

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Allergens produce serine proteases-dependent distinct release of metabolite DAMPs in human bronchial epithelial cells

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Summary

Background: The respiratory epithelium is a major site for disease interaction with inhaled allergens. Additional to IgE-dependent effects, allergens contain proteases that may stimulate human bronchial epithelial cells (HBECs) through protease-activated receptors, causing the release of mediators important in driving Th2-mediated immune responses.

Objective: We aimed to investigate whether different allergens induce metabolite DAMPs such as ATP and uric acid (UA) release in HBECs.

Methods: HBECs (BEAS-2B cell line) were exposed to different allergen extracts; house dust mite (HDM), *Alternaria alternata*, *Artemisia vulgaris* and *Betula pendula* and UA, ATP, IL-8 and IL-33 release were measured. Allergen extracts were heat-inactivated or pre-incubated with serine (AEBSF) or cysteine (E64) protease inhibitors to study the involvement of protease activity in ATP, UA and IL-8 release. HDM-induced release of UA was studied in a mouse model of allergic inflammation.

Results: All allergens caused dose-dependent rapid release of ATP and IL-8, but only HDM induced UA release from HBECs. HDM also caused release of UA in vivo in our mouse model of allergic inflammation. ATP release by all 4 allergen extracts was significantly reduced by heat-inactivation and by serine protease inhibitors. Similarly, the HDM-induced UA release was also abrogated by heat-inactivation of HDM extract and dependent on serine proteases. Furthermore, allergen-induced IL-8 mRNA expression was inhibited by serine protease inhibitors.

Conclusions and Clinical Relevance: ATP was released by all 4 allergens in HBECs supporting the role of ATP involvement in asthma pathology. However, HDM stands out by its capacity to cause UA release, which is of interest in view of the proposed role of UA in early initiation of allergic asthma. Although serine proteases may be involved in the activity of all the studied allergens, further work is warranted to explain the differences between HDM and the other 3 allergens regarding the effects on UA release.

KEYWORDS

alarmins, allergens, asthma, bronchial epithelial cells, proteases

1 | INTRODUCTION

Human bronchial epithelial cells (HBECs) play a vital role as a physically and biologically active barrier between inhaled allergens and the immune system. HBECs express pattern recognition receptors (PRRs) and protease-activated receptors (PARs) to recognize immune stimuli such as inhaled viruses and proteolytic allergens.¹⁻³ Certain allergens contain proteases, and the protease activity may promote a Th2 response.⁴ House dust mite (HDM) is a common allergen, and up to 85% of asthmatics are sensitized to HDM.⁵⁻⁸ Exposure to allergens is a causative factor in sensitization, in acute asthma and in development of a chronic inflammation of the airways.⁹ Previous reports have suggested that proteases present in many allergens contribute to the pathogenesis of atopic asthma.^{10,11}

During cell stress, tissue injury or pathogenic infections, cell-derived and humoral molecules are released interacting with immune cells and triggering inflammation. Early released molecules alert our body's defence towards an approaching danger.^{12,13} A selection of such initial response molecules has, therefore, been called different names alluding to imminent danger, endogenous danger signals, danger-associated molecular patterns (DAMPs) or alarmins.^{12,13} Metabolite DAMPs such as extracellular ATP and UA may alert the immune system of early damage events.⁸ There is an increasing body of experimental evidence for roles of alarmins such as IL-33, ATP and uric acid (UA) in the pathogenesis of asthma and allergic diseases mainly involving Th2 immune responses.¹⁴⁻²⁰ Allergen-challenged asthmatic patients also have increased levels of UA and ATP in their airways.^{16,19} Furthermore, ongoing Th2-type airway inflammation has been inhibited by blocking of ATP supported the role of ATP.¹⁹ These evidences support the important role of extracellular UA and ATP in different stages of Th2-type immune responses and inflammation in the airways. Experimental in vivo studies in mice actually suggest that UA may be a major factor in initiation, pathophysiology and immunoinflammatory features of allergic asthma.^{16,20} Bronchial epithelial cells are a likely source of alarmins in vivo. However, few studies have examined acute effects of allergen-induced alarmin release in human bronchial epithelium. Hence, comparing alarmin release after stimulation with different allergens is of interest.

In the present study, we have examined the release of ATP, UA and IL-8 in HBECs in response to 4 different allergens of relevance to asthma, namely HDM, *Artemisia vulgaris* (*A. vulgaris*), *Betula pendula* (*B. pendula*) and *Altenaria alternata* (*A. alternata*). The role of heat-inactivation and class-specific protease inhibitors in alarmin release and IL-8 mRNA expression and protein release was also studied. We demonstrated that all 4 allergens induced extracellular ATP release in a concentration-dependent manner. However, only HDM induced UA release supporting a special importance of this allergen and this alarmin in allergic asthma. Heat-inactivation of the present allergen extracts reduced the release of IL-8, ATP and UA. A serine protease inhibitor reduced the release of ATP and UA but not IL-8 release. Although involvement of proteases is a shared UA- and ATP-releasing feature of the present allergens, our findings reveal

major differences among allergens regarding their capacity of releasing different alarmins in HBECs.

2 | MATERIAL AND METHODS

2.1 | Allergen extract preparations

Whole HDM extract was purchased from GREER Laboratories (Lenoir, NC, USA). Allergen extracts from *A. vulgaris*, *B. pendula* and *A. alternata* were prepared by mixing 30 mg of grains (Allergon, Ängelholm, Sweden) into 1 mL of RPMI (1640) medium and incubate for 30 minutes at 37°C. This mixture was centrifuged for 20 minutes at 3345 g. Extracts were obtained after filtration (0.2 µm pore) of the supernatant.

2.2 | Measurement of protease activity

Proteolytic activity was assayed by using azocasein (Sigma-Aldrich, Stockholm, Sweden) as a substrate. Briefly, 10 µL volume of allergen extracts was used for the assay. After incubation with or without 1 mM dithiothreitol (DTT) and further incubation with or without E64 (50 µM) and/or AEBSF (100 µM), a suitable dilution of enzyme solution was added to 50 µL of azocasein (2%, wt/vol) in reaction buffer (Tris buffer containing MgCl₂ [final concentration, 10 mM]). Then, the mixture was incubated at 37°C for 24 hours. The reaction was terminated by adding 240 µL of 10% (vol/wt) trichloroacetic acid and incubated for 15 minutes, followed by centrifugation at 8000 × g, at 4°C for 10 minutes. In total, 125 µL of the supernatant was neutralized by adding 125 µL of 1M NaOH, and the absorbance at 440-450 nm was measured using a spectrophotometer.

