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Dust mite-derived Der f 3 activates a pro-inflammatory program in airway epithelial cells via PAR-1 and PAR-2

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ABSTRACT

Protease activity of allergens has been suggested to be involved in the pathogenesis of allergic diseases. The major allergen Der f 3 from *Dermatophagoides farinae* harbors serine protease activity, but its immunopathogenesis remains unclear. This study aims to explore the effect of Der f 3 on the airway epithelial barrier and on the molecular pathways by which Der f 3 induces inflammation. RNA-seq was performed to identify differentially expressed genes in bronchial airway epithelial cells (AEC) between native Der f 3 and heat-inactivated (H) Der f 3, coupled with real-time PCR (RT-PCR) and ELISA for validation. Unlike other protease allergens such as that induce Th2-promoting alarmins (IL-25, IL-33, TSLP) in AECs, Der f 3 induced pro-inflammatory cytokines and chemokines including IL-6, IL-8 and GM-CSF, which are known to promote Th17 response. These pro-inflammatory mediators were induced by Der f 3 via the MAPK and NF-κB pathways as well as the store-operated calcium signaling. Gene silencing with small interfering RNA in A549 and BEAS-2B cells indicated that activation of AECs by Der f 3 was mainly dependent on protease-activated receptor 2 (PAR-2), while PAR-1 was also required for the full activation of AECs. Double knock-down of PAR-1 and PAR-2 largely impaired Der f 3-inducecd IL-8 production and subsequent signaling pathways. Our data suggest that Der f 3 induces pro-inflammatory mediators in human epithelial cell lines via the PARs-MAPK-NF-κB axis. Our results provide a molecular mechanism by which Der f 3 may trigger the Th17-skewed allergic response toward house dust mites.

1. Introduction

Certain aeroallergens from house dust mites (HDM), fungi, cockroach and pollen are identified as cysteine, serine or aspartic proteases (Gunawan et al., 2008; Shen et al., 2007; Takai et al., 2005; Wünschmann et al., 2005). Several lines of evidence indicate that these proteases facilitate allergen sensitization (Reed and Kita, 2004). Deletion of a single protease allergen in *Aspergillus fumigatus* caused a decrease in airway hyperreactivity and allergic immune response in a murine inhalation model (Namvar et al., 2015). Removal of proteases from the extracts of cockroach (Sudha et al., 2008) or fungus (Kukreja et al., 2008) was reported to decrease airway inflammation and airway hyperresponsiveness in mouse models of allergic asthma. Several mechanisms by which the protease activity of these allergens promotes sensitization have been proposed (Wills-Karp et al., 2010). First, by digesting tight junction proteins of airway epithelial cells (AECs), protease allergens disrupt epithelial barrier and thus increase permeability, allowing entry of various allergens and associated immune activators into the underneath mucosal tissues. Dendritic cells then are activated, take up antigens, and present antigens to T cells (Cousins, 2017). Second, protease allergens can directly activate AECs to induce inflammatory cytokines, chemokines, and other immune modulators. Through activation of protease-activated receptors (PARs), they can induce alarmins such as TSLP, IL-25, and IL-33 that promote differentiation of naïve $CD4^+$ T cell into Th2 effector cells (Kouzaki et al., 2009, 2013; Snelgrove et al., 2014). Third, allergen-derived proteases can also exert direct effects on adaptive immune responses. The cysteine protease allergen papain directly activates naïve T cells through

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PAR-2 to initiate a chemokine/cytokine program and propel Th2 immunity (Liang et al., 2012).

PARs are proteins located on transmembrane cell surfaces and belong to the G protein-coupled receptor (GPCR) family. Four members (PAR-1, PAR-2, PAR-3, and PAR-4) have been described (Hollenberg and Compton, 2002). All four PARs were expressed in human AECs (Asokananthan et al., 2002) and documented contributing to the inflammatory processes (Atzori et al., 2009; Moraes et al., 2008; Su et al., 2005). PARs can be activated by its irreversible cleavage at the conserved sites mainly by serine proteases. These proteases cleave PARs at the extracellular N-terminal domains and expose tethered ligands that bind intramolecularly to trigger multiple GPCR-dependent and -independent signaling pathways. However, a growing number of proteases have been identified to cleave PARs at divergent sites to activate distinct patterns of receptor signaling and trafficking, referred as "biased signaling of PARs" (Zhao et al., 2014). Through biased signaling, PARs may be activated by a diverse array of proteases induced in different patho-physiological conditions.

HDM (e.g., *Dermatophagoides sp.*) are the most common aeroallergens, affecting over 15–20% people in industrialized countries (Zock et al., 2006) and up to 90% of atopic patients in Asia (Tham et al., 2016). *D. farinae* and *D. pteronyssinus* are the major sources of HDM allergens associated with allergic diseases such as asthma, rhinitis and atopic dermatitis. It is debatable how Der p 1 influences T cell differentiation. Some reported that Der p 1 via its protease actitivy promotes both Th1 and Th2 responses (Kikuchi et al., 2006), while others showed that it promotes Th2 response by suppressing the Th1-inducing cytokine IL-12 production by dendritic cells (Ghaemmaghami et al., 2002), decreasing the Th1-cytokine response through cleavage of CD23 on B cells (Hewitt et al., 1995) and CD25 on T cells (Schulz et al., 1998). The cysteine protease activity of Der p 1 (Takai et al., 2005) reduced the barrier function of the skin (Nakamura et al., 2006) and disrupted airway epithelium by digesting tight junction proteins (Wan et al., 1999). Der p 1 also induced in AECs, production of pro-inflammatory cytokines and chemokines such as IL-6 and IL-8 via its protease activity (Shi et al., 2010). The serine protease activity of the cockroach allergen Per a 10 (Kale et al., 2017) and the dust mite allergen Der p 9 (King et al., 1998) induced production of IL-6 and GM-CSF in AECs.

Der f 3 from *D. farinae* (Heymann et al., 1989) is a group III allergen which shares 80% homology with Der p 3, a trypsin-like enzyme (Stewart et al., 1992). Group III allergens play a significant role in allergic disease through their serine protease activity (Cheong et al., 2003). However, unlike group I allergens with the cysteine protease activity, our knowledge on these serine proteases including Der f 3 is relatively limited.

Here, we report the effects of Der f 3 on human bronchial epithelial cells (i.e., airway epithelial cells, AECs) at the cellular and molecular levels. We illustrate that (i) Der f 3 disrupted the barrier formed by AECs and potentially activated several intracellular signaling cascades including NF-κB, MAK kinases, and calcium mobilization; (ii) Through these pathways, Der f 3 induced pro-inflammatory cytokines and chemokines, which are known to promote the Th17 response such as IL-6, GM-CSF, IL-8, and IL-1β; (iii) However, Der f 3 did not induce alarmins, the promotor of the Th2 response such as IL-33, IL-25, and TSLP at either the mRNA or protein level; and (iv) Activation of AECs by Der f 3 was mainly dependent on PAR-2, while PAR-1 was also required for the full activation of AECs. These results provide evidence that Der f 3 directly activates AECs, which likely triggers the *in vivo* immune response against HDM.

