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Degradation of bacterial permeability family member A1 (BPIFA1) by house dust mite (HDM) cysteine protease Der p 1 abrogates immune modulator function

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ABSTRACT

Bacterial permeability family member A1 (BPIFA1) is one of the most abundant proteins present in normal airway surface liquid (ASL). It is known to be diminished in asthmatic patients' sputum, which causes airway hyperresponsiveness (AHR). What is currently unclear is how environmental factors, such as allergens' impact on BPIFA1's abundance and functions in the context of allergic asthma. House dust mite (HDM) is a predominant domestic source of aeroallergens. The group of proteases found in HDM is thought to cleave multiple cellular protective mechanisms, and therefore foster the development of allergic asthma. Here, we show that BPIFA1 is cleaved by HDM proteases in a time-, dose-, and temperature-dependent manner. We have also shown the main component in HDM that is responsible for BPIFA1's degradation is Der p1. Fragmented BPIFA1 failed to bind *E. coli* lipopolysaccharide (LPS), and hence elevated TNFα and IL-6 secretion in human whole blood. BPIFA1 degradation is also observed in vivo in bronchoalveolar fluid (BALF) of mice which are intranasally instilled with HDM. These data suggest that proteases associated with environmental allergens such as HDM cleave BPIFA1 and therefore impair its immune modulator function.

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1. Introduction

Asthma is a common airway inflammatory disease that currently affects over 17.7 million adults (age ≥ 18) and 6.3 million children (age < 18) in the United States [1,2]. Asthma poses a tremendous economic burden, with costs exceeding $56 billion annually [3]. The symptoms of asthma are characterized by intermittent airflow obstruction and airway inflammation, producing chest tightness, wheezing, and coughing. In genetically susceptible individuals, exposure to a wide variety of environmental stimuli, including allergens such as HDM, can induce and/or exacerbate the disease [4,5]. Structural and inflammatory changes throughout the airway wall lead to bronchial thickening and edema as well as increased mucus production and bronchoconstriction, all of which contribute to the episodic airflow obstruction typical of asthma [6].

HDM, a major perennial allergen, is a significant cause of allergic rhinitis and allergic asthma—the most common type of asthma. HDM is ubiquitous in human habitats; therefore it is the most important source of indoor allergens [7,8]. Over the past three decades, many important allergen groups from HDM have been identified and characterized at the molecular level [9]. HDM components that are thought to produce allergic inflammation include immunogenic epitopes, structural polysaccharide chitin from the exoskeleton of HDM, microbial adjuvant compounds, ligands originating from mite-associated compounds, and proteases [10,11]. These components manifest diverse biological functions depending on their molecular structures. Protease activity is a common feature of many human allergens, including fungi, pollen, animal dander, and bee venom. HDMs and their fecal pellets contain several proteolytic enzymes [11]. To date, a handful of cysteine and serine protease allergens have been cloned, including Der p 1, Der p 3, Der p 6 and Der p 9 [12]. A significant body of evidence suggests that the proteolytic enzyme activities of these allergens enhance IgE synthesis and promote T_H2 inflammatory responses [13]. Therefore, the design of new and more effective therapeutic approaches for treating allergic asthma may rely on the development of drugs that suppress or confer resistance to HDM-mediated protein degradation.

The bacterial permeability family member A1 (BPIFA1) gene was originally found to be expressed in murine embryonic nasal epithelium and adult trachea and bronchi [14]; it was later found in enriched human sputum and tracheobronchial secretions, as well as in apical secretions from cultured human tracheobronchial epithelial cells [15–20]. BPIFA1 and its related family members share structural similarity with

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the N-terminal half of the Bactericidal/permeability-increasing (BPI) protein [21], which binds and neutralizes endotoxic activity of lipopolysaccharides (LPS) from gram negative bacteria. Similar to BPI, the antimicrobial function of BPIFA1 has been supported by both in vitro and in vivo studies. Co-incubation with BPIFA1 can reduce the growth of a variety of Gram-negative bacteria [22–24]: BPIFA1$^{-/-}$ mice showed increased susceptibility to *Pseudomonas aeruginosa* (*P. aeru*) and significantly decreased survival rates compared to their wildtype (WT) littermate controls [25]. When human BPIFA1 was transgenically knocked into mouse airway epithelium, lung *Mycoplasma pneumoniae* load dropped significantly [22]. On the other hand, compared to BPI, BPIFA1 bears unique features that make it a multi-functional protein: 1) BPIFA1 has an additional helix (α6) in its C terminus that occludes the equivalent lipid-binding site in BPI. This helix interacts with an extended loop between β2 and β3. It has been speculated that the helix will move away upon lipid ligand binding [26]. (2) BPIFA1 contains an 18-residue domain (G22-A39, termed the "S18 region" in a previous publication [27]) that is not present in BPI or other BPI-type proteins. Although this region was present in the 20–256 residue construct used for crystallization, it was disordered in the resolved structure and could not be visualized within the electron density maps [21]. By binding to the β subunit of the epithelial sodium channel (ENaC) via the S18 region, BPIFA1 negatively regulates sodium transport, maintaining proper airway surface liquid (ASL) volume. These data imply that BPIFA1 is a structurally diversified, multi-functional protein: elucidating the ultimate function of each domain may reveal a novel mechanism of action.

Beyond its well-established anti-microbial activity and more recently reported functions in airway physiology/pathology, BPIFA1 has been shown to function as an innate immunity modulator, as demonstrated in asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) [28,29]. Specifically, in the context of asthma, BPIFA1 deficiency has been linked to the severity and progression of the disease. For example T_H2 cytokine IL-13 was previously reported to significantly reduce BPIFA1 expression and secretion from human primary epithelial cells, rendering a host susceptible to *M. pneumonia* infection [30]. BPIFA1$^{-/-}$ mice exhibit enhanced eosinophilic inflammation when challenged with ovalbumin (OVA); restored BPIFA1 concentration in these animals attenuated the inflammation phenotype, partially by reducing eotaxin-2 production from alveolar macrophages [31]. Our recent findings with asthmatic patients' sputum and asthmatic human bronchial epithelial cultures (HBECs) suggest that BPIFA1 levels are reduced in sputum samples from asthma patients and in apical/basolateral secretions of asthmatic HBECs, which leads to Ca^{2+} flooding through Orai1 and airway smooth muscle (ASM) hyper-contractility [32]. Our finding not only confirmed the association between BPIFA1 deficiency and asthma pathology, but also raised new research questions regarding (1) why BPIFA1 levels decreased in asthmatic airways, apart from transcriptional regulation of IL-13, and (2) how lack of BPIFA1 contributes to the development of airway inflammation. Here, we therefore tested the hypothesis that HDM proteases cleave BPIFA1, causing loss-of-function, which, in turn, contributes to hyperactivation of Ca^{2+} signal in airway epithelial cells and therefore increased secretion of pro-inflammatory cytokines.

2. Material and methods

2.1. Cell lines and cell culture

Human epithelial cell line Calu-3 was purchased from ATCC. These cells were maintained in Minimum Essential Alpha (MEM α; Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 0.1% Penicillin-Streptomycin (Thermo Fisher). HBECs were obtained from freshly excised bronchial specimens derived from normal and asthmatic subjects and were harvested as previously described under protocol #03-1396, approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board [33]. Informed consent was obtained from all donors or authorized representatives of the donors. Briefly, HBECs were extracted from excess surgical pathology or autopsy specimens procured through cooperating surgeons and pathologists using protocols following relevant regulations. Airways were dissected by removing all excess connective tissue and cutting into 5–10 cm segments. Specimens were dissociated over a period of 4–24 h, depending on the size of the tissue, in a 15-ml tube containing 9 ml tissue wash medium (Joklik Minimum Essential Medium <JMEM> supplemented gentamicin <50 μg/ml > and amphotericin <0.25 μg/ml>) plus 1 ml protease solution (1% Protease XIV with 0.01% DNase <10× stock>, Sigma Aldrich). HBECs were then cultured at an air-liquid interface in a modified bronchial epithelial growth medium with 5% CO_2 at 37 °C and were used 3–4 weeks after seeding on 12-mm T-clear inserts (Corning). For HDM exposure, HBECs were treated with DPBS (Hyclone) or 100 μg/ml HDM (Greer) dissolved in DPBS (50 μl total volume) apically. 24 h post-treatment, any apical residual solution was removed, and the apical side of HBECs was washed with 100 μl DPBS for 30 min. The apical lavage, basolateral media, and cell lysate were then collected and subjected to downstream applications.