The protein concentrations were measured using bicinchoninic acid (BCA) assay (Pierce® BCA Protein Assay Kit detection, Fischer Scientific AB, Sweden), according to the manufacturer's instructions.

2.3 | In vivo experiment

Mice received intranasal (i.n) administration of 25 µg HDM extract 3 days/week for 3 weeks to induce allergic airway inflammation. Mice received saline as control. The Animal Ethics Committee at Lund University approved all the experiments performed (Ethical number: M36-13). Bronchoalveolar lavage (BAL) of the lungs was performed with phosphate-buffered saline (PBS) at 5 hours after last HDM challenge. Total cell counts, protein and UA levels were measured in bronchoalveolar lavage fluid (BALF).

2.4 | Cell cultures and treatments

The human bronchial epithelial cell line, BEAS-2B, was purchased from ATCC (Walkersville, MD, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin and streptomycin from Life Technologies (Stockholm, Sweden) as previously described.²¹ All incubations were carried out in 5% CO₂ in air at 37°C. For the experiments, cells were seeded and

grown to 80%-90% confluency in collagen-coated (Advanced BioMatrix, Carlsbad, CA, USA) 12-well culture plates (Nunc Technologies, Carlsbad, CA, USA) and exposed to different concentrations of allergen extracts: HDM (5-40 µg/mL), *A. vulgaris* (125-1000 µg/mL), *B. pendula* (10-500 µg/mL) and *A. alternata* (10-100 µg/mL) for 1, 6 and 24 hours. In separate experiments, optimal concentrations of each allergen extract were heat-inactivated at 65°C for 30 minutes or pre-incubated with either serine (AEBSF) or cysteine (E64) protease inhibitors from Sigma-Aldrich (Stockholm, Sweden) at room temperature for 30 minutes. Cell lysate and cell-free supernatant were collected for further analysis.

2.5 | Uric Acid measurements

Amplex Red Uric Acid Assay Kit (Life Technologies, Eugene, Oregon, USA) was used to measure UA levels in cell supernatants. The UA levels were measured using a microplate reader (96-well plate) according to manufacturer's instruction. Detection limit of the assay was 7.8 µM.

2.6 | ATP measurements

ATP Kit SL (BioThema luminescent assay, Handen, Sweden) was used to analyse ATP levels in samples according to manufacturer's instructions.

2.7 | RNA isolation and Real-Time qPCR

Total RNA was extracted from BEAS-2B cells using a RNA extraction kit, NucleoSpin RNA II (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions; 1 µg of mRNA was reverse-transcribed to cDNA (Precision Nanoscript Reverse Transcription Kit, PrimerDesign, Southampton, UK); and real-time quantitative PCR was performed. In brief, an Mx3005P qPCR system (Stratagene, La Jolla, CA, USA) with standard cycling parameters was used to perform thermo-cycling and real-time detection of PCR products. The following primer sequences (PrimerDesign, Southampton, UK) were used: CXCL8: CAGAGACAGCAGAGCACAC (forward) and AGCTTGAAGT-CATGTTTACAC (reverse). Quantitative normalization was performed on the expression of ubiquitin C (UBC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Group comparisons were normalized to control sample using the comparative $\Delta\Delta C_t$ method.²²

2.8 | ELISA of IL-8 and IL-33 protein release in cell supernatants

IL-8 (detection limit of the assay is = 31.25 pg/mL) and IL-33 (detection limit of the assay is = 23.5 pg/mL) levels were measured in cell supernatants using a specific ELISA with matched antibodies from R&D Systems (Minneapolis, USA) according to the manufacturer's descriptions.

2.9 | Statistical analysis

Data were analysed using the software GraphPad Prism version 6.0 (San Diego, CA, USA) and expressed as mean value with SEM.

Significant variations between the groups were analysed using ANOVA followed by Dunn's multiple comparison test, while the Mann-Whitney test was used to analyse differences between unpaired groups. *P* values of less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Allergen extracts induce IL-8 extracellular protein release by HBECs

Allergens induce pro-inflammatory cytokines such as IL-6 and IL-8, potentially contributing to recruitment of immune cells to the airways.²³ Initial experiments were performed to determine whether different aeroallergens caused cytokine release from bronchial epithelial cells. BEAS-2B cells were incubated for 6 and 24 hours with different concentrations of HDM: *A. vulgaris*, *B. pendula* and *A. alternata*. Then, IL-8 release into the culture medium was measured. These data showed that, HDM extract stimulated BEAS-2B cells to release IL-8 (Figure 1A), at higher concentration of HDM (40 µg/mL) the effect was decreased. *A. vulgaris*, *B. pendula* and *A. Alternata* caused dose-dependent release of IL-8, especially at 24 hours (Figure 1B-D).

3.2 | All 4 allergen extracts induce ATP release in HBECs

Extracellular ATP acts as a danger signal as well as a mediator of inflammation.²⁴ To investigate the release of ATP, HBECs were exposed to different concentrations of the allergen extracts: HDM (5-40 µg/mL), *A. vulgaris* (125-1000 µg/mL), *B. pendula* (10-100 µg/mL) and *A. alternata* (10-100 µg/mL). Levels of ATP in the cell-free supernatant were analysed at 1 hour post-stimulation. We demonstrated that ATP release was significantly and dose-dependently increased by stimulation with all 4 allergens compared to medium alone (Figure 2A-D).

3.3 | HDM-induced airways allergic inflammation in mice

HDM-induced inflammation was assessed by total and differential counts of cells collected in BALF. In response to HDM, overall BALF cell counts and protein content were increased in HDM group compared to saline controls (Figure 3A,B); however, in HDM-challenged mice they had significantly more eosinophils in the BALF compared to saline group (Figure 3C).

