGB1 enhances human eosinophil survival and acts as a chemoattractant [7]. A potential contribution of HMGB1 to asthma pathogenesis, in part, may be explained by the interaction between HMGB1 and eosinophils. In the present study, we found that sputum expressions of HMGB1 were significantly enhanced in asthmatics with sputum eosinophils and showed significant correlations with sputum *IL-5* and *IL-13* expressions which are well-known cytokines as eosinophil chemo-attractants. Moreover, by blocking HMGB1 activity, eosinophilic airway inflammation, non-specific AHR, and pathological changes improved significantly in the murine model of asthma.

Another interesting finding of the present study is that blocking HMGB1 activity resulted in significant decreases of RAGE expressions on the CD11b-CD11c⁺ cells. RAGE, a member of the immunoglobulin superfamily, is highly expressed in normal lung, particularly in bronchiolar epithelium, alveolar macrophages, and type I alveolar epithelial cells [23, 24], whereas RAGE is present at low to undetectable levels in other tissues. As mentioned earlier, RAGE triggering by HMGB1 regulates several important functions that maintain lung homeostasis, including cell activation, differentiation, and migration [15–17]. Moreover, most of the cell types existing in human lung co-express HMGB1 and RAGE suggesting an autocrine/paracrine loop [8]. All together this suggests that an abnormal regulation of RAGE participates in the pathogenesis of asthma characterized by chronic eosinophilic inflammation and airway remodeling. In this sense, a decrease in RAGE expressions induced by the HMGB1 blockage may provide the

possible clue for the development of pharmacological intervention to alleviate chronic inflammation and potential airway damage in asthmatics. So far, there has been no report suggesting the underlying mechanisms for our observation that blocking HMGB1 activity attenuated RAGE expressions on the CD11b-CD11c⁺ cells. To understand underlying mechanisms, the effect of anti-IgE on FcεRI (high affinity receptor of IgE) is worthwhile to be considered. It was reported that a treatment with anti-IgE led to rapid and dramatic falls in FcεRIα receptors on circulating basophils [25]. Although detailed mechanisms should be searched, we tentatively think that a down-regulation of RAGE (receptor of HMGB1) by blocking HMGB1 activity is an example of a negative feedback which is observed in case of FcεRIα (receptor on IgE) and blocking IgE activity.

Recent studies performed in asthmatics suggest that there is a significant correlation between HMGB1 and neutrophilic inflammation found in airways [25–28]. It is well known that HMGB1 serves as a chemo-attractant to neutrophils and prevents apoptosis of neutrophils. Moreover, asthma, especially severe asthma, is characterized by airway epithelial cell damage [29] from which HMGB1 is released. Thus, it is possible that HMGB1 is associated with neutrophilic airway inflammation found in asthmatics. We do not exclude this possibility and think that our observations can be compatible with findings in recent publications mentioned above. The hypothesis we tested in the present study was that HMGB1 contributes to eosinophilic inflammation in asthma. Then we confirmed our hypothesis using not only human samples but also an experimental model of murine asthma characterized by pure eosinophilic airway inflammation. The main difference between findings in recent publications and ours is that asthmatics with more eosinophilic airway inflammation and milder severity were enrolled in our study. Actually, we could not find asthmatics with so-called neutrophilic (sputum neutrophils > 61.0%) or mixed granulocytic (sputum eosinophils > 3.0% and sputum neutrophils > 61.0%) airway inflammation [30] among asthmatics enrolled in our study. We may say that effect of HMGB1 on eosinophils is important in mild asthmatics, whereas effect of HMGB1 on neutrophils in addition to effect on eosinophils is important in severe asthmatics. Taken together, blocking HMGB1 activity which can attenuate both eosinophilic and neutrophilic inflammation in the airway may be a promising treatment modality for not only mild but also severe asthmatic patients.

In conclusion, our data suggest that HMGB1 plays a key role in the pathogenesis of clinical and experimental asthma characterized by eosinophilic airway inflammation.

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Conflict of interest: Authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The characteristics of subjects enrolled

Figure S1. A protocol for a murine model of asthma.

Figure S2. HMGB1 levels in BAL fluid (murine model of asthma).

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1 **The role of high mobility group box-1 (HMGB1) in the pathogenesis of asthma**

2

3 **Supplementary materials**

4

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6 Kyung-Up Min, Heung-Woo Park

7

8 **Detailed Methods**

9 ***Study subjects***

10 A total of 50 asthmatics and 15 normal controls were enrolled at Seoul National University Hospital, Seoul, Korea. A diagnosis of
11 asthma was made when a subject with symptoms of dyspnea or wheezing showed reversible airway obstruction, as detailed by the
12 guidelines issued by the National Institute of Health (Reference 12 in the manuscript). The exclusion criteria applied were a smoking
13 history of > 10 pack years and the presence of an abnormality by simple chest radiography. All asthmatics enrolled were treatment-
14 naïve and their severity of asthma was determined by the guidelines issued by the National Institute of Health (Reference 12 in the
15 manuscript). The characteristics of subjects enrolled were shown in Table 1.