2.2. Detection of protein degradation, antibodies, and immunoblot analysis

Recombinant human BPIFA1 (GenScript) in DPBS (Hyclone) was incubated with HDM at indicated times and concentrations. The resulting products were resolved by NuPAGE 4–12% bis-tris protein gels and subject to immunoblot using goat anti-hBPIFA1 or sheep anti-mBPIFA1 (both 1:2000; R&D Systems); Peroxidase-conjugated donkey anti-goat (1:3000; Santa Cruz Technologies), or donkey anti-sheep (1:3000; R&D Systems) IgG (H + L) from commercial sources. Protease inhibitors aprotinin, E64D, phenylmethylsulfonyl fluoride (PMSF; Sigma-Millipore), and protease inhibitor cocktail (PIC; Roche) were purchased from indicated commercial sources.

2.3. mRNA extraction and quantitative real-time PCR (qRT-PCR)

Total mRNA was extracted from cells using RNaEasy reagent (Qiagen). Equal amounts of RNA (1 μg) were used for reverse transcription using a cDNA synthesis kit (BioRad). The following TaqMan probes were used: human BPIFA1 (assay ID: Hs00213177_m1) and human GAPDH (assay ID: Hs02786624_g1) (Thermo Fisher). qRT-PCR was performed on the Applied Biosystems 7500 fast machine (Thermo Fisher) as follows: one cycle of initial denaturation (95 °C for 4 min), 45 cycles of amplification (95 °C for 10s and 60 °C for the 30s). Data are presented as relative mRNA levels normalized to the level of GAPDH; the value from the untreated cells was set as 1. PCR assays were performed three times with duplicate samples, which were used to determine the mean ± SEM.

2.4. Animal exposure to HDM and bronchioalveolar fluid (BALF) collection

Experiments were performed on 8–10-week C57BL/6J mice of both genders obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in American Association for Accreditation of Laboratory Animal Care accredited facilities at North Carolina Central University under standard environmental conditions (12-h light-dark cycles at 23 °C). Mice were housed over hardwood shavings. Food and tap water were provided ad libitum. All animal studies were reviewed and received prior approval by the North Carolina Central University Institutional Animal Care and Use Committee.

For HDM exposure, *Dermatophagoides pteronyssinus* lyophilized HDM extract (Greer Laboratories) was resuspended in DPBS at a concentration of 1 mg (dry weight)/ml. The HDM suspension was delivered in the morning between 9:00 and 12:00 AM by intranasal instillation in a single 20-μl volume while the mice were lightly anesthetized with

isoflurane (2.5%). 24 h after instillation, mice were sacrificed and BALF was collected.

2.5. BPIFA1 fragment purification

BPIFA1 (250 μg/ml) was incubated with biotin-labeled HDM (100 μg/ml, Thermo Fisher) for 24 h at 37 °C. The resulting solution was then centrifuged at 8000 xg for 5 min to remove any large debris and applied to a NeutroAvidin column pre-washed 3 times with 1× DPBS (Gibco). The sample volume was 200 μl and the protein-beads mixture was incubated at room temperature for 30 min before being spun down at 500 g for 5 min. The flow-through was then applied to a second NeutroAvidin column to remove any residual biotin-HDM from BPIFA1 fragments, following the same procedure. BPIFA1 protein alone was used as control. Immunoblots against BPIFA1 and biotin-HDM were performed to monitor the quantity of protein before and after purification and to confirm the removal of HDM. Purified BPIFA1 or its fragments were then used for LPS binding assay or human whole blood incubation.

2.6. LPS binding assays

A modified, enzyme-linked immunosorbent assay (ELISA)-based LPS binding method was used to detect interactions between LPS and BPIFA1 as described previously [23]. Briefly, 96-well plates were coated overnight with purified LPS (400 ng) from *Escherichia coli* 055:B5 (Millipore Sigma). Wells were washed and blocked with 1% bovine serum albumin (BSA)-PBS for 1 h; 400 ng of purified BPIFA1 was then

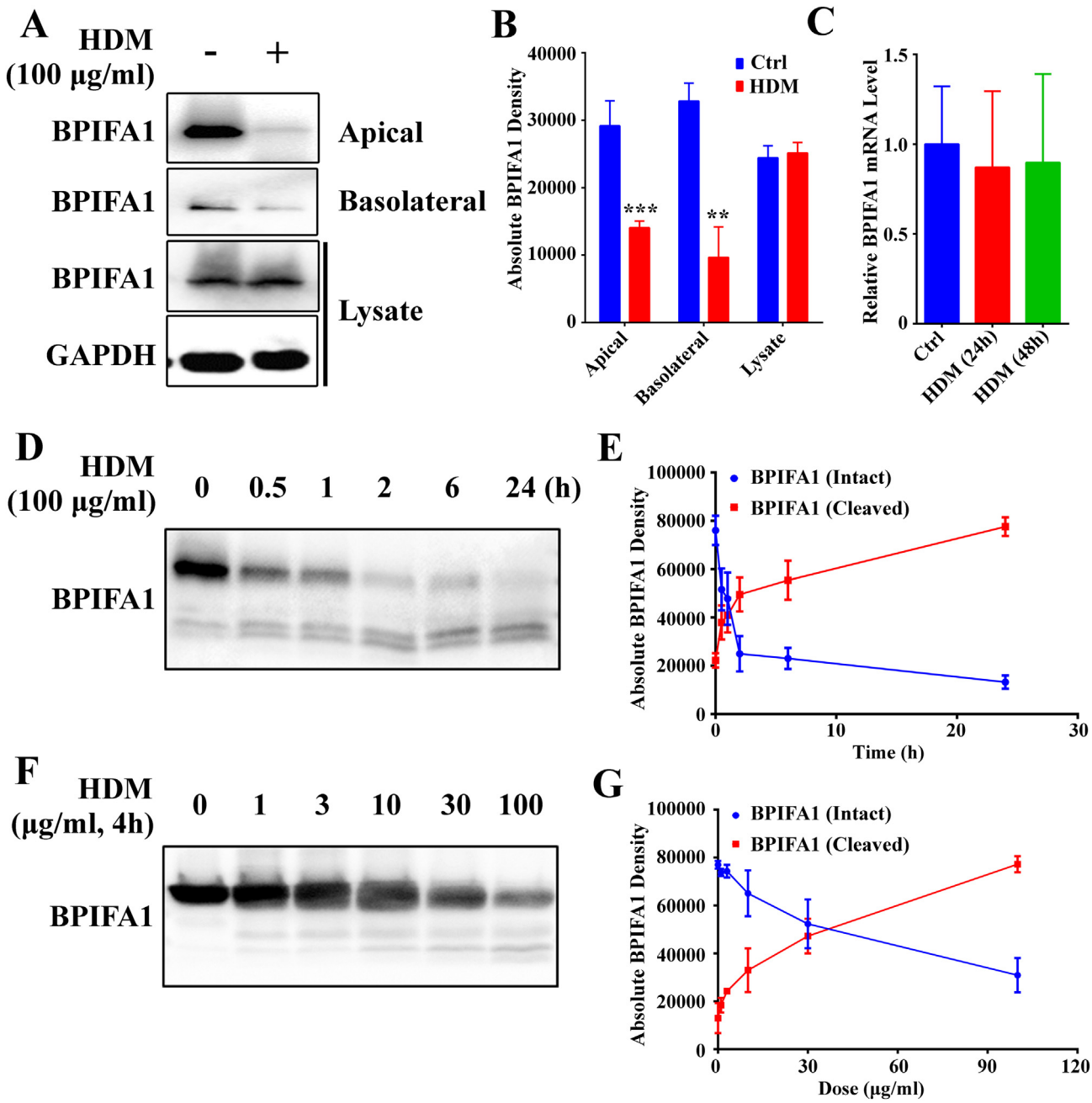

Fig. 1. HDM degrades recombinant BPIFA1. (A) and (B) are representative immunoblot and densitometry showing HDM decreases BPIFA1 in apical lavage and basolateral media of HBECs ($n = 6$). (C) HDM does not change BPIFA1 mRNA expression ($n = 4$). (C) and (D) Representative immunoblots and densitometry showing BPIFA1 is cleaved by HDM in a time-dependent manner ($n = 5$). (E) and (F) Representative immunoblots and densitometry showing BPIFA1 is cleaved by HDM in a dose-dependent manner (n = 4). Data in B, C, E, and G are mean ± s.e.m. The data were analyzed using student's *t*-test in B, one-way ANOVA followed by Tukey post-hoc analysis in C. ** indicates $P < 0.01$, *** indicates $P < 0.001$ vs. control.

added to each well in triplicate. PBS was used as a control for this experiment. An antibody specific to human BPIFA1 (R&D Systems), diluted 1:5000 with BSA, was used to detect the LPS-bound BPIFA1. Horseradish peroxidase (HRP)-conjugated anti-goat antibody was used as the secondary antibody to detect binding recombinant BPIFA1. Enzyme activity was detected using a TMB Ultra 1-step assay (Pierce Biotechnology) and the reaction was stopped with H_2SO_4 (Thermo Fisher). Absorbance was detected at OD 450 nm in a BioTek spectrophotometer (BioTek).