3.4 | HDM-induced UA release in HBECs and in a model of allergic airway inflammation

UA has been forwarded as an essential initiator and amplifier of pulmonary allergy.¹⁶ Stimulation of HBECs with different concentrations of HDM induced UA release at 6 hours. At 24 hours, UA

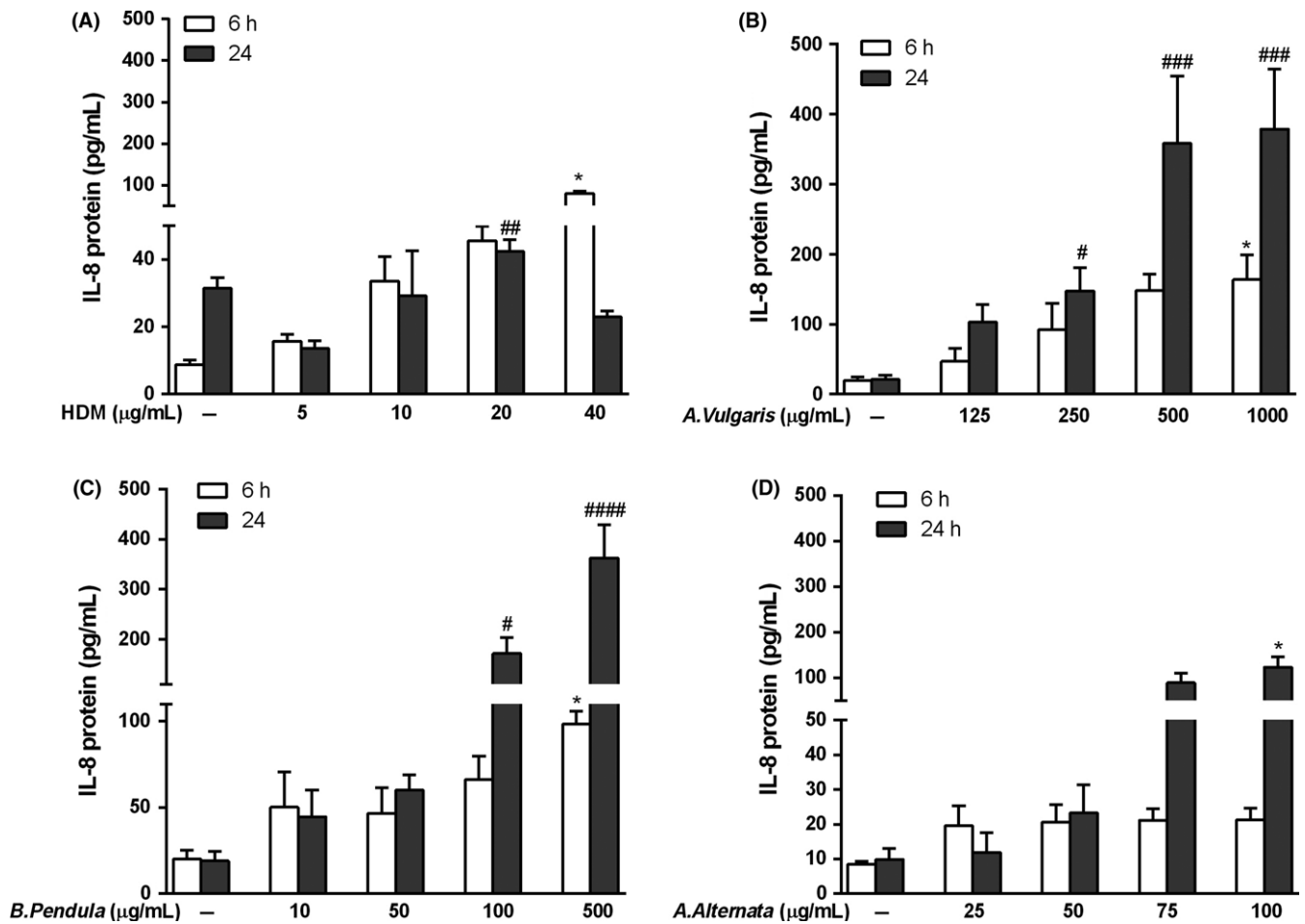


FIGURE 1 Allergen extracts induce IL-8 extracellular protein release by HBECS. HBECS were treated with various concentrations of HDM (A), *A. vulgaris* (B), *B. pendula* (C) and *A. alternata* (D) extracts for 6 h and 24 h. IL-8 protein levels in supernatants were measured by ELISA. Data are presented as mean $\pm$ SEM, $n = 4$ –6 from 3 independent experiments. * $P < .05$, ** $P < .01$ (6 h) and # $P < .05$, ## $P < .01$ (24 h) compared to respective control

levels were increased with higher concentrations of HDM (10–40 $\mu\text{g/mL}$) (Figure 4A). Further, to confirm that HDM-induced UA release in HBECS, we measured UA levels in BALF in our mouse model.²⁵ Exposure to HDM induced UA release in BALF compared with saline 5 hours after last challenge (Figure 4B). Our results suggest that HDM induced UA release in HBECS and in vivo. In contrast, UA release was not detected or below the detection limit in controls and in HBECS exposed to *A. vulgaris*, *B. pendula* and *A. alternata* extract (Figure 4C,D).

3.5 | Heat-inactivation of allergen extracts reduces metabolite DAMPs release and IL-8 expression in HBECS

Many aeroallergens consist of proteolytic activities that can influence cytokine production. Heat-inactivation of allergen extracts is known to affect their protease activity. Heat-inactivation of allergens was used to test whether alarmin release and IL-8 expression were due to allergen protease activity. We treated optimal concentrations of 4 different allergen extracts: HDM (20 $\mu\text{g/mL}$), *A. vulgaris*

(500 $\mu\text{g/mL}$), *B. pendula* (100 $\mu\text{g/mL}$) and *A. alternata* (100 $\mu\text{g/mL}$) for 30 minutes at 65°C before stimulation of HBECS.²⁶ Our data showed that UA release was fully abrogated by heat-inactivation of HDM allergen extract compared to active HDM extract (Figure 5A). ATP release by all 4 allergen extracts was significantly reduced by heat-inactivation (Figure 5B). Heat-inactivation of the allergen extracts tended to variably reduce IL-8 mRNA and protein compared to active allergens at 6 and 24 hours (Figure 5C–F).

3.6 | Specific protease activity of allergen extracts determined by the azocasein assay

Next, we examined the protease activity of 4 different allergen extracts using azocasein as a substrate. Allergen extracts were incubated with serine (AEBSF) or cysteine protease (E64) inhibitors or a reducing agent, dithiothreitol (DTT) alone or in combination. Our data showed that all 4 allergen extracts show protease activity to various degrees (Figure 6 A–D). This activity was enhanced by DTT in HDM and *B. pendula*, which indicates that the cysteine protease component of these 2 extracts was stronger compared to *A. vulgaris*