2. Materials and methods

2.1. Reagents

The human PAR-2 agonist peptide (AP) SLIGKVD-NH2 was purchased from Abcam (Cambridge, UK). *E. coli* lipopolysaccharide (LPS) was from Sigma (St. Louis, MO, USA). DMEM, Trypsin-EDTA and buffers were obtained from Gibco (Carlsbad, CA, USA). Protease inhibitors (AEBSF, a serine protease inhibitor, E64, a cysteine protease inhibitor, EDTA, a metalloprotease inhibitor) were purchased from Sigma (St. Louis, Mo., USA).

The following antibodies from Cell Signaling Technology (Beverly, MA) were used: rabbit anti-human phospho-ERK1/2 antibody, rabbit anti-human phospho-JNK antibody, rabbit anti-human phospho-p38 MAPK antibody, mouse anti-human IκBα antibody, rabbit anti-human NFAT-1 and mouse anti-human GAPDH antibody.

2.2. Recombinant Der f 3 expression and refolding

A full-length Der f 3 cDNA was cloned into pET44a through a PCR-based cloning strategy. Primers DF3F and DF3R, and *Dermatophagoides farinae* cDNA library were used. A Thr27Arg mutation was generated as reported (Nishiyama et al., 1995) in order to facilitate auto-maturation of pro-Der f 3. We removed the signal peptide but added Strep-tag II at both the N- and C-terminus by PCR (primers: P4-F and Strep-R), and cloned it into pET44a at *Nde* I/*Xho* I sites. Amplification conditions were 1 cycle of 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 40 s; and 1 cycle of 72 °C for 7 min. The PCR primers were listed in Table 1. We sequenced the construct and confirmed that the cloned cDNA matched the published Der f 3 protein sequence in the GenBank (UniProtKB P49275) except for the Thr27Arg mutation we created.

Recombinant Der f 3 protein (here after Der f 3) was induced using 0.1 mM isopropyl-D-thiogalactoside (Sigma, MO, USA) in *E. coli* BL21 (Novagen, Germany). *E. coli* cells were harvested by centrifugation at 10,000 g for 30 min, resuspended in the buffer containing 100 mM Tris-HCl and 10 mM EDTA (pH 9.0) and sonicated. Der f 3 was found in inclusion bodies, which was precipitated by centrifugation at 10,000 g for 10 min (Cui et al., 2009). The inclusion bodies were denatured in 20 mM Tris-HCl (pH 8.0) containing 8 M urea, and Der f 3 was refolded by dialysis sequentially in the urea gradient (6 M, 3 M, 1.5 M Urea, and PBS), after which it was purified by affinity chromatography using StrepTrapHP according to the manufacturer's manual (GE healthcare, USA). The LPS level in the recombinant Der f 3 preparation was determined to be less than 20 ng/mg protein by the endotoxin quantification assay (Invitrogen, California, USA).

2.3. Amino acid sequencing

For N-terminal sequencing, the recombinant Der f 3 was separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride

Table 1
Primers used for PCR in this study.

Name	Sequence (5'-3')
DF3F	TTCATATGATGATTTTAACCATTGTCGTGTTATTGG
DF3R	TACTGCAGTTATTACTGTGAACGTTTTGATTCAATCCAATC
P4-F	GGAGATATACATATGGCTTGGAGCCATCCGCAGTTTGAAAAAACACCGATTC
Strep-R	CCGCTCGAGTTATTACTTCTCGAACTGCGGGTGGCTCCACTGTGAACGTTTTGAT

(PVDF) membrane (Bio-Rad), with 3-cyclohexylamino-1-propanesulfonic acid (pH 11) containing 10% (vol/vol) methanol. The membrane was stained with Coomassie blue, from which the protein bands were excised, which were sequenced by commercial services on a PPSQ31 A sequencer (SHIMADZU) (Biotech Pack Scientific, Beijing, China).

2.4. Protease activity measurement

The protease activity of Der f 3 was measured using the fluorogenic substrate Boc-Q-A-R-MCA (Peptide Institute, Japan) in 50 mM Tris-HCl, pH 8.0. Different amounts of Der f 3 were incubated with the substrate at 37 °C for 30 min, and the amount of 7-amino-4-methylcoumarin (AMC) released was measured using a fluorometer (Excitation at 380 nm, emission at 460 nm, Thermo Fisher, USA). For protease inhibition assays, Der f 3 was incubated with the inhibitors for 30 min at 37 °C before addition of the substrate.

2.5. Culture and stimulation of airway epithelial cells (AECs)

The human pulmonary epithelial cell lines A549, Calu-3 and BEAS-2B were acquired from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine and 100 μg/ml penicillin/streptomycin. Cells were incubated for 6~15 h in serum-free DMEM before stimulation.

2.6. Gene expression profiling by RNA-seq and bioinformatic analysis

For an unbiased evaluation of Der f 3-induced gene expression in AECs, BEAS-2B cells were stimulated with 1 μg/ml of Der f 3 or heat-inactivated Der f 3 (H-Der f 3) for 3 h and the gene expression levels was measured by RNA sequencing (RNA-seq) using Illumina sequencing technology (Mega Genomic, Beijing, China). Prior to mapping reads to the reference database, we filtered all sequences to remove low quality reads. The remaining reads were aligned to human reference genome (ftp://ftp.ensembl.org/pub/release88/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.toplevel.fa.gz) using TopHat v1.0. Genes whose count per millions (CPM) was below 1 were removed from further processing, and the counts were analyzed for differential expression using Cuffnorm.

2.7. ELISA and real-time PCR

Concentrations of IL-8 and IL-6 in the culture supernatants of AECs were determined by ELISA (BioLegend, California, USA). Total RNA was extracted using TRIzol reagent (Invitrogen, California, USA). First strand cDNA was synthesized using RT Master Mix (Takara, Shiga, Japan) and real-time qPCR was performed with the SYBR Green (Invitrogen, California, USA) using LightCycler 480II (Roche, Basel, Switzerland). The qPCR primer sequences are listed in Table 2.

2.8. Western blotting

AECs grown in 6-well plate were stimulated with 1 μg/ml of Der f 3 for different time periods. After washing with ice-cold PBS, the cells were lysed in RIPA lysis buffer (Solarbio, China) containing PhosSTOP (Roche, Switzerland) and harvested. Cell suspensions were placed on ice for 20 min and centrifuged at 10,000 g for 10 min at 4 °C. Protein samples (30 μg/lane) were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad). The membrane was blocked with 5% skim milk in PBS for 1 h at room temperature. After washing 3 times with PBS-0.3% Tween 20 (PBST), the membrane was incubated with the primary antibodies at appropriate dilution with gentle agitation overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Invitrogen, California, USA). For the measurement of nuclear translocation of NFAT and p65 (NF-κB), nuclear and cytosolic proteins were extracted as described (Lee et al., 2006).

2.9. siRNA interference

siRNA transfections were conducted in 12-well plates. Cells were seeded at the density of 5×10^5 cells per well one day prior to transfection. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen, California, USA) according to the manufacturer's instructions. Briefly, 40 pmol of specific siRNA (Ribobio, Guangzhou, China) in 100 μl Opti-MEM (Invitrogen, California, USA) was combined with 2 μl of RNAiMAX in 100 μl of Opti-MEM and incubated for 10 min at room temperature. The siRNA-lipid complex was added to the cells. Next day, the cells were pooled and split into 2~3 wells and stimulated a day later. For double siRNA KD, combinations of the same amount of siRNA (30 pmol for each siRNA) were incubated with the recommended transfection reagent prior to adding to cells in culture. The siRNA sequences are listed in Table 3.