2.7. Human whole blood incubation

Human whole blood from healthy donors was purchased from a commercial source (VWR) and diluted 1:5 with RPMI 1640 (Thermo Fisher) containing 100 IU penicillin +100 μg streptomycin/ml (Thermo Fisher) in polypropylene reaction vials (BD Biosciences). Incubation with BPIFA1 or BPIFA1 fragments in the presence of LPS was performed for 24 h at 37 °C and 5% CO2 in humidified air; vials were shaken and centrifuged for 2 min at 400 *g*. The cell-free supernatants were stored at −80 °C until cytokine measurement by ELISA.

2.8. ELISA of cytokines

TNFα and IL-6 were determined by ELISA (BioLegend) following the manufacturer's instructions.

2.9. Measurement of intracellular Ca^{2+} activity

Changes in cytosolic Ca^{2+} concentration were measured using 5 μM fluo-4 dye (Thermo Fisher) loaded into Calu-3 cells in the presence of 1 mM probenecid and 1 x powerload (both from Thermo Fisher) for 1 h at 37 °C. Cultures were then rinsed, and media were replaced with a standard Ringer's solution; fluorescence intensities were read every 15 or 30 s using a Tecan Infinite Pro plate reader for fluo-4 (excitation: 494 ± 5 nm; emission: 516 ± 5 nm). A fluorescent baseline was established before the thapsigargin (TG, 5 μM, Thermo Fisher) was added to the wells, and changes in fluorescence were normalized to the baseline (F/F_0). $\Delta F/F_0$ represents average peak fluorescent intensity changes of three independent experiments.

2.10. Statistical analysis

All data are presented as the mean ± SEM for n experiments. Differences between means were tested for statistical significance using paired or unpaired *t*-tests or their non-parametric equivalent as appropriate to the experiment. Differences between groups were judged using ANOVA. From such comparisons, differences yielding $P < 0.05$ or smaller were judged to be significant. GraphPad Prism software was used for statistical analysis.

3. Results

3.1. The reduction of BPIFA1 protein levels is due to HDM protease activities

We previously measured sputum BPIFA1 levels in healthy donors, asthmatic patients, subjects with chronic obstructive pulmonary disease (COPD), and atopic individuals without asthma, with the latter two cohorts serving as disease controls. Immunoblot analyses indicated significantly decreased BPIFA1 protein levels in asthmatic patients' samples compared to the other donors [32]. In the same vein, treatment of healthy HBECs for 24 h apically with 100 μg/ml HDM extract resulted in decreased BPIFA1 protein levels in apical lavages and basal lateral media—but not in the lysates of these cells (Fig. 1A and B). These findings suggest that only secreted BPIFA1 protein is affected by HDM treatment. We then examined BPIFA1 mRNA levels after HDM treatment. Up to 48 h post-treatment, no significant change was observed (Fig. 1C). This evidence suggests that allergens such as HDM that are associated with protease activity may partially contribute to the decrease of BPIFA1 protein levels in the airways of allergic asthma patients. To confirm this speculation, we performed time- and dose-course studies by incubating purified human recombinant BPIFA1 with HDM at the indicated time and dose ranges. As expected, HDM cleaves BPIFA1 in both time- and dose-dependent manners (Fig. 1D–G). The half-life of BPIFA1 in the presence of 100 μg/ml HDM is roughly 0.83 h, whereas the EC_{50} of HDM at 4 h is 32.4 μg/ml. Taken together, these data indicate that reduction of BPIFA1 protein levels in the asthmatic airway is likely due to HDM protease activity and that this proteolytic effect is time- and dose-dependent without changing BPIFA1 mRNA levels.

3.2. HDM-mediated BPIFA1 degradation is temperature-insensitive

Because HDM is a major perennial allergen that causes allergic rhinitis and allergic asthma, we wanted to test whether HDM exerts its function at different temperature conditions. We incubated 10 μg/ml of recombinant BPIFA1 with 0, 30, and 100 μg/ml HDM at 4, 25, and 37 °C for 4 h, respectively. The immunoblot results show that HDM cleaves more than 50% of BPIFA1 at 100 μg/ml regardless of temperature, whereas a lower concentration of HDM requires higher temperature (25 °C and 37 °C) to work (Fig. 4). A study has shown that most US homes have detectable levels of dust mite allergen in a bed; further, levels previously associated with allergic sensitization and asthma are common in US bedrooms [34]. Our data corroborate this finding, suggesting that HDM-mediated BPIFA1 degradation is temperature-insensitive and that, as long as a sufficient amount of HDM is present, protein substrate such as BPIFA1 can be cleaved at low as 4 °C, which likely leads to the perennial allergy problems.

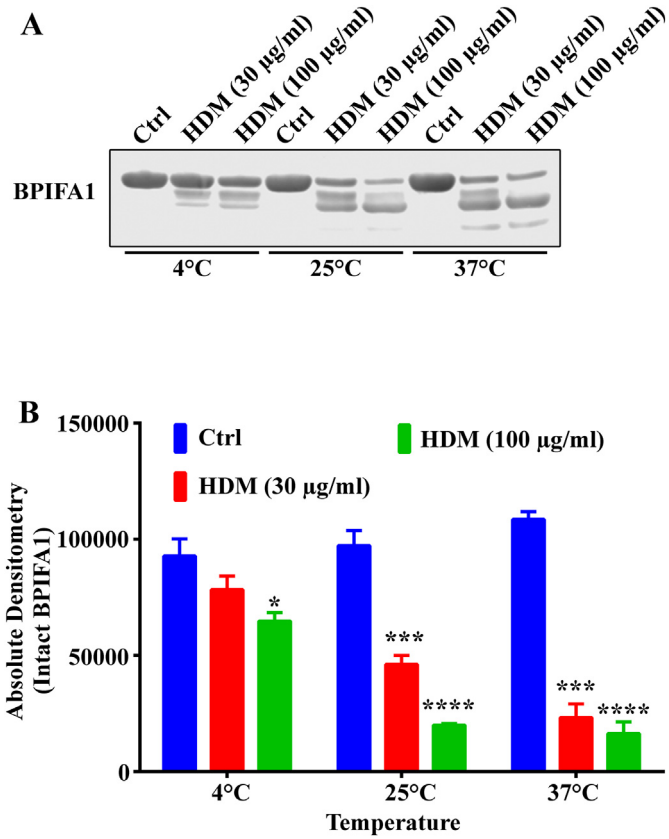

Fig. 2. HDM-mediated BPIFA1 degradation is temperature insensitive. (A) and (B) are representative immunoblot and densitometry showing HDM degrades recombinant BPIFA1 at indicated temperatures (n = 6). Data in B are mean ± s.e.m. The data were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. * indicates $P < 0.05$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$ vs. control.