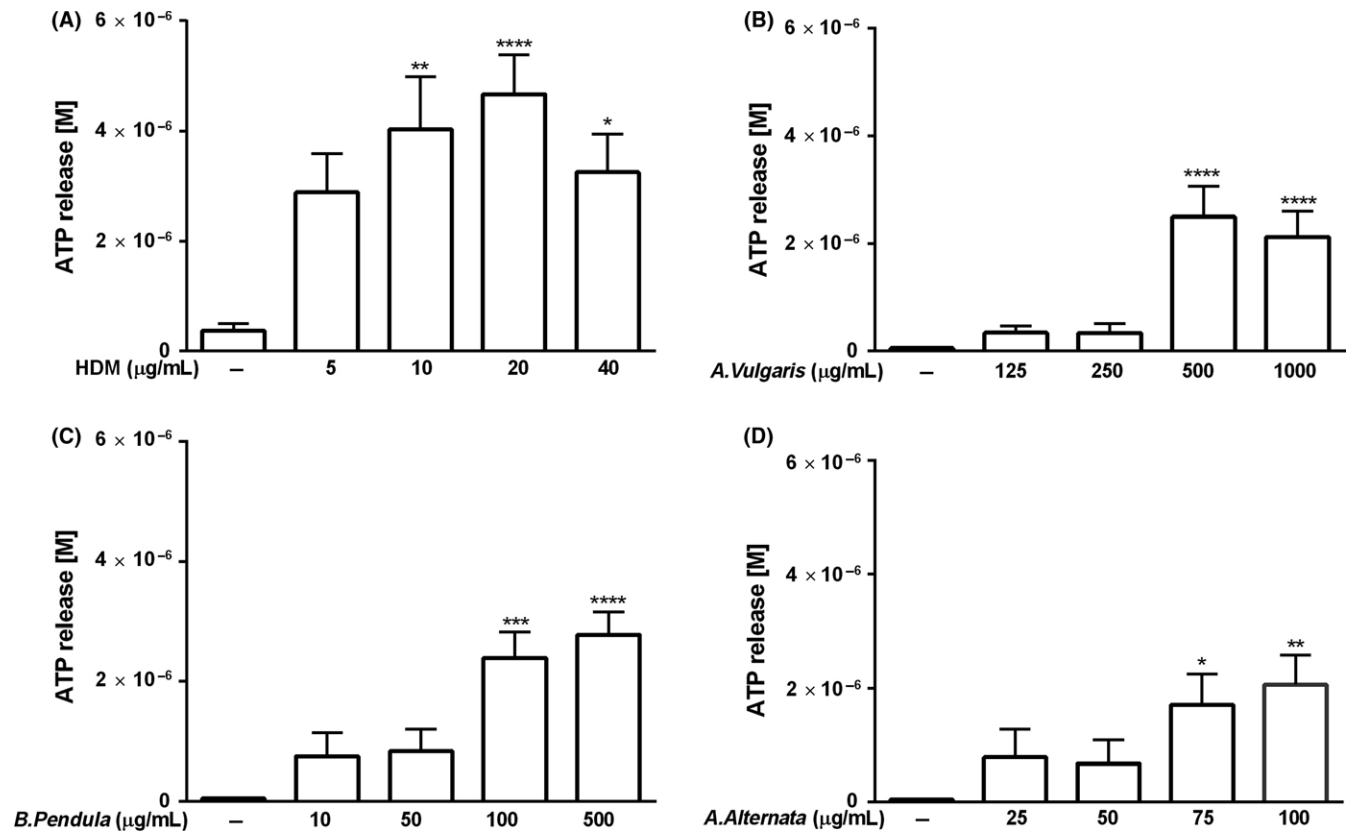


FIGURE 2 Allergen extracts induce ATP release in HBECs. HBECs were stimulated with various concentrations of HDM (A), *A. vulgaris* (B), *B. pendula* (C) and *A. alternata* (D) extracts for 1 h. ATP levels were measured in supernatants. Data are presented as mean ± SEM, n = 12 from 5 independent experiments. *P < .05, **P < .01, ***P < .001, ****P < .0001 compared to respective control

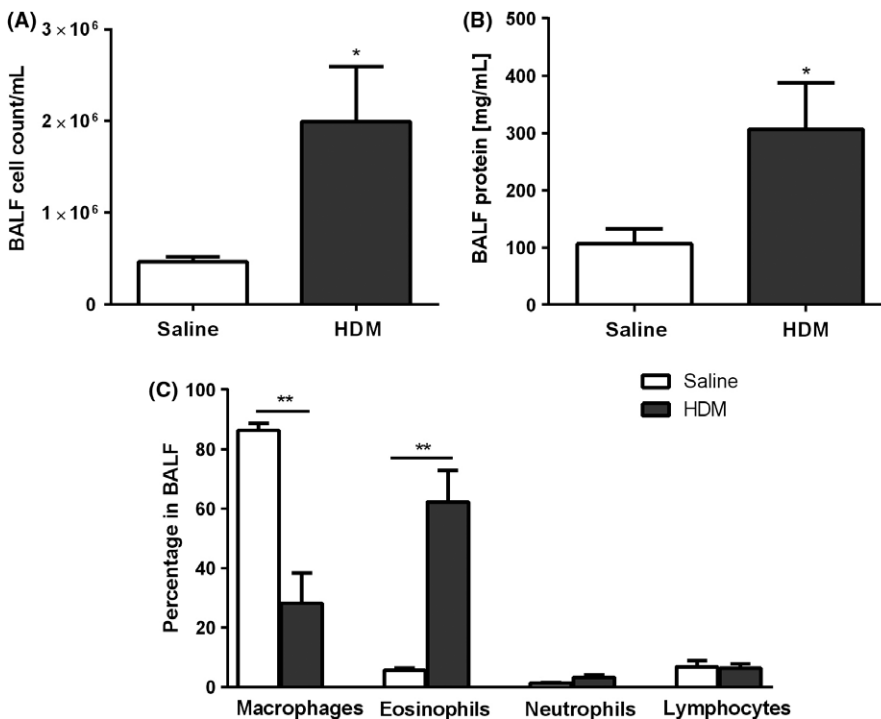


FIGURE 3 HDM-induced airways inflammation in mice. HDM induces inflammatory cell recruitment to the airways 5 h after the last challenge. HDM administration increased total BALF cells (A), increased BALF protein content (B) and increased eosinophils compared to the non-allergic saline exposed group (C). Data are presented as mean ± SEM (n = 4-6 mice in each group). *P < .05, compared to saline control

and *A. alternata*. The protease activity was inhibited by both serine and cysteine protease inhibitors in 3 of the allergens (HDM, *B. pendula*, and *A. vulgaris*) to different magnitude (Figure 6 A-C). Further,

the protease activity of all 4 allergens was corrected to μg of protein (Figure S2). Interestingly, 4 different allergen extracts of protease activity per μg of protein followed the same pattern as IL-8 gene