2.10. Measurement of transepithelial electrical resistance (TEER) and permeability

AECs were cultured in Transwell plates as described (Kim et al., 2005): Briefly, 5×10^5 Calu-3 cells were seeded onto Transwell (Costar, Cambridge, MA) cell culture inserts fitted with 0.4 μm-pore-size membranes and were cultured for at least 5 days while the medium was replaced every other day. Transepithelial electrical resistance (TEER) was measured with EVOM2 (World Precision Instruments, FL, USA).

Measurement of transepithelial permeability was performed as described previously (Gavard et al., 2008). FITC-Dextran (4KD, 1 mg/ml) (Sigma, Mo., USA) was added to the upper chamber, and the FITC-dextran concentration in the lower chamber was measured up to 6 h. The transferred FITC-Dextran concentration from each lower chamber was determined using a fluorescence plate reader (Thermo Fisher, USA) with excitation and emission wavelengths of 492 nm and 518 nm,

Table 2
Sequences of primers used for real-time PCR.

Gene	Sense (5'-3')	Antisense (5'-3')
PAR1	CATTTGCTTCGGACCCACAA	TGCTGGGATCGGAACTTTCT
PAR2	CAGTGGCACCATCCAAGGAA	CAGGGCCATGCCGTTACTT
PAR3	GCAAAGCCAACCTTACCCATT	GAGGTAGATGGCAGGTATCAGT
PAR4	CCATGCTGCTGATGAACCTC	ACTGAGCCATACATGTGACCA
IL-1β	TGGTGTTCTCCATGTCCTTT	GAAGACAAATCGCTTTTCCA
IL-6	CAAAGAGGCACTGGCAGAAA	CTGCACAGCTCTGGCTTGTT
IL-8	CTTGGCAGCCTTCCTGATTT	TTCCTTGGGGTCCAGACAGA
GM-CSF	CTGCACAGCTCTGGCTTGTT	AATCTGGGTTGCACAGGAAGT
CXCL1	CCCCAAGAACATCCAAAGTGT	TGGATTTGTCACTGTTCAGCA
CCL20	CAACTTTGACTGCTGTCTTGGATA	TTGACTTTTTTACTGAGGAGACGC
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

Table 3
Sequences for siRNA used for knockdown of PARs.

Gene	Sense (5'-3')	Antisense (5'-3')
PAR1	AGUGCCAGAGGUACGUCUAAUU	UAGACGUACCUCUGGCACUUU
PAR2	CCACUGUCUUCCUUCCAAUUU	AUUGGAAGGAAGACAGUGGUU
PAR3	CAUCCGGACACUUAAUGCAUU	UGCAUUAAGUGUCCGGAUGUU

respectively.

2.11. Statistical analysis

Statistical analysis was performed by using Prism 5 (GraphPad Software). Data were presented as mean ± SEM. We assumed the normal distribution and used the Student's *t* test for paired observations. For time-dependent measurements (TEER and permeability assay), the mean response for each experimental group was compared with its respective control utilizing Student's *t* test. A difference was considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Der f 3 is a serine protease

Recombinant pro-Der f 3 (29 kDa) was expressed in *E. coli*. Monomers and dimers were detected in inclusion body (data not shown). Its maturation was achieved after dialysis (Supp Fig. 1A). As previously described (Stewart et al., 1992), we confirmed the protease activity of Der f 3 (Fig. 1A), which was heat-labile at 56 °C (Fig. 1B). We verified Der f 3 as a serine protease using the irreversible class-specific inhibitors for cysteine and serine proteases, E64 and AEBSF, and the metalloprotease inhibitor EDTA (Fig. 1C).

3.2. Der f 3 induces expression of cytokines and chemokines in AECs

Previous studies demonstrated that the proteinase activity of allergens can alter epithelial cell morphology and cause epithelia cell detachment (Kauffman et al., 2000; Tomee et al., 1998). Here Der f 3 disrupted the 2D monolayer of BEAS-2B cells (Supp. Fig. 2A); and it also disrupted the epithelial barrier formed by polarized Calu-3 cells and accordingly increased permeability (Supp. Fig. 2, B and C). To further assess whether Der f 3 induces pro-inflammatory genes in AECs, we performed RNA-seq after stimulating BEAS-2B cells with Der f 3 for 3 h, using the heat-inactivated Der f 3 (H-Der f 3) as a control. BEAS-2B cells were chosen for the superior sensitivity to Der f 3 stimulation compared to Calu-3 (Supp. Fig. 3). Since Der f 3 protein solution contains LPS (below 20 ng per mg protein), we investigated the effect of LPS contamination in Der f 3 on the stimulation of BEAS-2B cells. Although H-Der f 3 induced little expression of the genes, LPS at 1 μg/ml did not activate BEAS-2B in the serum-free conditions under which the Der f 3 stimulation was performed (Supp. Fig. 4). Der f 3 induced expression of several pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, IL-8 (here also noted as CXCL8), and granulocyte monocyte colony-stimulating factor (GM-CSF) above the level induced by H-Der f 3 (Fig. 2A and B). We confirmed the RNA-seq results by qPCR (Fig. 2C) and ELISA (Fig. 2D). We also found that the genes involved in cytoskeleton, signal transduction, cell metabolism etc. were induced by Der f 3 stimulation (Table 4).

3.3. Der f 3-induced IL-8 production depends on MAP kinases, NF-κB, and store-operated calcium signaling

We next investigated the signaling pathways activated by Der f 3 in AECs. Both MAP kinase and NF-κB pathways were activated by Der f 3 in a time-dependent manner. After a 15 min incubation with Der f 3, ERK1/2 phosphorylation reached the peak while the phosphorylation of JNK and p38 peaked at 60 min. As for the NF-κB pathway, both phosphorylation of p65 and degradation of IκBα peaked at 60 min after Der f 3 stimulation (Fig. 3A). To determine the contribution of different signaling pathways in induction of IL-8 by Der f 3, AECs were pre-incubated with specific pharmacologic inhibitors for 2 h before exposure to Der f 3. As shown in Fig. 3B, the ERK (U1026), JNK (SP600125), or NF-κB (BAY11-7082) inhibitor significantly decreased the Der f 3-induced IL-8 production by 90%, while the maximal inhibition of about 50% reduction in IL-8 production was achieved by p38 (LY2228820). These results show that the Der f 3 induces IL-8 expression in a MAP kinase and NF-κB-dependent manner.

Allergens in the extract from dust mites or cockroaches provoked intracellular Ca^{2+} signals in an airway epithelial cell line (Jairaman et al., 2015), and a recent study demonstrated that Der p 3 in the mite extract was essential for activation of store-operated Ca^{2+} channels through PAR-4 (Lin et al., 2018). Here Der f 3 induced activation of the calcium-dependent signaling molecule NFAT-1 measured by its nuclear translocation in DMEM with or without calcium (Supp. Fig. 5, A and B). However, IL-8 production induced by Der f 3 was not affected the extracellular calcium level (Supp. Fig. 5C), while chelating intracellular Ca^{2+} with BAPTA-AM abolished IL-8 expression (Supp. Fig. 5D), thus indicating that Der f 3-induced IL-8 is store-operated Ca^{2+}-dependent signaling, rather than extracellular calcium influx.