3.3. HDM-mediated BPIFA1 degradation is blocked by E64D, a pan cysteine protease inhibitor

More than 20 groups of HDM allergens have been identified to date, at least four of which are known to express catalytically competent proteinase activity [13,35,36]. These components exert profound effects on airways, which promotes allergic sensitization [13,37]. Identifying key components in HDM that are responsible for BPIFA1 degradation could enable us to further elucidate the potent allergenicity of HDM and inform specific treatment strategies. Based on the known protease components in HDM and their properties, we carried out a "short gun" protease inhibitor screening using protease inhibitors against a broad range of important proteases of well-defined activity. We incubated 10 μg/ml of recombinant BPIFA1 and HDM with or without PIC (1×), E64D, PMSF, and aprotinin respectively, at 37 °C for 4 h, and subjected the products to immunoblot. PIC, which is a broad-spectrum protease inhibitor cocktail, partially inhibited HDM-mediated BPIFA1 degradation at an arbitrary 1× working concentration. A similar effect was seen with E64D (5 μM), a pan cysteine protease inhibitor, whereas serine protease inhibitor PMSF (5 μM) and trypsin inhibitor aprotinin (5 μM) both failed to prevent HDM mediated BPIFA1 degradation (Fig. 3A and B). These data point to possible component(s) in HDM that could underly BPIFA1 degradation to a group of cysteine proteases. To further confirm the specificity of cysteine protease inhibitor E64D for blocking HDM-mediated BPIFA1 degradation, we performed an E64D dose-course experiment with a fixed amount of recombinant BPIFA1 protein (10 μg/ml) and HDM (100 μg/ml). The inhibitory effect was detectable at a concentration of 3 μM, with nearly complete block of BPIFA1 occurring at 30 μM (Fig. 3C and D). Therefore, these results support the conclusion that BPIFA1 proteolysis can be attributed to an HDM-associated cysteine protease(s).

3.4. Der p1 is the culprit responsible for BPIFA1 degradation

The various HDM allergens of different mite species have been classified according to structurally similar homologs [13]. Many of these groups in HDM have been identified as having protease activity (groups 1, 3, 6, and 9). Group 1 HDM allergens exhibit cysteine protease activity, and are responsible for more than 50% of sensitization of HDM-allergic subjects [38]. Because our findings suggested that HDM-mediated BPIFA1 degradation is blocked by a pan cysteine protease inhibitor, E64D (Fig. 3), we further examined which group 1 allergen is responsible for BPIFA1 degradation. 10 μg/ml of recombinant BPIFA1 protein was incubated with or without 100 μg/ml of HDM, Der p1, and Der f1 for 24 h, respectively. Immunoblot results showed that both HDM and Der p1 cleaved BPIFA1 completely, whereas Der f1 had no effect on BPIFA1 at all (Fig. 4A and B). Moreover, the cleavage pattern of Der p1 also resembled that of HDM, suggesting that Der p1 is the active

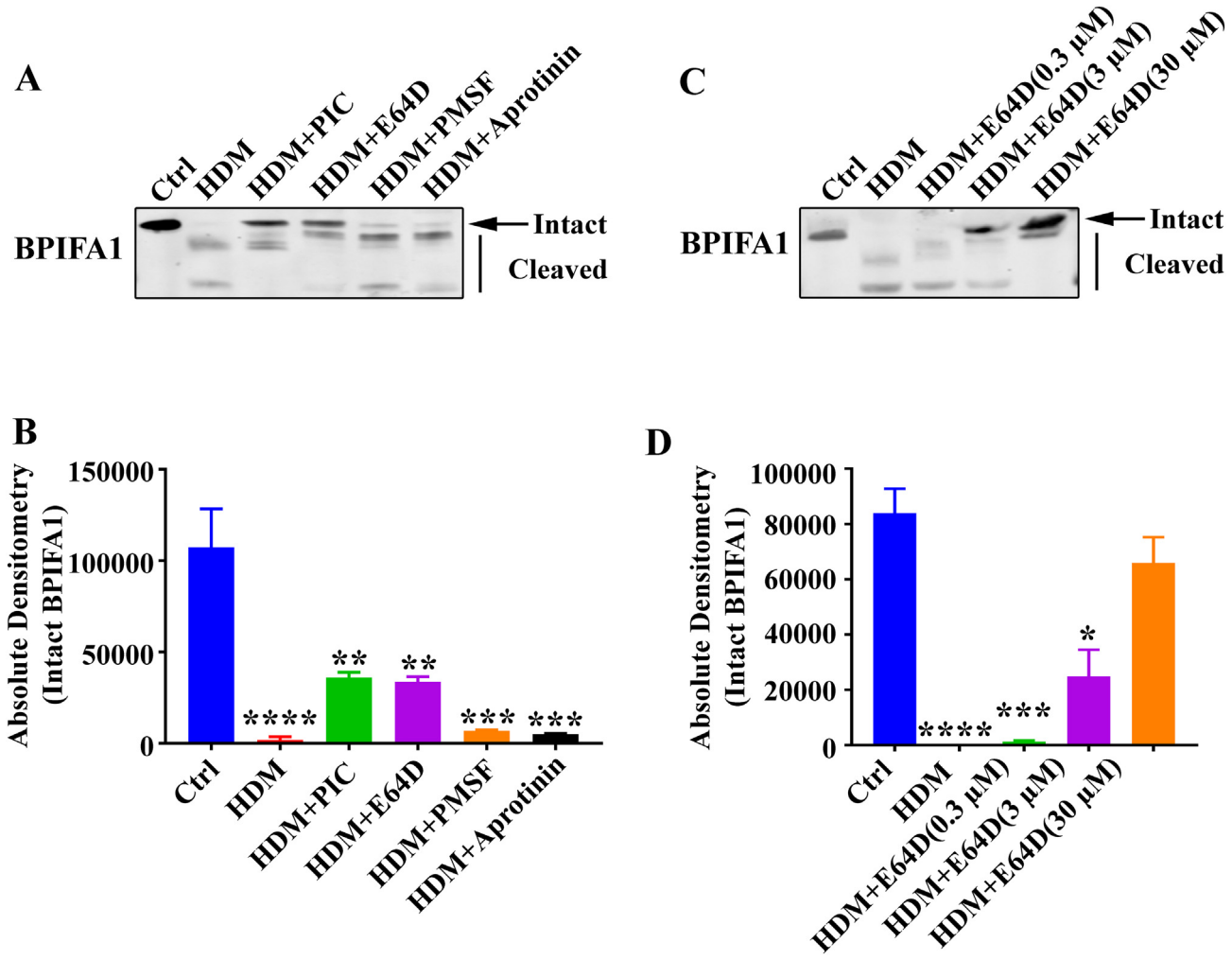

Fig. 3. HDM-mediated BPIFA1 degradation is blocked by E64D, a pan-cysteine protease inhibitor. (A) and (B) are representative immunoblot and densitometry showing the effects of different types of protease inhibitors on blocking HDM -mediated BPIFA1 degradation (n = 6). (C) and (D) are representative immunoblots and densitometry showing cotreatment with recombinant BPIFA1 with an increasing dose of E64D sufficiently blocked HDM-mediated degradation (n = 6). Data in B and D are mean ± s.e.m., and were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$ vs. control.

component in HDM that degrades BPIFA1. To further confirm the specificity of Der p1 cleavage, we again performed an E64D dose-course experiment with a fixed amount of recombinant BPIFA1 protein (10 μg/ml) and Der p1 (100 μg/ml). E64D showed a dose-dependent effect on blocking Der p1-mediated BPIFA1 degradation, with the lowest effective dose at 0.3 μM (Fig. 4C and D). Taken together, these results suggest that Der p1 is the protease component of HDM that is responsible for BPIFA1 degradation.

3.5. HDM degrades BPIFA1 in vivo

To determine whether BPIFA1 cleavage occurs in vivo, we next tested whether BPIFA1 is cleaved in mouse BALF when exposed to an increased dose of HDM. We instilled 1 μg, 10 μg, and 50 μg HDM into mice airways respectively. 24 h after installation, BALF was collected and subject to immunoblot. Similar to what we observed in our in vitro studies, HDM decreased the amount of full-length BPIFA1 in a dose-dependent manner (Fig. 5A, upper panel; Fig. 5B), without significant impact on total protein amount (Fig. 5A, lower panel). However, unlike human BPIFA1, we did not detect lower molecular weight fragments in these mice BALF samples. We speculate that this could be due to the sequence difference between human and mouse BPIFA1 protein. Previous research showed that human and mouse BPIFA1 proteins share ~72% identity [39]. Mouse BPIFA1 has an extra 24 amino acid sequence inserted in the corresponding N-terminal "S18" region in human protein [27], with other gaps and amino acid changes spread along the sequence. These residue differences may affect BPIFA1 being recognized and bound by HDM and therefore lead to a different cleavage pattern.