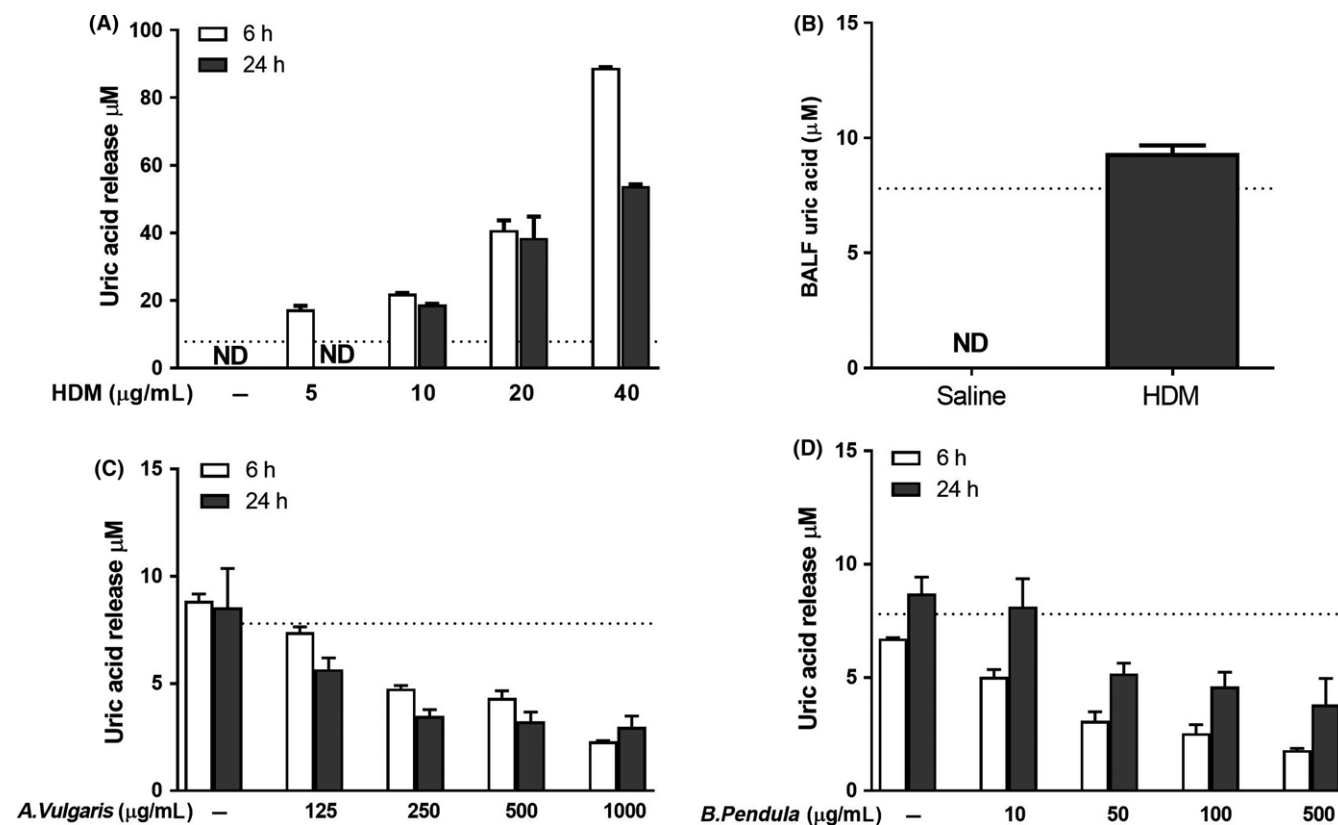


FIGURE 4 HDM-induced UA release in HBECs and in a model of allergic airway inflammation. HBECs were incubated for 6 h and 24 h with various concentrations of HDM (A), *A. alternata* (non-detected), *A. vulgaris* (C) and *B. pendula* (D). UA concentration was measured in supernatants. Data are presented as mean $\pm$ the SEM, $n = 6$ from 3 independent experiments. The UA release was confirmed in an in vivo mouse model. BALF UA in HDM-challenged mice compared to controls (saline) group after 5 h (B). $n = 5$ mice in each group. ND (non-detected)

expression after allergen stimulation in bronchial epithelial cells (Figure 5D).

3.7 | Treatment of allergen with serine protease inhibitor reduced metabolite DAMPs release and IL-8 mRNA expression in HBECs

Allergens contain serine and cysteine proteases.¹⁰ As the heat-inactivation of allergen extracts reduced the UA and ATP release and IL-8 expression (Figure 5), we hypothesized that proteases may play a role in alarmin and IL-8 expression. Hence, we examined the effects of cysteine and serine proteases on the present allergen responses. Allergen extracts were pre-incubated with cysteine protease inhibitor (E64) or serine protease inhibitor (AEBSF) before being added to the cells. HDM-induced UA release was completely blocked by AEBSF but not with E64 at 24 hours (Figure 7A). ATP release by all allergens was reduced by both AEBSF and E64 but the reduction being only significant with AEBSF (Figure 7B). Further, allergen-induced IL-8 mRNA was inhibited by AEBSF but not by E64 (Figure 7C), suggesting that allergen serine proteases may mediate IL-8 transcription. In contrast, IL-8 protein release was not inhibited by protease inhibitors (Figure 7D). These data suggest that allergen-induced alarmin release and IL-8 mRNA expression in HBECs partly reflect effects of allergen serine proteases.

4 | DISCUSSION

Interaction between common aeroallergens and airway epithelium exacerbates and maintains airway inflammation in allergic asthma.^{27,28} In the present study, we demonstrated that 4 different aeroallergens of significance in asthma variably induced the release of alarmin, UA and ATP, from HBECs and that this activity was inhibited by serine protease inhibitors. Our findings support the view that the biological effects of asthma allergens, in part, reflect their proteolytic activity. However, important differences emerge among the allergens regarding their alarmin-releasing capacities.

Alarmins such as extracellular ATP and UA may alert the immune system of early damage events.⁸ These 2 alarmins are also released at HDM inhalation challenges in asthmatic subjects.^{16,19} In HDM-induced allergic disease in mice, UA is reported to be causally involved in sensitization, Th2-type inflammation, eosinophilia and airway hyperresponsiveness (AHRs).^{8,16,20} Supporting a role of ATP, Th2 sensitization to inhaled antigens and ongoing Th2-type airway inflammation has been inhibited by blocking of ATP.¹⁹ Taken together these data support the potential importance of extracellular UA and ATP in different stages of Th2-type immune responses and inflammation in asthma. Hence, the comparative capacity of allergens and their modes of inducing release of alarmins from a major target cell such as the bronchial epithelium are of interest.