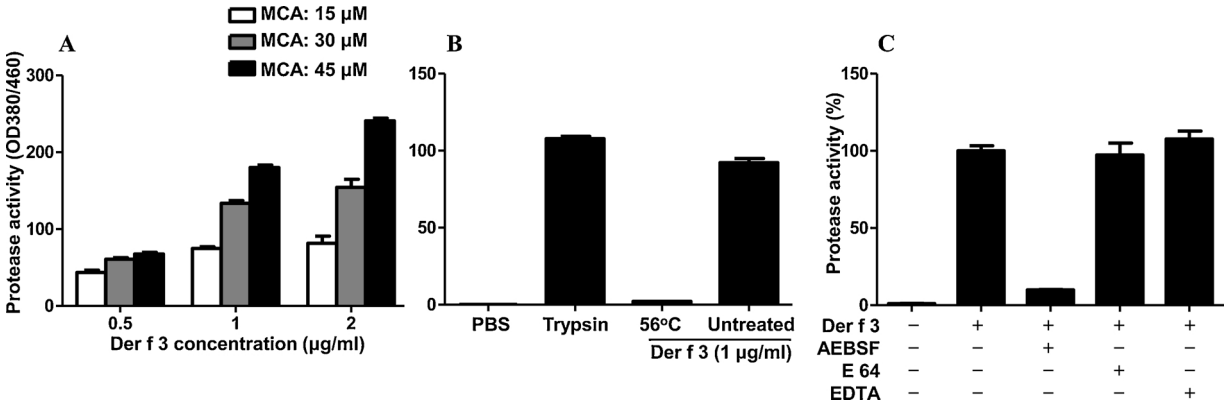


Fig. 1. Der f 3 displays serine protease activity. (A) The protease activity of the indicated amount of Der f 3 was measured fluorometrically using Boc-Q-A-R-MCA as the substrate. (B) The Der f 3 protease activity is abolished by the 1 h heat treatment at 56 °C. (C) A serine protease inhibitor (AEBSF, 5 μM) but not a cysteine protease (E64, 2.5 μM) or a metalloprotease (EDTA, 0.5 mM) inhibitor inhibited the protease activity of Der f 3. Der f 3 (1 μg/ml) was incubated with the respective inhibitor for 30 min before the assay. Concentration of the substrate in B and C is 15 μM. Data are representative of three independent experiments.

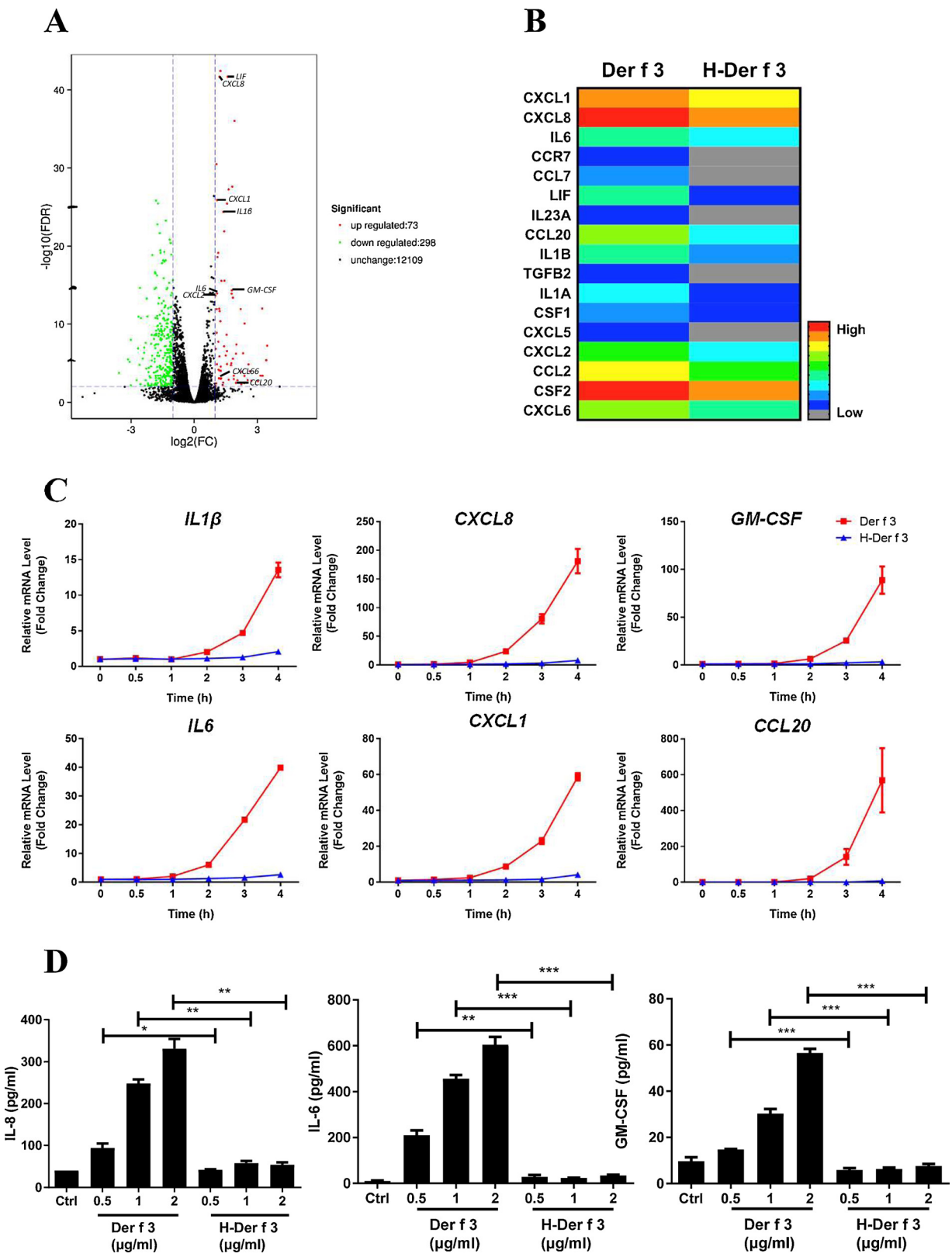
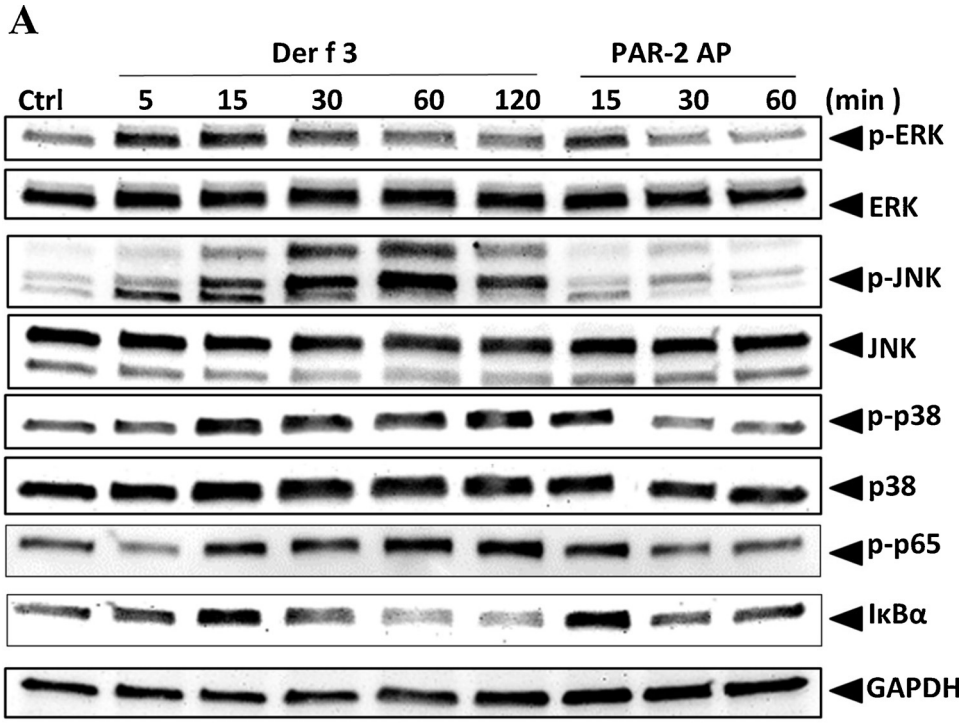