A decrease in full-length BPIFA1 protein in vivo can be attributed to transcriptional downregulation rather than post-translational degradation. To exclude the interference of transcriptional regulation, we adopted an ex vivo method using cell-free BALF from mice and incubate it with an increased amount of HDM extract for 24 h at 37 °C. Consistent with the results from in vivo studies, increasing doses of HDM resulted in a decrease trending of full-length BPIFA1 (Fig. S2), with no small fragments. Although this does not rule out transcriptional regulation of BPIFA1 by HDM, it indicates that HDM-mediated protein degradation also plays a role in the downregulation of BPIFA1.

3.6. HDM cleavage impairs BPIFA's ability to bind E. coli LPS and suppress Ca^{2+} influx, resulting in enhanced release of pro-inflammatory cytokines from human whole blood cells

To assess the effect of HDM cleavage on BPIFA1, we first purified HDM-cleaved BPIFA1 using neutravidin and biotin-labeled HDM. Immunoblot results showed that biotin-HDM cleaves BPIFA1 in the same pattern as untagged HDM, and total BPIFA1 or BPIFA1 fragment amounts did not change after two rounds of purification (Fig. S1, upper panel), whereas biotin-HDM was retained on neutravidin beads after the first round of incubation (Fig. S1, lower panel). We then examined intact and cleaved BPIFA1 for their ability to bind to LPS from *E. coli*. and found that when cleaved by HDM, BPIFA1 binding to LPS was significantly reduced (Fig. 6A). Since previous research shows that BPIFA1

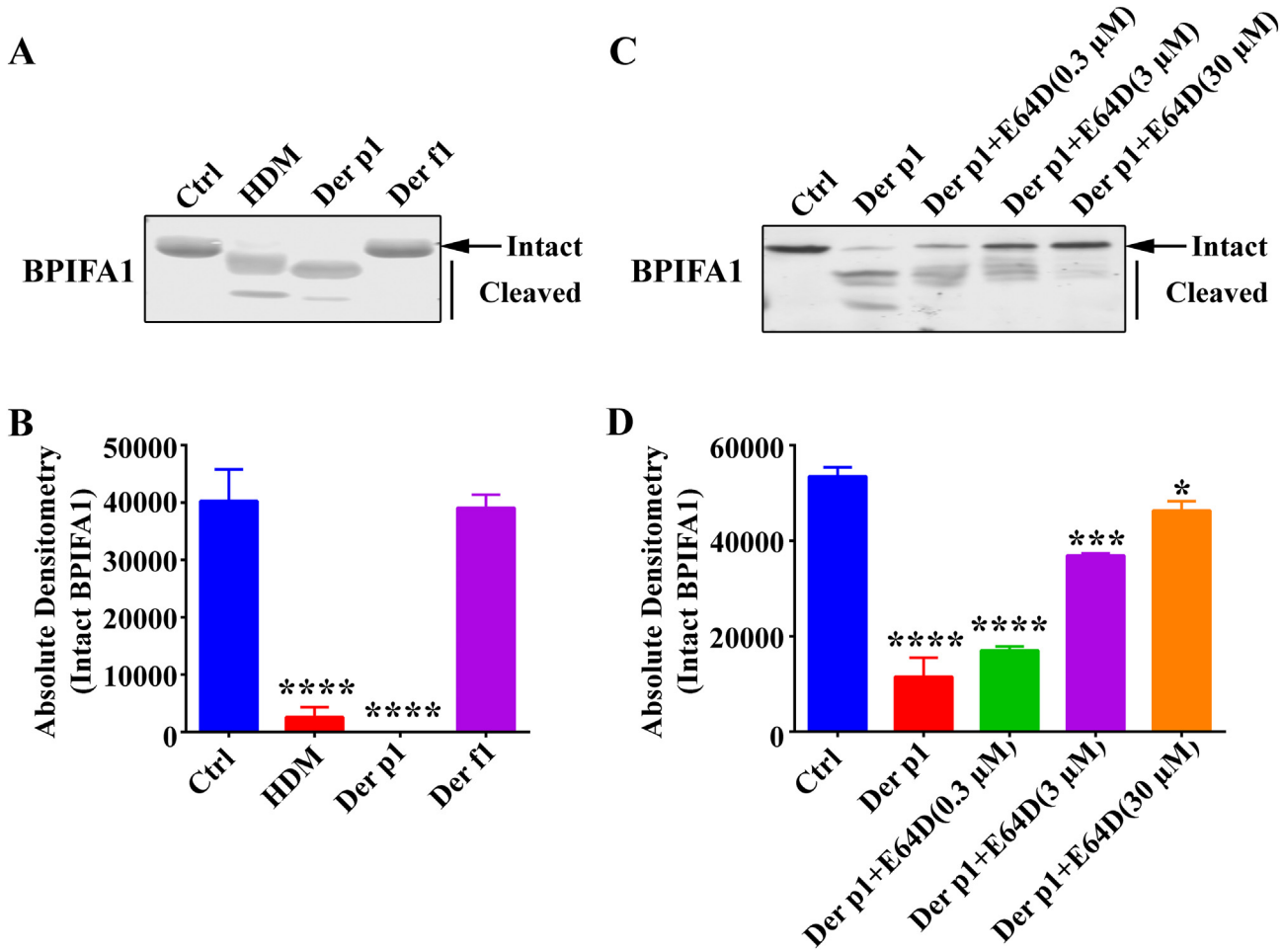

Fig. 4. Der p1 is the culprit in BPIFA1 degradation. (A) and (B) are representative immunoblot and densitometry showing cysteine protease Der p1, but not Der f1, degrades recombinant BPIFA1 (n = 6). (C) and (D) are representative immunoblots and densitometry showing Der p1-mediated BPIFA1 degradation is blocked by E64D (n = 5). Data in B and D are mean ± s.e.m., and were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. * indicates $P < 0.05$, *** indicates $P < 0.001$ and **** indicates $P < 0.0001$ vs. control.

binds to the α4 region of LPS [24], we conclude that HDM impairs this ability by cutting into the α4 helix of BPIFA1.

We also evaluated whether HDM cleavage affects BPIFA1's ability to regulate Ca^{2+} influx, in light of our previous finding that BPIFA1 inhibits Ca^{2+} influx in airway smooth cells by binding to Orai1 channel [32]. When incubated with Calu-3 cells, intact BPAIF1 protein decreased the TG-induced cytosolic Ca^{2+} increase by half, whereas HDM-cleaved BPIFA1 completely lost this inhibitory effect (Fig. 6B and S2), suggesting that HDM also cleaves BPIFA1 in its c-terminal α6 region.

A method that is widely used to study the activation of the innate immune system is in vitro stimulation of human whole blood using LPS. LPS-stimulated whole blood generally exhibits massive activation of a battery of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-10, and interferon (IFN)-γ; it is therefore considered a credible tool to study sepsis and endotoxemia ex vivo [40]. Because our LPS binding assay suggests that HDM cleavage impairs BPIFA1's ability to bind to LPS, we incubated LPS-stimulated human whole blood in the presence of intact or HDM-cleaved BPIFA1 to investigate its effects on cytokine secretion pattern. As shown in Fig. 6C and D, 100 ng/ml LPS induced surges of TNFα and IL-6 secretion, which was inhibited by co-treating human whole blood with intact BPIFA1. This inhibitory effect was partially (TNFα) or completely (IL-6) abolished with HDM-cleaved BPIFA1, suggesting that when cleaved by HDM, BPIFA1 lost its function to modulate the innate immune response.

4. Discussion

Allergens associated with HDM (*D. pteronyssinus* and *D. farinae*) are a major cause of allergic diseases worldwide. Prevalence data for HDM allergen sensitization as many as 130 million people in the general population worldwide to roughly 50% among asthmatic patients [9]. Given its high prevalence, it is necessary to understand the mechanisms involved in orchestrating the allergic response. Research from the past two decades of studying HDM and its components has suggested that allergenic effects in HDM allergy are triggered and perpetuated through two main routes: the IgE-dependent allergic response and the innate immune system [41,42]. The protease activity of some allergens has been shown to primarily contribute to their allergenicity via the IgE-dependent route [43]. This is best exemplified by the major HDM allergen Der p 1, which elicits IgE responses in more than 80% of patients who are sensitive to *D. pteronyssinus* and is the most immunodominant allergen involved in the expression of IgE-mediated dust mite hypersensitivity [44]. Some of the substrates of Der p1 include human cell surface molecules such as the low-affinity IgE receptor (CD23/FcεRII), the loss of which from IgE-secreting B cells is thought to promote and enhance IgE immune responses by ablating an important feedback inhibitory mechanism that normally limits IgE synthesis [45,46]. CD40 on dendritic cells (DCs) is also cleaved by Der p1, resulting in decreased IL-12 production, thus making DCs less responsive to stimulation through the CD40L-CD40 pathway and inducing generation of DCs that favor a T_H2 response [47].