■ CM
□ Active allergens
▨ HI-allergens

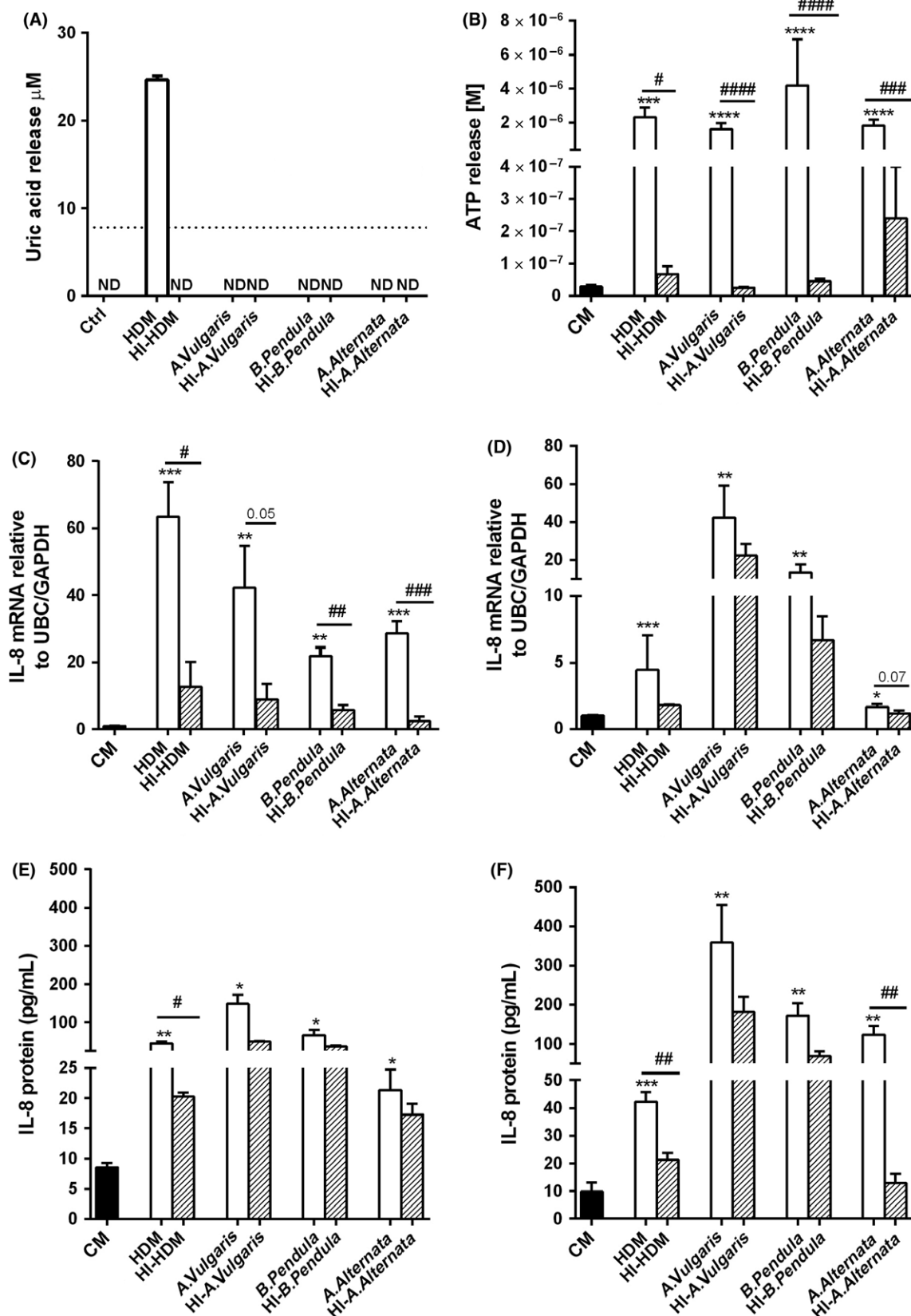


FIGURE 5 Heat-inactivation of allergen extracts reduces metabolite DAMPs release and IL-8 expression in HBECs. HBECs were treated with optimal concentrations of active and heat-inactivated HDM (20 $\mu\text{g/mL}$), *Artemisia vulgaris* (500 $\mu\text{g/mL}$), *Betula pendula* (100 $\mu\text{g/mL}$) and *Alternaria alternata* (100 $\mu\text{g/mL}$) allergen extracts. UA concentrations were measured after 24 h (A). ATP concentrations were measured after 1 h stimulation (B), IL-8 mRNA expression and protein levels were measured at 6 h (C, E), and 24 h (D, F). ND (non-detected). Data are presented as mean $\pm$ SEM, $n = 5-12$ from 3-6 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ compared to respective control and # $P < .05$, ## $P < .01$, ### $P < .001$, #### $P < .0001$ compared to active and heat-inactivated allergen extracts

The 4 allergens examined in the present study exhibited similarities regarding effects on ATP release. Exposure of HBECs to the different allergens (HDM, *A. vulgaris*, *B. pendula* and *A. alternata*) thus induced extracellular release of ATP in a dose-dependent manner. It was previously shown that *A. alternata* extract induced the release of ATP from bronchial epithelial cells,²⁹⁻³¹ but our findings suggest that ATP-releasing capacity is a common characteristic of different

allergens. These results support the notion that allergen interaction with bronchial epithelial cells is a major mechanism for the proposed ATP-mediated immunopathology in asthma.¹⁹

The most striking finding in this study was that only HDM of the 4 allergens tested induced UA release in HBECs. HDM induced UA release also in our mouse model of HDM-induced allergic inflammation. As the present effect occurred in bronchial epithelial cells