16 ***Sputum induction, processing and RNA extraction in asthmatics***

17 After measuring basal values of forced expiratory volume in 1 second (FEV1), all asthmatics were administered 200 µg of inhaled
18 salbutamol (Ventolin™, GlaxoSmithKleine, Brentford, UK). An ultrasonic nebulizer was used to administer 4.5% saline at an output of

19 4.5mL/min (Omron Co., Tokyo, Japan) for 5 min up to four times. After each saline administration, subjects were asked to rinse their
20 mouths and encouraged to expectorate into a sterile Petri dish. The samples were treated with a 4% solution of the mucolytic agent,
21 dithiothreitol. A total cell count was done and cell concentrations were then adjusted to 1.0×10^6 cells/ml. Cytospins were prepared by
22 adding 60 μ l of these cell suspensions to Shandon II cytocentrifuge cups (Shandon Southern Instruments, Sewickley, PA) and spun for
23 5 min at 500 rpm. Slides were stained with Diff Quik solution (Sysmex Co., Kobe, Japan) to determine the differential cell counts for
24 leukocytes, bronchial epithelial cells, and squamous cells. To do the differential cell count, 300 nucleated cells per slide were counted
25 and the results were expressed as percentages of intact round nucleated cells, excluding the squamous epithelial cells. In addition,
26 sputum samples were mixed with 1ml Trizol (Gibco, Carlsbad, CA). RNA extraction was done using phenol chloroform extraction
27 and ethanol precipitation according to the manufacturer's instructions (Gibco, Carlsbad, CA). Total RNA was quantified by measuring
28 the optical density (OD) at 260nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

29 ***Semi-quantitative reverse transcription polymerase chain reaction for HMGB1 and inflammatory cytokines in sputum cells***

30 RNA (2 μ g) was reverse-transcribed using an oligo(dT) and AMV reverse transcriptase (Promega, WI, USA). The polymerase chain
31 reaction (PCR) was done in total volume of 50 μ l containing 10 μ l of cDNA product, 1 μ M of each primer, 1.2 μ l of 50mM MgCl₂, 1 μ l
32 of 5mM dNTP mix and 2U of Taq polymerase. The house keeping gene, *β -actin*, was also amplified as an internal control. Reverse
33 transcription PCR (RT-PCR) was done with the following primers:

34 human TNF- α sense (5'- TTACCCCTCCTTCAGACACCC-3')

35 human TNF- α anti-sense (5'-CAGCCTATTGTTTCAGCCTCCGT-3')

36 human IL-5 sense (5'-GTGTATGCCATCCCCACAGAAA-3')

37 human IL-5 anti-sense (5'-CAGTACCCCTTGCACAGTTTG-3')

38 human HMGB1 sense (5'-TGAAGATATGGCAAAAGCGG-3')

39 human HMGB1 anti-sense (5'-CTCCCAGTTTCTTCGCAACA-3')

40 human β -actin sense (5'GTCACCAACACTGTGCCCCAT-3')

41 human β -actin anti-sense (5'- CTCAAAGTCCAGGGGGATGT-3')

42 Equal amounts of PCR products were run on a 2 % agarose gel and visualized by ethidium bromide staining. The image was scanned
43 and densitometric analysis was done by measuring the signal intensity of each band. The values were normalized to the β -actin
44 expression and are expressed as fold increase.

45 ***Measuring the airway hyperresponsiveness in a murine model of asthma***

46 Non-specific AHR was assessed as a change in airway function after challenge with aerosolized methacholine via the airway.
47 Anesthetization was achieved with 80 mg/kg of pentobarbital sodium injected i.p. The trachea was then exposed through midcervical
48 incision and tracheostomized, and an 18-gauge metal needle was then inserted. Mice were connected to a computer-controlled small
49 animal ventilator (flexiVent, SCIREQ, Montreal, Canada), and each mouse was quasi-sinusoidally ventilated with a nominal tidal
50 volume of 10 ml/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung

51 volume similar to that occurring during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator
52 to a water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To
53 determine the differences in airway response to methacholine, each mouse was challenged with increasing concentrations of
54 methacholine (2.5-50 mg/ml in saline) in an aerosol form. The data needed to calculate R_L was collected continuously following each
55 methacholine challenge. Maximum R_L values were selected to express changes in airway function, which was represented as the
56 percent change from baseline after saline aerosol treatment.