Despite a growing body of evidence concerning possible HDM/Der p1 substrates, only a handful has been identified and characterized. Besides the previously discussed CD23 and CD40, studies performed with protein substrates such as α1-antitrypsin [48] and insulin β-chain [49] have shown that Der p 1 has a preference for small hydrophobic residues (Val/Ala) in the S_2 position, charged residues (Glu/Arg) in the S_1 position, and small hydrophilic/hydrophobic residues (Ser/Ala) in the S_1' position. Emerging in silico methods—in particular, a novel bioinformatics tool called prediction of protease specificity (PoPS; http://pops.csse.monash.edu.au/home.html)—have helped identify two salient molecules of immunological interest: DC-SIGN (CD209) and DC-SIGNR (CD299: L-SIGN) [50]. Otherwise, the lack of effective ways to screen for or predict potential protease substrates remains a bottleneck to our understanding of HDM/Der p1 allergenicity. To the best of our knowledge, the ability of HDM/Der p1 to degrade BPIFA1 has not been previously reported. However, neutrophil elastase, which is abundant in the lungs of cystic fibrosis (CF) patients, has been shown to degrade BPIFA1 [51]. This degradation contributes to increased ENaC activity and ASL dehydration in CF HBECs [19]. Our findings add new evidence that the substrates of HDM/Der p1 proteases are not limited to cell surface molecules. Secretory proteins, such as BPIFA1, are also subject to proteolytic effects, which implies potential unidentified candidate proteins, as well as underlying regulatory mechanisms.

The data presented in this study demonstrate that BPIFA1 is subject to proteolytic degradation by HDM, leading to its loss of function in modulating Ca^{2+} influx, *E.coli* LPS binding and the *E.coli* LPS-induced TNFα and IL-6 production in whole human blood cells. Further, our data suggest that HDM degrades BPIFA1 protein at a broad range of

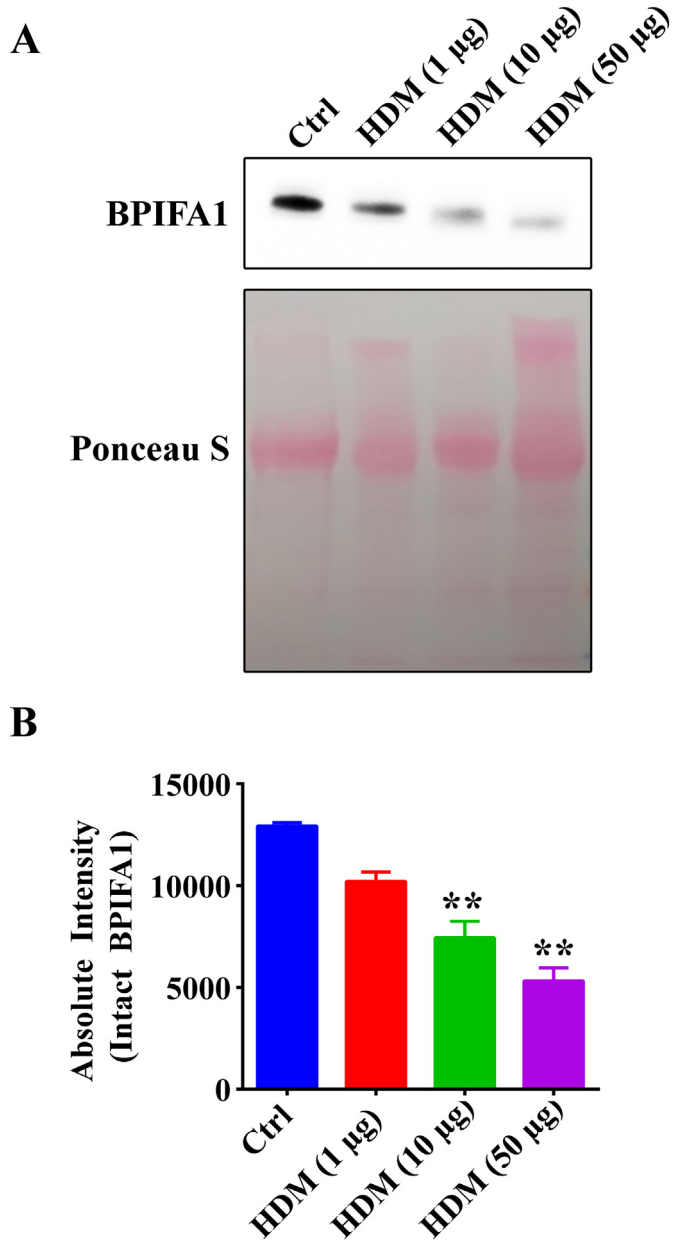

Fig. 5. HDM degrades BPIFA1 in vivo. (A) and (B) are representative immunoblot (A, upper panel) and Ponceau S. stain (lower panel) for total protein and densitometry; (B) shows that HDM degrades BPIFA1 in mouse BALF (n = 6 mice/group). Data in B are mean ± s.e.m., and were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. ** indicates $P < 0.01$ vs. control.

doses and various ambient temperatures (Figs. 1 and 2), which reflects the diverse HDM amount and environmental conditions that people are exposed to daily basis. Although it is experimentally challenging to reproduce these variable exposure conditions to reliably predict the quantity of HDM or associated components that end up in the airway, evidence shows that HDM is found in the sputum and even deep lungs [52], which supports our hypothesis that HDM directly interacts and degrades BPIFA1. We have also demonstrated that the main component responsible for HDM-mediated BPIFA1 degradation is Der p1, a cysteine protease (Figs. 3 and 4). Future studies should to be carried out to identify the cleavage sites of HDM/Der p1 within BPIFA1, thereby enabling the generation of HDM/Der p1-resistant mutants. *Re*-introducing these mutant proteins into patients' airways presents a possible avenue for a novel asthma treatment strategy. Functional studies using full-length and cleaved BIPFA1 protein indicate that HDM cleavage impairs several functions of BPIFA1, including LPS binding, Ca^{2+} regulation, and cytokine secretion (Fig. 6). These findings suggest the existence of multiple HDM/Der p1 cleavage sites throughout the BIPFA1 protein; HDM/Der p1 will "hit" all of these sites given sufficient time and dosage. It will be critical to establish the sequence of these events so that we can gain more knowledge on the HDM-BPIFA1 dynamic and identify the most "vulnerable" part of BPIFA1 protein. Another question that stems from these functional studies of BPIFA1 is whether the LPS binding and cytokine modulating are essentially the same function. It is commonly believed that apically secreted BPIFA1 normally 'mops up' excess bacterial LPS; following allergen exposure, reduced BPIFA1 levels increases LPS, leading to exaggerated inflammatory cytokine secretion and allergic response [31]. In our study, when incubated with whole human blood in the absence of *E.coli* LPS, full-length BPIFA1 did not significantly affect either TNFα or IL-6 secretion. However, in the presence of LPS, full-length—but not HDM-cleaved—BPIFA1 effectively inhibited the secretion of these cytokines (Fig. 6C and D). We can only speculate from these data that HDM simultaneously impairs the domains in BPIFA1 that are responsible for binding to LPS and regulating cytokine secretion. To further delineate the relationship between the structure and function of BPIFA1, we will need to test whether $BPIFA1^{\Delta\alpha4}$ —a BPIFA1 mutant that lacks the LPS binding α4 region—exerts the same immune-modulating functions as the full-length protein, under both basal and LPS challenged conditions.