Fig. 2. Der f 3 induces expression of cytokines and chemokines in AECs. (A) RNA-seq analysis was performed on Der f 3-treated BEAS-2B cells. Shown is a volcano plot of *P* value (FDR) versus fold-change (FC) comparing gene expression in the Der f 3-treated group over the H-Der f 3-treated group; red (up-regulation) and green dots (down-regulation) indicate a false discovery rate (FDR) of < 0.05. (B) FPKM (Fragments per kilo base per million mapped reads)-normalized expression of cytokines and chemokines. (C) Confirmation of the RNA-seq data by qPCR. BEAS-2B cells were stimulated as indicated, and the expression levels of the indicated genes were measured by qPCR. (D) Der f 3 induces IL-8 and IL-6 in AECs. BEAS-2B cells were stimulated as indicated, and the expression levels of the indicated genes were measured by ELISA. Data represent mean ± standard error of the mean (SEM) of three separate experiments. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
Most significantly upregulated genes upon Der f 3 stimulation in AECs.

Gene	#ID	FDR	log2FC	regulated	eggNOG_class_annotation	GO_annotation
OPN1SW	ENSG00000128617	0.002044	3.032432	up	Signal transduction mechanisms	Molecular Function: G-protein coupled receptor activity; Biological Process: G-protein coupled receptor signaling pathway; Cellular Component: integral component of membrane;
NPPA	ENSG00000175206	0.001474	2.539256	up	Nucleotide transport and metabolism	Molecular Function: hormone activity; Cellular Component: extracellular region;
CNTF	ENSG00000242689	5.43E-07	2.324648	up	Signal transduction mechanisms	Cellular Component: cytoplasm; Biological Process: growth;
TMPRSS9	ENSG00000178297	0.005029	2.238029	up	Posttranslational modification, protein turnover, chaperones	Molecular Function: serine-type endopeptidase activity; Molecular Function: protein binding; Biological Process: proteolysis;
MAB21L3	ENSG00000173212	1.17E-12	2.20771	up	Signal transduction mechanisms	–
MYH7B	ENSG00000078814	4.12E-08	2.006898	up	Cytoskeleton	Molecular Function: motor activity; Molecular Function: ATP binding; Cellular Component: myosin complex;
ST6GALNAC2	ENSG00000070731	9.22E-37	1.916658	up	Carbohydrate transport and metabolism	Biological Process: protein glycosylation; Molecular Function: sialyltransferase activity;
SERPINF1	ENSG00000132386	0.001083	1.881323	up	Defense mechanisms	–
PARP15	ENSG00000173200	2.50E-28	1.811324	up	Transcription	Molecular Function: NAD + ADP-ribosyltransferase activity;
LONRF2	ENSG00000170500	1.02E-05	1.773628	up	Posttranslational modification, protein turnover, chaperones	Molecular Function: metal ion binding;
CASR	ENSG00000036828	5.69E-28	1.636773	up	Signal transduction mechanisms	Molecular Function: G-protein coupled receptor activity; Biological Process: G-protein coupled receptor signaling pathway; Cellular Component: integral component of membrane;
CEBPD	ENSG00000221869	3.64E-26	1.566986	up	Transcription	Molecular Function: sequence-specific DNA binding transcription factor activity; Biological Process: regulation of transcription, DNA-templated;
UGT8	ENSG00000174607	2.98E-07	1.560736	up	Carbohydrate transport and metabolism	Biological Process: metabolic process; Molecular Function: transferase activity, transferring hexosyl groups;
ELF3	ENSG00000163435	2.25E-09	1.543004	up	Transcription	Molecular Function: DNA binding; Molecular Function: sequence-specific DNA binding transcription factor activity; Cellular Component: nucleus; Biological Process: regulation of transcription, DNA-templated; Molecular Function: sequence-specific DNA binding;
C10orf11	ENSG00000148655	1.80E-09	−1.50167	down	RNA processing and modification	–
MYLK2	ENSG00000101306	1.53E-18	−1.52591	down	Cytoskeleton	Molecular Function: protein kinase activity; Biological Process: protein phosphorylation;
RAD51B	ENSG00000182185	1.82E-10	−1.55524	down	Replication, recombination and repair	Molecular Function: DNA helicase activity; Molecular Function: single-stranded DNA binding; Molecular Function: ATP binding; Biological Process: DNA replication; Biological Process: DNA repair;
TMPRSS5	ENSG00000166682	2.43E-07	−1.59087	down	Posttranslational modification, protein turnover, chaperones	Molecular Function: serine-type endopeptidase activity; Biological Process: proteolysis; Cellular Component: membrane;
MESDC1	ENSG00000140406	7.46E-20	−1.59395	down	Cytoskeleton	–
RAPSN	ENSG00000165917	1.08E-06	−1.76669	down	Extracellular structures	Molecular Function: protein binding; Biological Process: synaptic transmission; Molecular Function: acetylcholine receptor binding; Molecular Function: protein anchor;
RAB40AL	ENSG00000102128	3.20E-17	−1.8332	down	Signal transduction mechanisms; Intracellular trafficking, secretion, and vesicular transport	Molecular Function: GTPase activity; Molecular Function: GTP binding; Biological Process: small GTPase mediated signal transduction; Biological Process: intracellular signal transduction;
AL592183.1	ENSG00000273748	2.60E-06	−1.87909	down	Cytoskeleton	–
TAS2R13	ENSG00000212128	3.10E-15	−1.88058	down	Signal transduction mechanisms	Molecular Function: G-protein coupled receptor activity; Biological Process: G-protein coupled receptor signaling pathway; Cellular Component: integral component of membrane; Biological Process: sensory perception of taste;
PLGLB2	ENSG00000125551	0.000518	−2.12002	down	Posttranslational modification, protein turnover, chaperones	–
HIST1H1C	ENSG00000187837	4.56E-07	−2.37996	down	Chromatin structure and dynamics	Cellular Component: nucleosome; Molecular Function: DNA binding; Cellular Component: nucleus; Biological Process: nucleosome assembly;

FDR, False Discovery Rate; FC, fold change; eggNOG, evolutionary genealogy of genes: Non-supervised Orthologous Groups; GO, Gene ontology.