57 ***Real-time PCR for HMGB1 in mouse lungs***

58 Amplification of *HMGB1* was done using an ABI 7500 real-time PCR system (Applied Biosystems, Foster city, USA) and SYBR
59 Green master mix (Applied Biosystems). The sequences of the primers used in this study are as follows:
60 HMGB1 sense (5'-GAGCCTTGTCAGCCTTTGCC- 3')

61 HMGB1 anti-sense (5'-AGCACCCGGATGCTTCTGTC- 3')

62 GAPDH sense (5'-AC GGCAAATTCAACGGCACAG- 3')

63 GAPDH anti-sense (5'-AGACTCCAC GACATACTCAGCAC- 3')

64 The data was analyzed using the software for the ABI 7500 (Applied Biosystems).

65 ***Cell preparation and immunostaining for microscopic detection of HMGB1***

66 Mouse BAL cells were put on a coated-slide using cytocentrifugation (Cytospin 3, Shandon, UK) at 500 rpm for 5 min at room

67 temperature with each slide holding 50,000 cells. Slides were fixed with 4% paraformaldehyde for 30 minutes, and then washed three

68 times with PBS and finally with distilled water. The slides were air-dried overnight. To detect HMGB1 in BAL cells, the slides were

69 placed in ice-cold 100% methanol for 5 minutes and then washed. Nonspecific reactions were blocked with 3% bovine serum albumin

70 in PBS. The slides were incubated with 60ul of 1:100 diluted rabbit polyclonal to HMGB1 (Abcam) for 1 hour at room temperature.

71 To bind the secondary antibody, a 1:1000 diluted goat anti-rabbit IgG Alexa 568 conjugated Abs (Invitrogen) was incubated for 1h at
72 room temperature and then wash off any excess secondary antibody using PBS. Finally, the slides were mounted with Vectashield
73 mounting medium including DAPI for nuclei staining (Vector laboratories, Inc., Burlingame, USA).

74 ***Isolation of the CD11b-CD11c⁺ cells from a murine model of asthma***

75 Perfused lung tissues were chopped and incubated for 15 min at 37°C in 0.5% EDTA. To obtain single cell suspensions, the chopped
76 lung tissue were ground on a 0.7 µm strainer with complete RPMI-1640 (cRPMI-1640) supplemented with 10% fetal bovine serum,
77 2mM L-glutamine, and 1% penicillin/streptomycin. Erythrocytes were lysed with ammonium chloride solution (StemCell
78 technologies, Vancouver, Canada). The cell pellets were washed twice with cRPMI-1640 and centrifuged at 1500 rpm for 10 min.
79 Total lung were separated into the CD11b-CD11c⁺ cells using anti-CD11b (FITC-CD11b) and anti-CD11c (PerCP-Cy5.5-CD11c)
80 (eBioscience, San Diego, CA). Labeled cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

81 ***Immunostaining***

82 Isolated CD11b-CD11c⁺ cells were stained for 20 min at 4°C with antibodies specific for RAGE (Chemicon International, Temecula,
 83 CA), TLR2 and TLR4 (eBioscience, San Diego, CA) diluted in 100 ml of PBS/10% FCS/0.1% NaN³. After two washings, cells were
 84 fixed in 1% PFA. Labeled cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

85 **Table 1. The characteristics of subjects enrolled**

86	Characteristics	Asthmatics		Control
		Sputum eosinophilia (+)	Sputum eosinophilia (-)	
87		(n = 30)	(n=20)	(n=15)
88				
89	Age, mean (± S.D.)	47.9 (± 11.2)*	49.8 (± 9.4)*	28.6 (± 6.7)
90	Male, n (%)	12 (40.0)	9 (45.0)	8 (53.3)
91	Atopy ^s (%)	10 (50.0)	8 (40.0)	0 (0)
92	FEV1 pred. % (± S.D.)	94.2 (± 17.2)	92.3 (± 13.1)	100.2 (± 12.3)

93	PC20 [†] , mean (± S.D.)	5.1 (± 4.9)	5.6 (± 4.8)	-
94	Severity			
95	mild, n (%)	23 (76.7)	16 (80.0)	-
96	moderate to severe, n (%)	7 (23.3)	4 (20.0)	-
97	Sputum profile			
98	neutrophil % (± S.D.)	17.7 (13.5)	19.1 (13.8)	14.6 (22.4)
99	eosinophil % (± S.D.)	22.8 (17.9)	1.1 (0.9)**	0.1 (0.7)**

100 * Significantly different (P < 0.05) compared to controls

101 ** Significantly different (P < 0.05) compared to asthmatics with sputum eosinophilia

102 [§] Skin prick test was performed with 11 common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, dog
103 fur, cat fur, *Aspergillus*, *Alternaria*, tree pollen mixture, grass pollen mixture, mugwort, ragweed, and cockroach). Atopy was defined
104 as a positive skin prick test response (allergen/histamine ratio > 1.0 plus a mean wheal size > 4 mm) to one or more allergens.