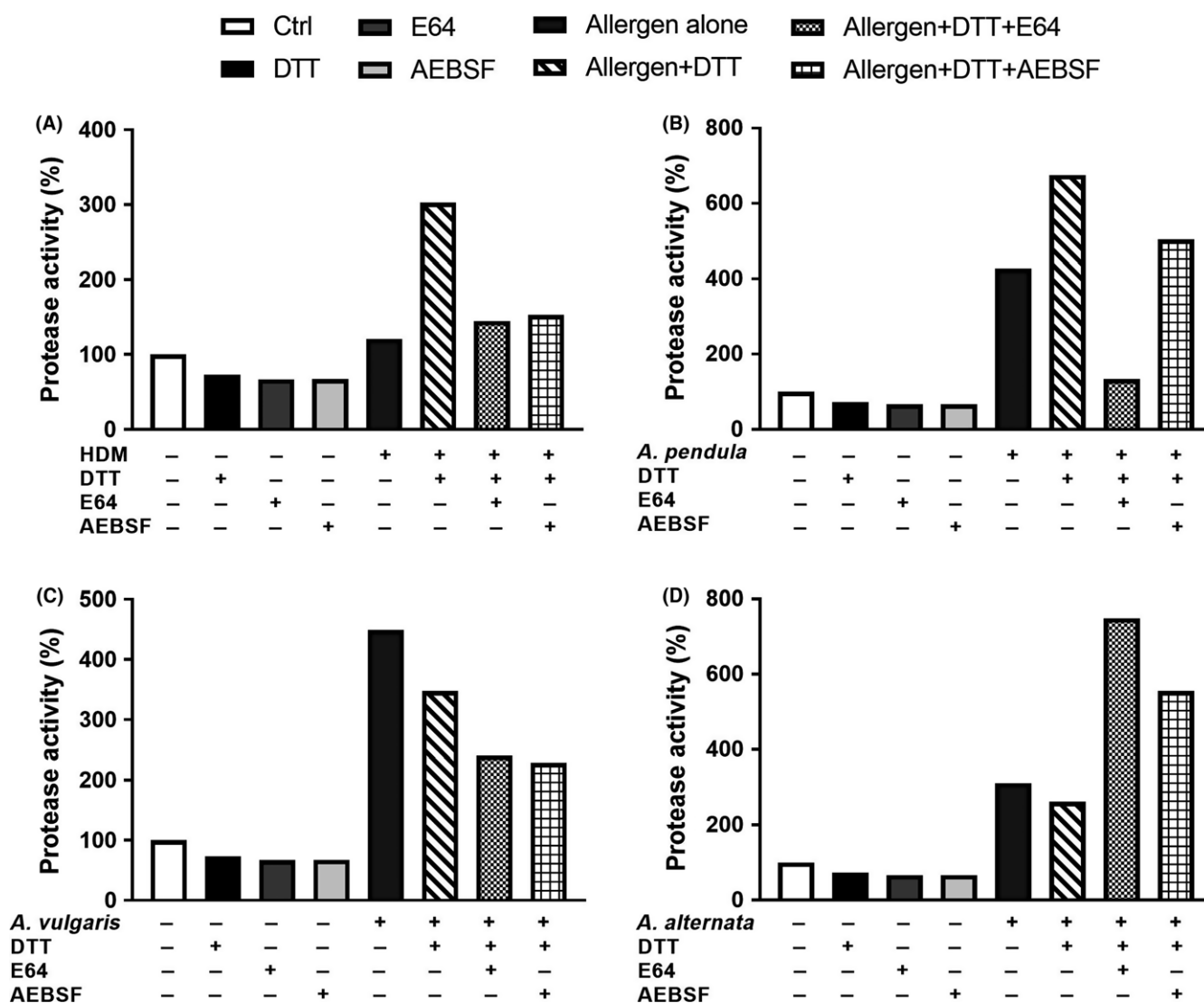


FIGURE 6 (A) HDM, (B) *B. pendula*, (C) *A. vulgaris* and (D) *Alternaria alternata* protease activity was measured with azocasein as a substrate. Allergen extracts were incubated with serine (AEBSF) or cysteine (E64) inhibitors or a reducing agent, dithiothreitol (DTT) alone or in combination. The absorbance was measured at 440-450 nm. Data are presented as percentage of protease activity