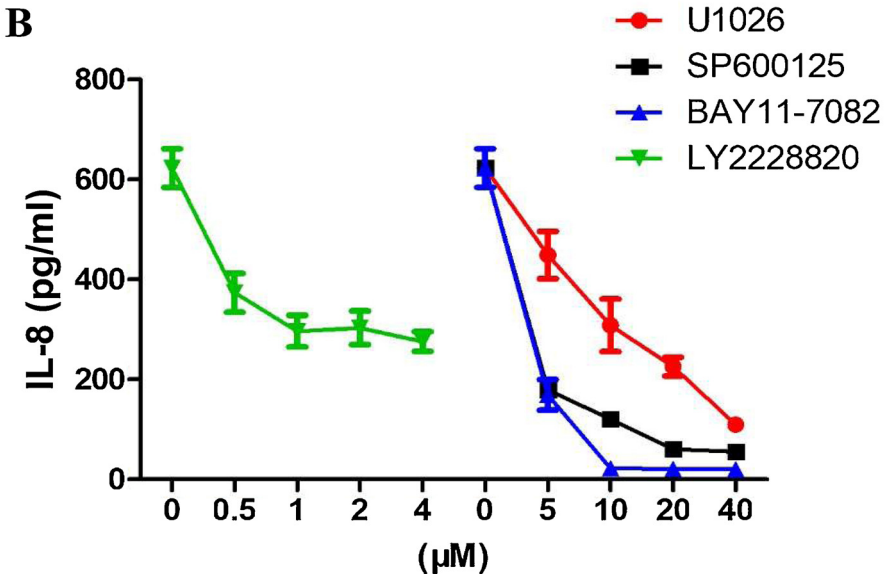


Fig. 3. Requirement of MAPK and NF-κB for the Der f 3 induced IL-8 secretion. (A) The effect of Der f 3 and PAR-2 AP on MAPK and NF-κB activity. BEAS-2B cells were incubated with medium alone (Ctrl), or with Der f 3 (1 μg/ml) or PAR-2 AP (50 μM) for various periods. Equal amounts of proteins were analyzed by western blotting. (B) BEAS-2B cells were pretreated with ERK1/2 inhibitor U1026 (5–40 μM), JNK inhibitor SP600125 (5–40 μM), NF-κB inhibitor BAY11-7082 (5–40 μM) and p38 inhibitor LY2228820 (0.5–4 μM) followed by treatment with or without Der f 3 for 24 h. The amount of IL-8 secretion was quantified by ELISA. Data are expressed as the mean of 3 independent experiments.

3.4. Der f 3 activates AECs via PAR-1 and PAR-2

To investigate whether Der f 3 activates the pro-inflammatory program through the serine protease activity, we pretreated Der f 3 with 5 μM AEBSF (an irreversible serine protease inhibitor) for 30 min before applying to BEAS-2B cells. Pretreatment with AEBSF significantly decreased the IL-8 production (Fig. 4A) as well as pro-inflammatory cytokines and chemokines expression (Fig. 4B). Since the protease activity of Der f 3 is essential for induction of inflammatory genes, we next investigated whether Der f 3 activates protease-activated receptors (PARs). PAR-1, -2, and -3 transcripts were detectable, but PAR-4 was not, by qPCR in BEAS-2B (Supp. Fig. 6). Therefore, we silenced PAR-1, -2, or -3 with siRNA in AECs and measured the levels of Der f 3-induced expression of inflammatory genes. Knockdown (KD) efficiency of siRNAs was measured by qPCR (Supp. Fig. 7). Der f 3-induced IL-8 expression was significantly decreased after PAR-1 or PAR-2 knockdown (KD), while PAR-3 KD did not cause any significant change (Fig. 5A). These results indicate that Der f 3 activates AECs mainly via PAR-2 and PAR-1. Furthermore, both production of IL-8 and activation of the MAPK and NF-κB pathways were decreased after double KD of PAR-1 and PAR-2 (Fig. 5B and C). Thus, we conclude that the full activation of AECs by Der f 3 requires both PAR-1 and PAR-2. In order to clarify the PAR-2-dependent cell activation by Der f 3, we further performed the desensitization assay, which showed that PAR-2 AP pretreatment decreased the Der f 3-induced IL-8 expression in both A549 and BEAS-2B cells (Supp. Fig. 8).