The findings of this study provide substantial in vitro and ex vivo evidence that HDM—especially its cysteine protease component Der p1—cleaves BPIFA1 and thereby negatively impacts a host of BPIFA1-mediated antimicrobial and anti-inflammatory functions. We also detected a dose-dependent decrease of full-length BPIFA1 protein in the presence of HDM in mouse BALF without significant change in total protein amount, indicating the substrate specificity of HDM. There was a discrepancy in HDM cleavage patterns between human and mouse BPIFA1 proteins, which we attribute to the amino acid sequence differences between these two species and possible HDM preference for amino acid residues around cutting sites. Our data will expand the knowledge regarding

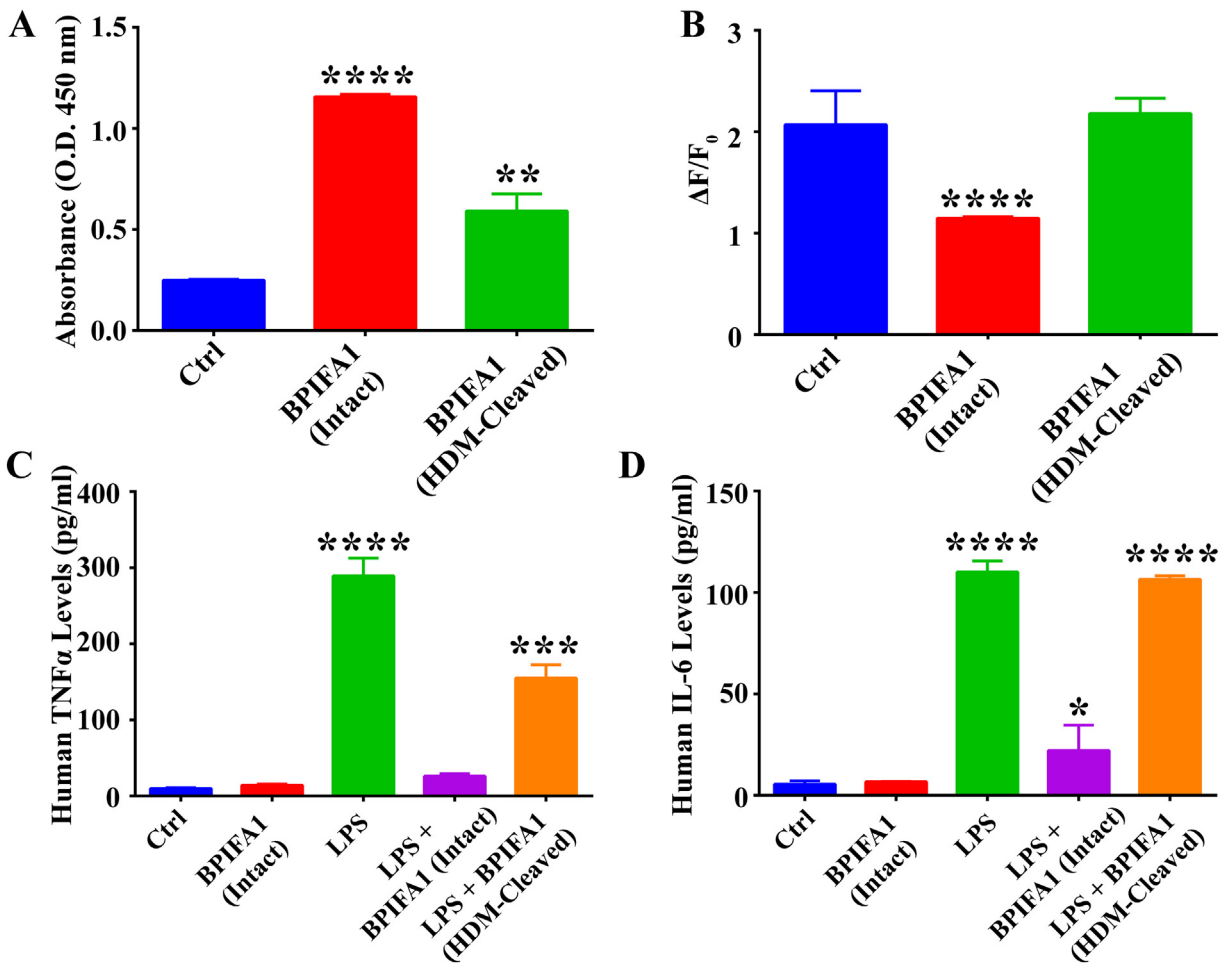

Fig. 6. HDM cleavage impairs BPIFA1's innate immune modulator function. (A) HDM cleavage reduces BPIFA1's ability to bind LPS. Binding of BPIFA1 to *E. coli* LPS as assessed by an ELISA (n = 4). (B) HDM cleavage impairs BPIFA1's ability to inhibit TG-induced cytosolic Ca^{2+} increases. Calu-3 cells were incubated with intact BPIFA1 or HDM-cleaved BPIFA1 proteins, and changes in the fluo-4 emission ratio over time were recorded. $\Delta F/F_0$ represents the average peak fluorescence intensity increase after TG addition (n = 5). (C) and (D) are ELISA readouts of TNFα and IL-6 produced by human whole blood cells in the presence or absence of BPIFA1 (n = 5). Data are mean ± s.e.m., and were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. * indicates $P < 0.05$, ** indicates $P < 0.01$, and **** indicates $P < 0.0001$ vs. control.

environmental allergen-induced airway diseases and potentially lead to novel protein/peptide-based biologics that are resistant to the highly proteolytic environment typical of many airway diseases.

Our finding that BPIFA1 reduces LPS induced IL-6 and TNFα production are timely in light of the current pandemic, since the fatal outcome observed with coronavirus 2019 (COVID-19) infection often results from diffuse alveolar damage, desquamation of pneumocytes, and hyaline membrane formation, indicating acute respiratory distress syndrome (ARDS) and multi-organ failure—both of which are associated with the hyperproduction of cytokines, also known as a cytokine storm or cytokine release syndrome. Clinical reports show that both mild and severe forms of disease result in changes in circulating leukocyte subsets and cytokine secretion, particularly IL-6, IL-1β, IL-10, TNF, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-induced protein 10 (IP-10), IL-17, Chemokine (C—C motif) ligand 7 (CCL7), and interleukin-1 receptor antagonist (IL-1ra) [53] [54]. Therefore, anti-cytokine interventions, such as blocking IL-6 production/secretion, appear to be potentially efficacious and proactive strategies against the adverse clinical outcomes caused by COVID-19. Our data showed that BPIFA1 reduces LPS induced IL-6 and TNFα production in whole human blood cells and that this inhibitory effect was abolished by HDM protease (Fig. 6C and D). Such results raise more questions for our future studies: First and foremost, can we develop BPIFA1 and its derived peptides into biologics to reduce IL-6-initiated inflammation in COVID-19 infected lungs and thereby prevent further damage? Based on current results, we speculate that peptides will be more preferable since they will be less susceptible to protease degradation, which is commonly seen in highly inflamed airways [55]. Secondly, since BPIFA1 (and other lung surfactant proteins) is subject to protease degradation in diseased airways, is there a correlation between the severity of the disease/infection and residual BPIFA1 amount? In other words, what is the interaction between COVID-19, BPIFA1, environmental proteases, and host immune response? Answering these questions will provide comprehensive information on how BPIFA1 and its derivative peptides could potentially be used for treating COVID-19-induced inflammation. These answers could also potentially open up a new avenue of research into airway inflammation, with a focus on developing alternative anti-inflammatory drugs based on proteins composing our immune defense mechanisms.

CRediT authorship contribution statement

Rui Zhang: performed experiments and analyzed the data. Jessika Trower: performed experiments and analyzed data. Tongde Wu: designed and performed experiments, analyzed data and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.08.214.