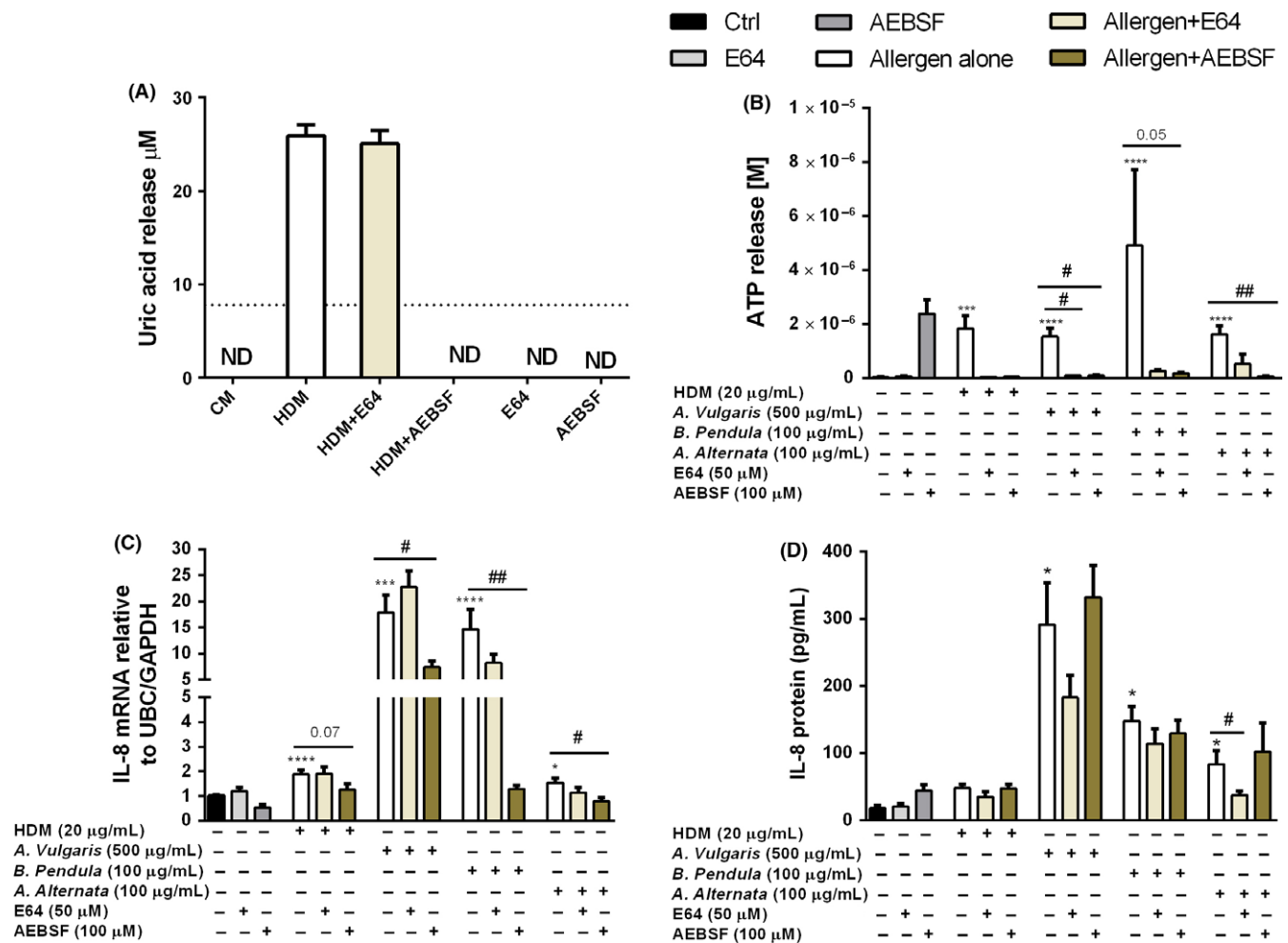


FIGURE 7 Treatment of allergen with serine protease inhibitor reduced metabolite DAMPs release and IL-8 mRNA expression in HBECs. HBECs were stimulated with an optimal concentration of HDM, *A. vulgaris*, *B. pendula* and *A. alternata* in the absence and presence of AEBSF (100 μM ; serine protease inhibitor) and E64 (50 μM ; cysteine protease inhibitor). Then, UA release was measured at 24 h (A), ATP was assayed in the supernatant at 1 h (B), IL-8 mRNA expression was assayed at 24 h (C) and IL-8 protein levels were measured in the supernatant at 24 h (D). ND (non-detected). Data are presented as mean $\pm$ SEM, $n = 5$ –12 from 3–6 independent experiments. * $P < .05$, *** $P < .001$, **** $P < .0001$ compared to respective control and # $P < .05$, ## $P < .01$ compared to allergen extracts alone and with inhibitors

without a disease background, our results are consistent with the demonstration by Kool, Willart¹⁶ that HDM-induced release of UA in mouse airways was not confined to animals that already had been sensitized to HDM but occurred also in naïve mice. This similarity between mouse lungs in vivo and human bronchial epithelium in vitro may support the hypothesis that the role of UA as delineated in mouse model studies¹⁶ could be relevant also to humans. Particularly intriguing is the possibility that HDM through its UA-releasing property in non-asthmatic epithelium could contribute to sensitization and initiation of HDM-induced asthma.

Lipopolysaccharides (LPS) contamination in allergens has been highlighted as an issue in many allergy models. Endotoxins are present in the environment and have been shown to modulate the allergic response through activation of TLR4.³² TLR4 thus plays a major role in Th2 immune responses to inhaled HDM allergens,³³ but it is not known whether TLR4 is involved in UA release. Hence, we also examined the effects of LPS in HBECs exposed for 24 hours. We

observed that UA release was not detected in HBECs stimulated with LPS (data not shown). Taken together, these findings indicate that HDM-induced UA release in HBECs may not reflect endotoxin components.

Apart from UA and ATP, IL-33 is a major alarmin cytokine from the IL-1 family, which plays a critical role in the initiation of Th2 immune response following viral infection or allergen exposure.³⁴ In our study, we exposed HBECs to 4 different allergen extracts for different time points and then measured IL-33 extracellular protein release. However, extracellular release of IL-33 was not induced by allergen treatments because it was below the detection limit (Figure S1).

Allergens can activate HBECs to secrete cytokines and chemokines such as IL-6 and IL-8 that contribute to airway inflammation in asthmatics.³⁵ Isolated HDM-derived proteases have been reported to produce marked and dose-dependent release of IL-8 from exposed epithelial cells.²⁹ Our studies showed that all allergens used in this study activate HBECs and produced IL-8.

We demonstrated that heat treatment of the allergen extracts reduced the allergen-induced release of alarmins in HBECs indicating involvement of heat-sensitive components. This observation is in accord with and extends previous observations on other epithelial effects of HDM and *A. alternata*.^{30,36,37} In the current study, we further observed that serine protease inhibitor (AEBSF) universally reduced ATP release and completely abrogated the HDM-induced UA release, whereas a cysteine protease inhibitor (E64) was without effect. These findings indicate that heat-labile, AEBSF-sensitive serine protease activity is likely involved when the present allergens induced alarmin release.

Allergens such as HDM, pollen, fungi and cockroach allergens contain proteases, and the proteolytic activities may injure epithelial cells. Ensuing bronchial epithelial restitution processes in vivo confers a range of inflammatory and remodelling effects in the airways.³⁸ Allergen proteases may also have non-injurious, pro-inflammatory roles in the respiratory tract³⁹ involving cytokines produced by epithelial cells.⁴⁰ Previous findings thus demonstrated that heat-sensitive proteases present in HDM, pollen, fungal and cockroach extracts activate HBECs and increase the release of pro-inflammatory cytokines via PAR-mediated mechanism.^{29,41-47} We demonstrated protease activity in all of the different allergen extracts used in our study. All 4 allergen extracts show protease activity to various degrees. *B. pendula*, *A. vulgaris* and *A. alternata* showed the highest protease activity, while HDM extract only had weak protease activity compared to control. Also, we found that the allergen protease activity followed the same pattern as IL-8 gene expression after allergen stimulation in bronchial epithelial cells (Figure S2). Further, the results of this study suggest that exposure of HBECs to 4 different allergen extracts variably causes release of alarmins (UA and ATP) and a pro-inflammatory cytokine such as IL-8. These activities, except IL-8 release, were largely dependent on heat-sensitive component activity involving serine rather than cysteine proteases. However, HDM also contains cysteine proteases such as Der p1 that might be capable of releasing alarmin and pro-inflammatory cytokines in the reducing environment of the lung.³⁶ Strikingly, HDM was distinguished by being the only allergen that evoked both ATP and UA release supporting the major role of this allergen in asthma including its initiation. Further studies are warranted to advance our understanding of mechanisms involved in the present differences between HDM and other aeroallergens.

In conclusion, the present study of comparison of 4 different allergens involved in pathogenesis of asthma show distinct differences in their ability to induce human bronchial epithelial alarmins revealing a distinct capacity of HDM to cause the release of both ATP and UA. Our results might indicate that allergen proteases thus may be an important target for future therapies in allergic diseases.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

S.R., H.A. and L.U. contributed to the design of the study and supervised the scientific work. S.R., M.M., H.A. C.A. and L.U. contributed to the acquisition, analysis and interpretation of the data. S.R. drafted the manuscript and M.M., H.A., C.A., L.B. and L.U. revised the manuscript. All authors have read and approved the submission of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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