4. Discussion

Allergic diseases induced by ubiquitous, innocuous environmental

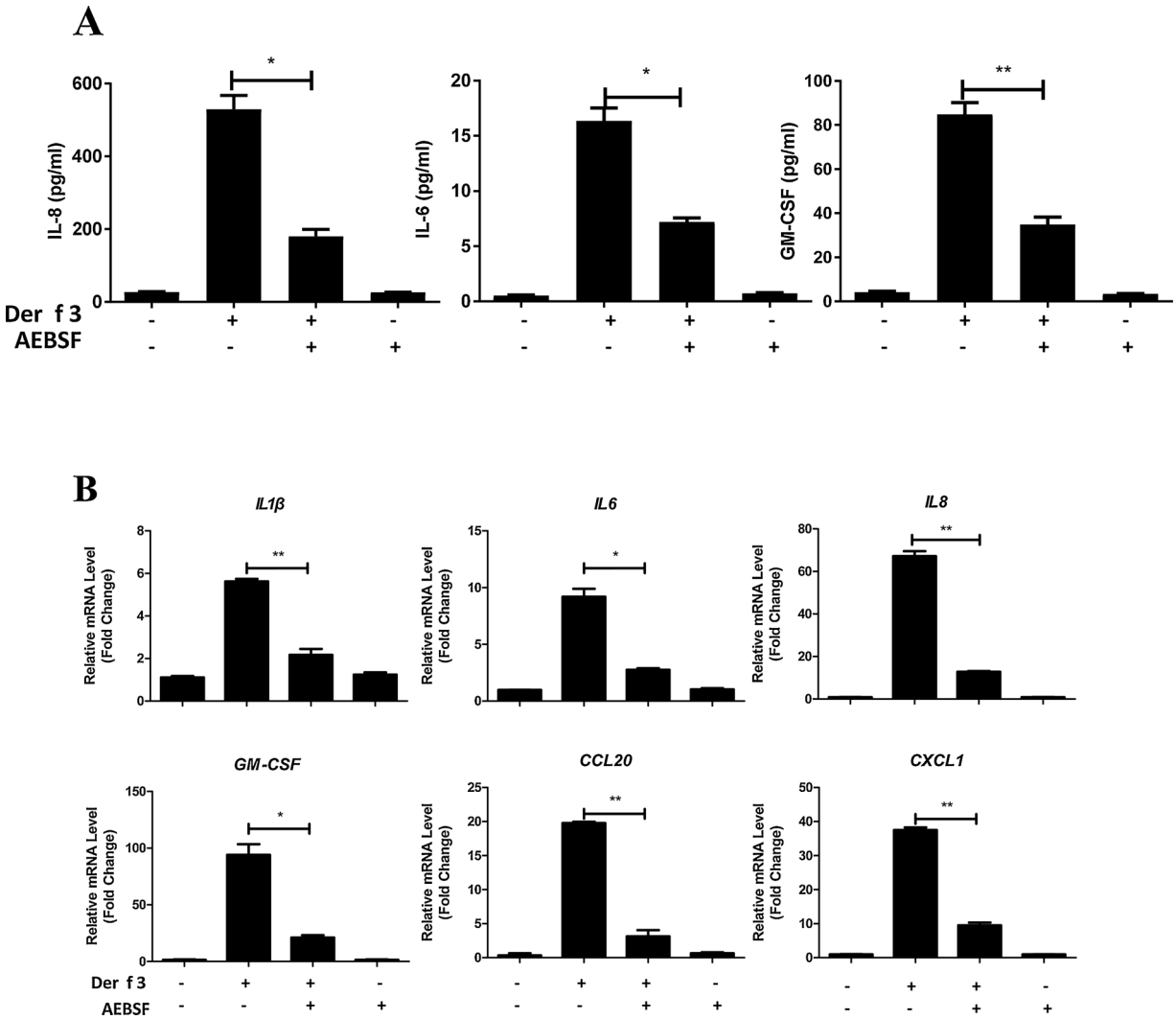


Fig. 4. Der f 3 activates AECs using its protease activity. (A) The serine protease inhibitor AEBSF abolished IL-8 and IL-6 production by Der f 3. Der f 3 (1 μg/ml) was preincubated with AEBSF (5 μM) before stimulation and the level of IL-8 in the culture medium was measured by ELISA. (B) Der f 3 induces expression of cytokines and chemokines in AECs via its protease activity. Der f 3 (1 μg/ml) was preincubated with AEBSF (5 μM) before stimulation and the mRNA level of the indicated genes was measured by qPCR. Data represent mean ± SEM of three separate experiments. (*$P < 0.05$, **$P < 0.01$).

allergens (Scheurer et al., 2015) affect 10–30% of the global population (Dave et al., 2011; Ring et al., 2012). Cumulative evidence indicates that allergenicity of certain allergens relies on their intrinsic properties, through which they affect diverse pathways of innate immune responses at the mucosal surfaces. Proteolytic activity (Kauffman et al., 2006; Kouzaki et al., 2009), engagement of pattern recognition receptors (Iwasaki and Medzhitov, 2004), molecular mimicry of TLR signaling complex molecules (Trompette et al., 2008) and lipid-binding activity (Thomas et al., 2005) of allergens have all been suggested to be important in allergenicity.

The N-terminal extension of full-length Der f 3 was divided into two functional parts, pre-sequence (1 st to 16th) for secretion and pro-sequence (17th to 27th) for refolding (Nishiyama et al., 1995). But Der p 3 cannot be generated without the pro-sequence in *E. coli* since the mature form of protein can digest the bacteria for expression (Lin et al., 2018). Alternative strategies need to be adopted to generate active recombinant Der f 3. It was reported that an threonine (27) at the C-terminus of the pro-region was replaced by arginine, Der f 3 underwent auto-cleavage during the refolding process by dialysis (Nishiyama et al., 1995). Here we produced recombinant Der f 3 accordingly and confirmed maturation after dialysis by N-terminal sequencing (Supp. Fig. 1B).

Although much attention has been placed on immune cells as the key factor in immune response, the concept that nonhematopoietic tissues are a critical source of cytokines and chemokines has been supported by a number of publications in recent years (Yin et al., 2018). The airway epithelium, in addition to being the first line of defense as a physical barrier against inhaled allergens, has recently been shown to play an immunomodulatory role in the lung (Gour and Lajoie, 2016). Cumulative evidence shows that AECs are the active participants in regulating immune response to environmental stimuli via the release of chemokines and cytokines. The current literature establishes the airway epithelium as an innate immune tissue that senses inhaled allergens through an armory of receptors and initiates innate and adaptive immunity (Gandhi and Vliagoftis, 2015).

Recent investigations revealed that proteases including trypsin, papain (Kouzaki et al., 2009), HDM extract (Kouzaki et al., 2013) and certain fungal allergens (Snelgrove et al., 2014) induce production of alarmins such as TSLP, IL-25 and IL-33 in AEC through activation of PAR-2, which underlies the development of a robust Th2 inflammation. However, in this study, we demonstrated that Der f 3 did not induce alarmins at either the mRNA or protein level; rather it induced a set of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, GM-CSF, IL-1β, CXCL1 and CCL20 in the bronchial epithelial cell (i.e.,