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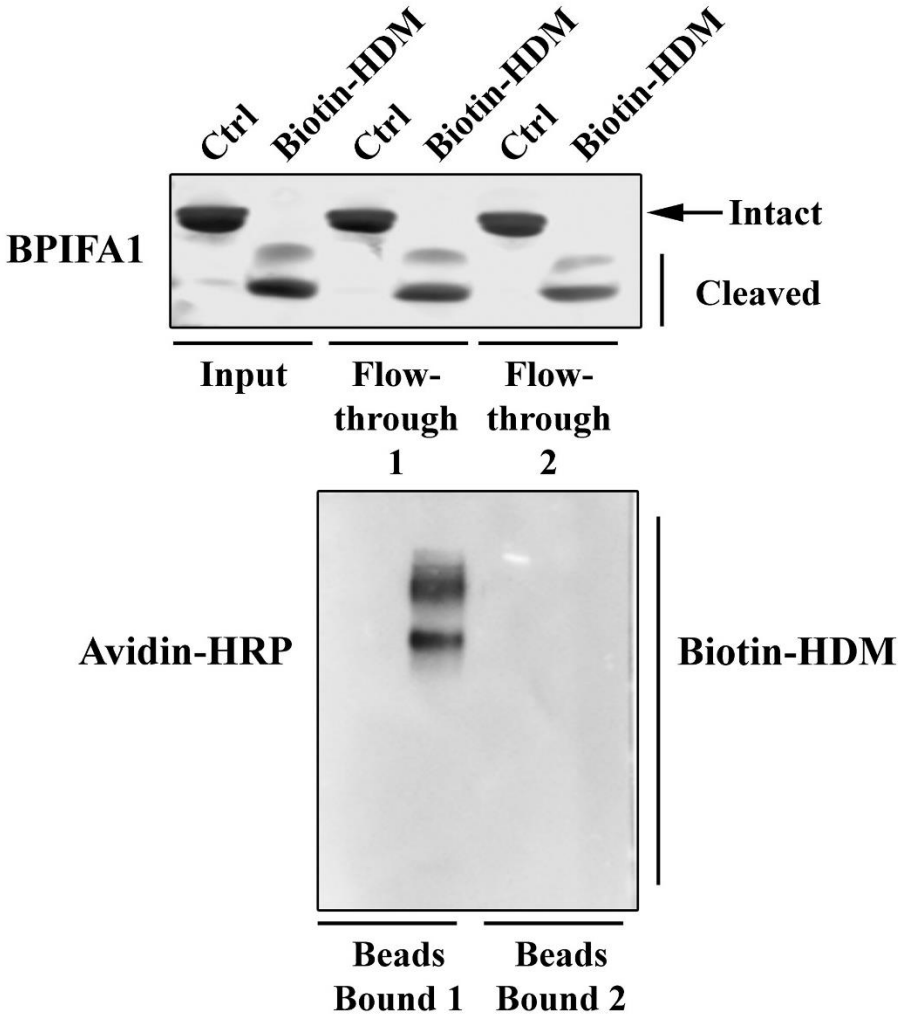
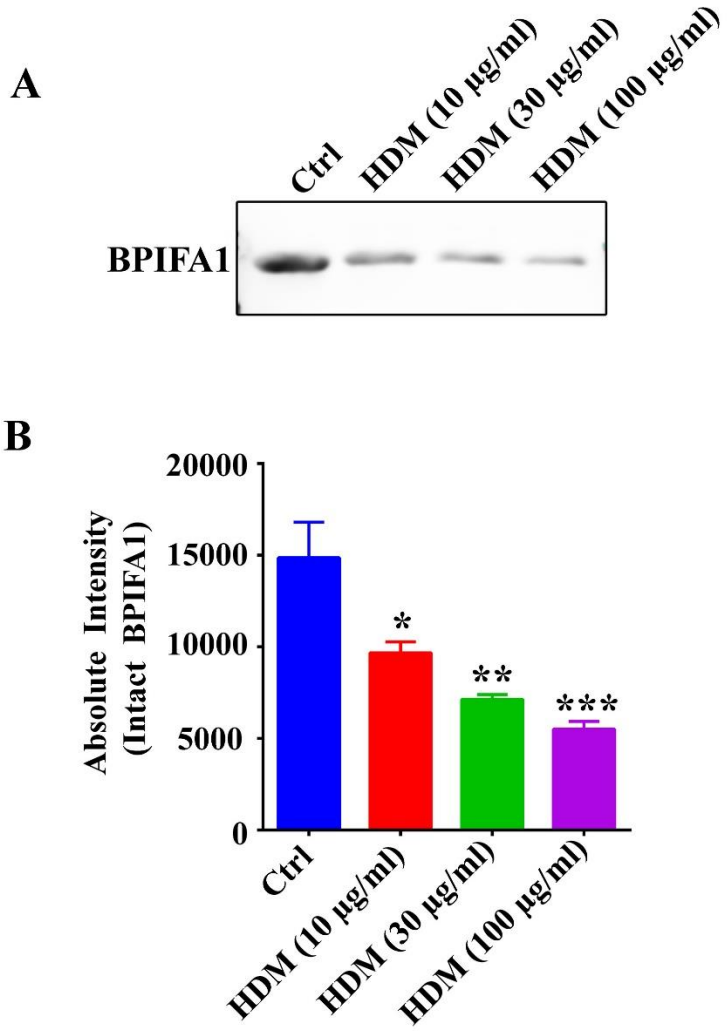
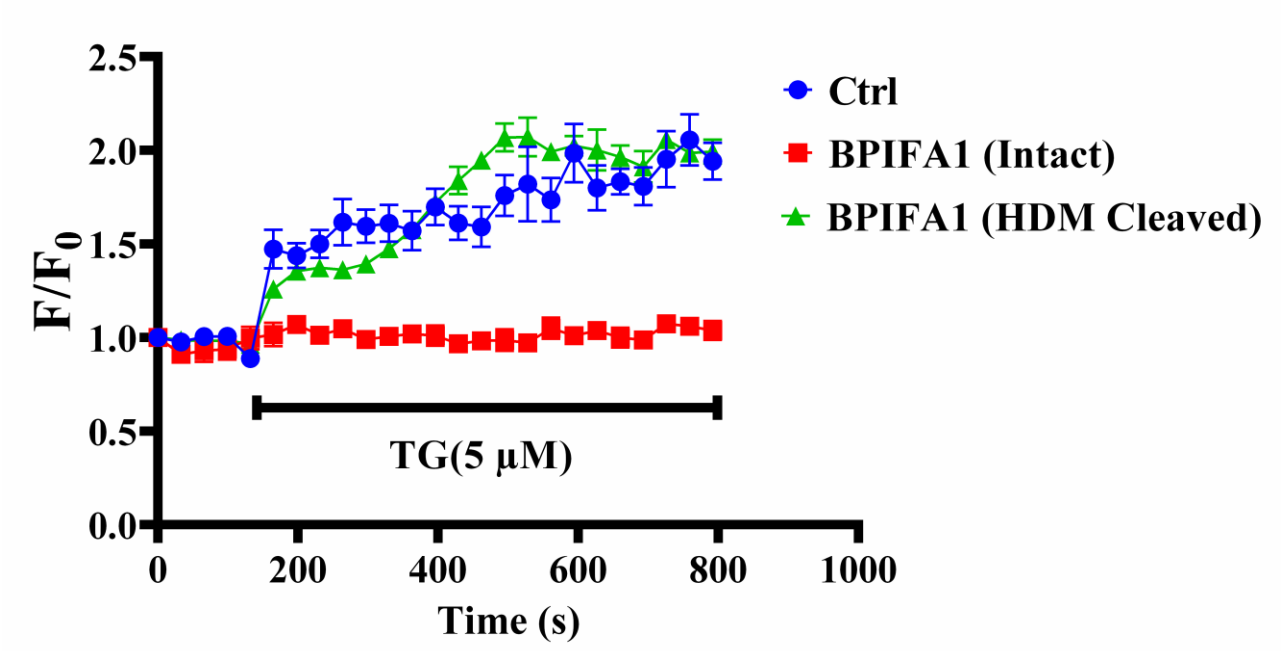


Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.

Figure Legends

Supplementary Figure 1. Immunoblots showing intact and HDM-cleaved BPIFA1 after neutravidin pulldown. Upper panel: full-length and cleaved bands of BPIFA1 was detected in the flow-through of the pulldown. Lower panel: biotin-HDM was bound to the neutravidin beads and was eliminated in the first round of purification.

Supplementary Figure 2. HDM degrades BPIFA1 in mouse BALF. (A) and (B) are representative immunoblot and densitometry showing a decrease of full-length BPIFA1 protein in mouse BALF instilled ex vivo with indicated doses of HDM extract at 37 °C for 24 h. Data in B are mean±s.e.m. (n = 6)., and were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. * indicates $P < 0.05$, ** indicates $P<0.01$, and *** indicates $P < 0.001$ vs. control.

Supplementary Figure 3. Average traces of fluo-4 fluorescence as an indicator of Calu-3 cells were incubated with intact BPIFA1 or HDM-cleaved BPIFA1 proteins, and changes in the fluo-4 emission ratio over time were recorded. The average peak fluorescent intensity increase after TG addition (n=5) was shown in Fig. 6B.