105 [†] mg/dL

106

107

108

109

110

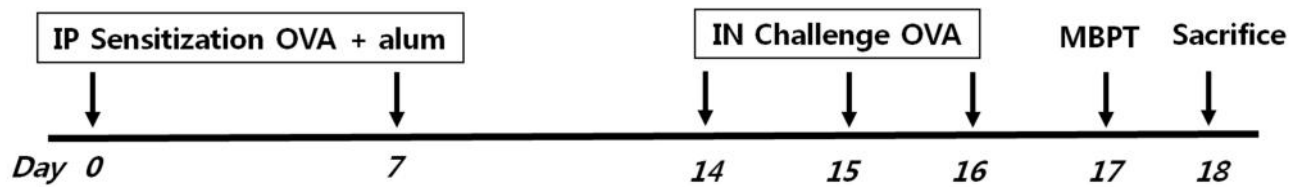
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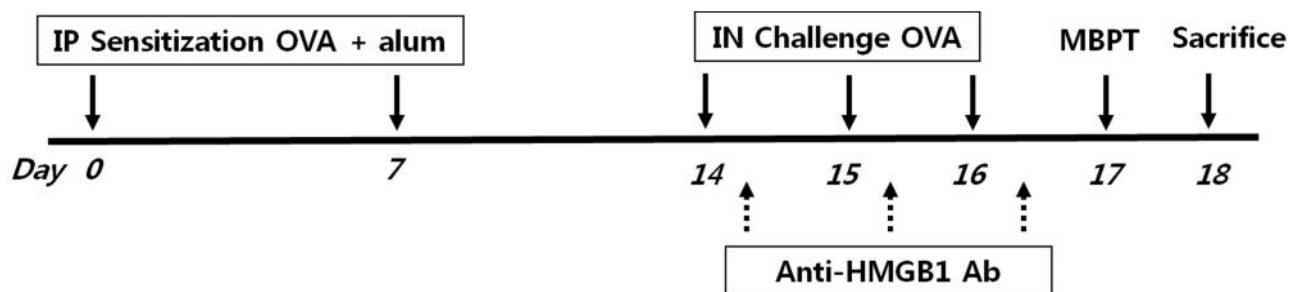
113

Figure 1. A protocol for a murine model of asthma

A.



B.



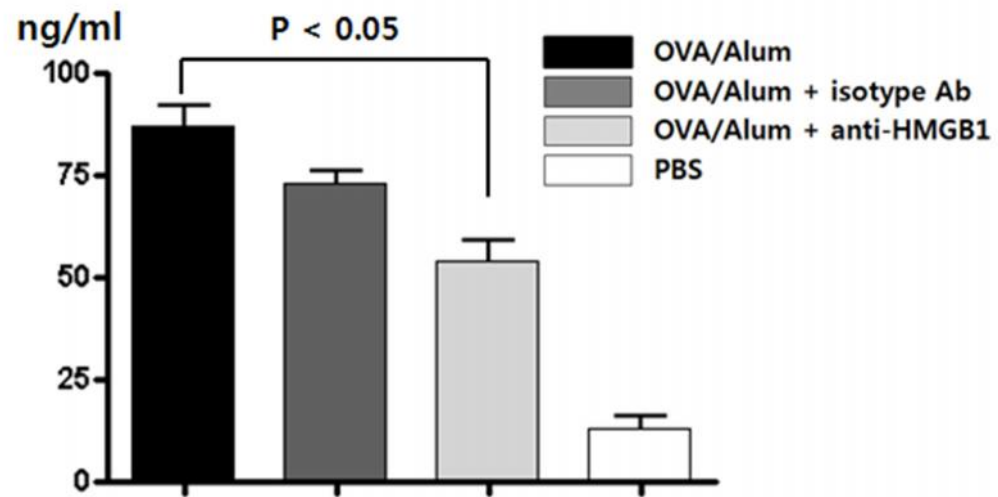
A. Mice (BALB/C, female, 6weeks) were sensitized intraperitoneally with OVA plus alum (day 1, 7) and then challenged intranasally with OVA alone (day 14, 15, 16). At day 17, MBPT was done and then mice were sacrificed 24 hours after MBPT.

B. To block HMGB1activity, anti-HMGB1 antibody (200 μ g /ml) was injected intraperitoneally 6 hours after every challenge (day 14, 15, 16).

(IP: intraperitoneal, IN: intranasal, OVA: ovalbumin, MBPT: methacholine bronchial provocation test)

1 **Figure 2. HMGB1 levels in BAL fluid (murine model of asthma)**

2



3

4 (OVA/Alum; asthmatic mice, PBS; control mice, isotype Ab; treated by isotype antibody, anti-HMGB1; treated by anti-HMGB1
5 antibody)

6

7