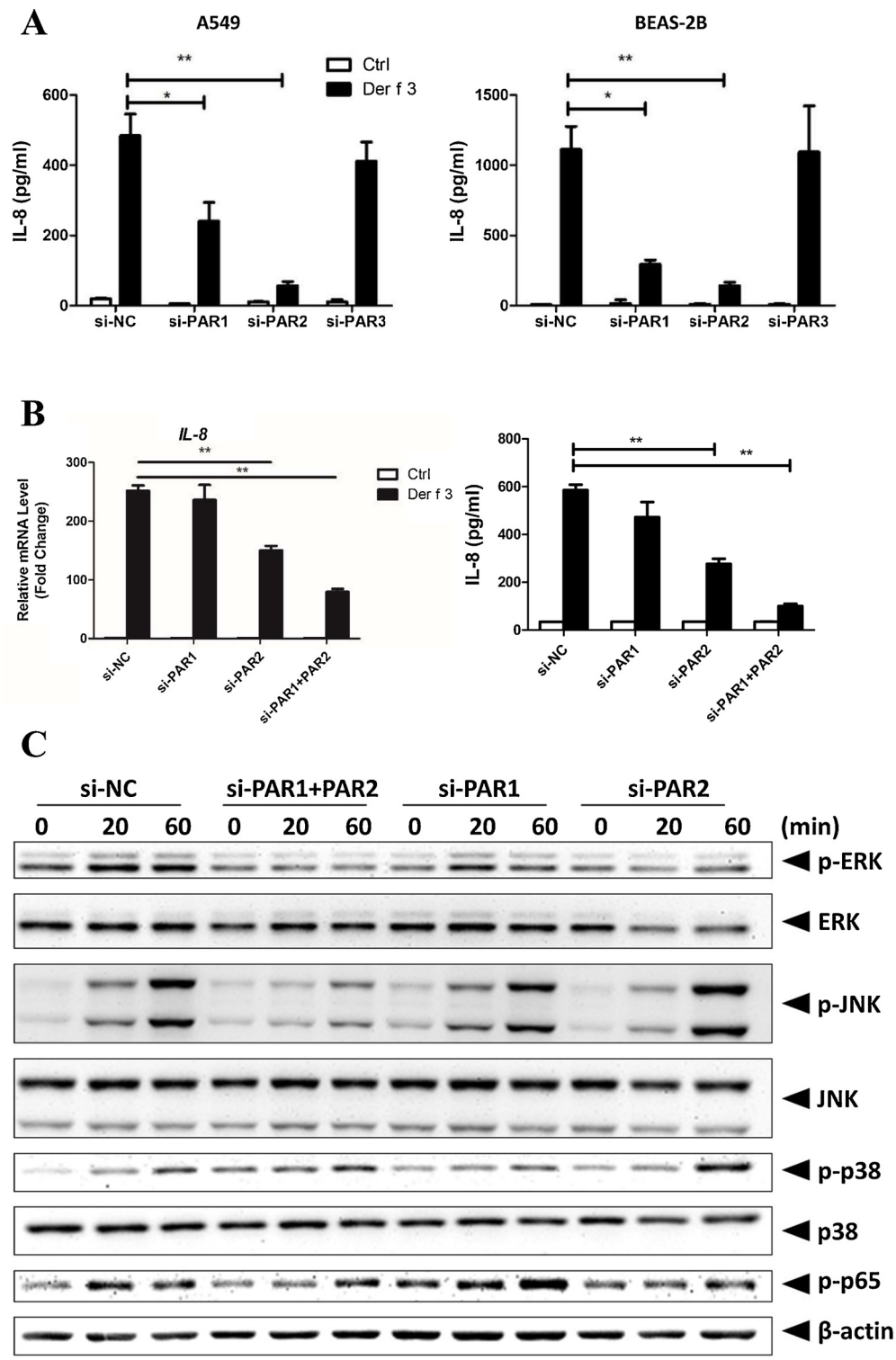


Fig. 5. Der f 3-induced activation of AECs is dependent on both PAR-1 and PAR-2. (A) The siRNA knockdown (KD) of PAR-1 or PAR-2 impaired IL-8 induction by Der f 3. A549 or BEAS-2B cells transfected with 40 pmol of siRNA were stimulated with Der f 3 and the level of IL-8 induction was measured by ELISA. (B) Quantitative analysis of IL-8 production showed significant decrease in the double-KD group compared with control group. BEAS-2B cells transfected with 60 pmol of siRNA (30 pmol si-PAR-1 and 30 pmol si-PAR-2 for double KD) were stimulated with Der f 3 and the level of IL-8 induction was measured by ELISA and qPCR. Data represent mean ± SEM of three separate experiments. (* $P < 0.05$, ** $P < 0.01$) (C) Activation of MAPK and NF-κB pathway after PARs KD in BEAS-2B cells was verified by western blotting. Si-NC, scrambled RNA that works as negative control for all PARs.

AECs) line BEAS-2B in a protease-dependent manner (Fig. 2). In line with this finding, several studies showed that proteolytic active allergens induced the release of IL-6, IL-8 and GM-CSF from bronchial (K. E. Lee et al., 2007; Matsuwaki et al., 2012) and alveolar epithelial cells (Kauffman et al., 2000; M.-F. Lee et al., 2010). As shown in Fig. 4, pretreatment with 5 μM AEBSF partly inhibited Der f 3-induced IL-8 production; but higher concentration of serine protease inhibitors further inhibited it (data not shown).

Allergens in the extract of dust mites or cockroaches provoked intracellular Ca^{2+} signals in an airway epithelial cell line (Jairaman et al., 2015), and Der p 3 in the mite extract was essential for activation of store-operated Ca^{2+} channels through PAR-4 (Lin et al., 2018). In this study we found that Der f 3 induced activation of the calcium-dependent signaling molecule NFAT-1 measured by its nuclear translocation in DMEM with or without calcium (Supp. Fig. 5, A and B), but IL-8 production induced by Der f 3 was not affected by the extracellular calcium level (Supp. Fig. 5C) while chelating intracellular Ca^{2+} with BAPTA-AM abolished IL-8 expression (Supp. Fig. 5D). Our findings thus indicate that Der f 3-induced IL-8 is store-operated Ca^{2+}-dependent signaling, rather than extracellular calcium influx.

Asthma is a phenotypically heterogeneous chronic disease of the airways and can be subdivided into two major endotypes; Th2-dominant and Th17 endotypes (Stokes and Casale, 2016). A mixed endotype of Th2/Th17 is also relatively common. The Th2 endotype is characterized by enrichment of eosinophils, basophils and mast cells (Ritchie et al., 2016), and is initiated via IL-25, TSLP and IL-33 released by epithelial cells (Wittekindt, 2017), while the Th17 endotype mainly by neutrophils. The Th17 and the Th2/Th17 mixed endotype are initiated via IL-1β, TGF-β and IL6, which induce recruitment of neutrophils via stimulation of IL-17 release from Th17 cells (Wittekindt, 2017). The Th17 and the mixed endotypes show more severe asthmatic symptoms and are resistant to the steroid treatment. Der f 3 induced the potent neutrophil chemoattractants IL-8 and CXCL1 (Saiman and Friedman, 2012) and the Th17-inducing cytokines IL-1β and IL-6 (Vroman et al., 2015). Our data here therefore suggest that Der f 3 likely promote the Th2/Th17 endotype.

Aeroallergen proteases can activate AECs via protease activated receptors (PARs) (Jacquet, 2011; Kauffman et al., 2006). All four PARs were expressed in human AECs (Hammad et al., 2003) and illustrated to contribute to the inflammatory processes (Atzori et al., 2009; Moraes et al., 2008; Su et al., 2005). The fungal allergen Pen c 13 induced IL-8 in AECs via PAR-1 and 2-dependent activation (Chiu et al., 2007). While for cockroach allergen, both PAR-2 and PAR-3 might play a role in CraA-induced IL-8 secretion from human airway epithelial cells (Kauffman et al., 2000; M.-F. Lee et al., 2010). Der p 3 and Der p 9, as serine protease allergens in HDM, induced an inflammatory response in A549 cells and this effect is at least in part mediated by PAR-2 (Adam et al., 2006; Sun et al., 2001). Our results indicate that PAR-2 is the major receptor for Der f 3 while PAR-1 is also required for the full activation of AECs by Der f 3 (Fig. 5).

Activated PARs can activate multiple GPCR-dependent and -independent pathways. Numerous studies identified the transcription factor NF-κB to be a critical modulator of inflammation in the pathogenesis of lung diseases including allergic asthma (Hayden et al., 2006; Pantano et al., 2008). CraA stimulation increases phosphorylation of both ERK1/2 and JNK (Kauffman et al., 2000; Lee et al., 2010), while Pen c 13 induces IL-8 expression by calcium-dependent signaling and that the increase in intracellular calcium levels is up-stream of ERK 1/2 activation (Chiu et al., 2007). In this study we found that the phosphorylation levels of p65, p38, ERK and JNK were all elevated in Der f 3-treated BEAS-2B cells (Fig. 3), and a similar pattern was observed in A549 cells after stimulated with the Der p 3 (Adam et al., 2006). In addition, we observed the nuclear translocation of the calcium-dependent signaling molecule NFAT-1 upon stimulation by Der f 3 via store-operated Ca^{2+} signaling (Supp. Fig. 5, A and B), which is similar to a previous finding with Der p 3 (Lin et al., 2018). However, our data suggest that Der f 3-activated PAR-1/2 integrates MAPKs, NF-κB, and store-operated calcium signaling in order to activate a pro-inflammatory program in AECs. These interesting findings sharpen our understanding of the molecular mechanism underlying the Der f 3 allergenicity. Yet it remains to be illustrated in the future whether and how this program contributes to allergic responses *in vivo*. Current treatment of allergic disease relies heavily on inhaled corticosteroids and anti-histamines to provide symptomatic relief but they do not provide a curative impact of these diseases. Our findings provide a molecular mechanism by which Der f 3 may trigger the allergic response toward house dust mites, and enrich evidence for better understanding of early events of allergic lung diseases, which may ultimately help developing new preventive and therapeutic targets in relevant allergic diseases in the future.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.02.018.

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Dust mite-derived Der f 3 activates a pro-inflammatory program in airway epithelial cells via PAR-1 and PAR-2

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Supplementary Materials Online

Supplementary Figure Legends

Supplementary Figure 1. Recombinant Der f 3. (A) SDS-PAGE of recombinant Der f 3. M, protein maker. Lane 1, without IPTG; Lane 2, 0.1 mM IPTG; Lane 3, supernatant of inclusion body; Lane 4, pellet of inclusion body; Lane 5, refolded Der f 3. (B) N-terminal sequence of mature Der f 3.

Supplementary Figure 2. Der f 3 disrupts epithelial barrier. (A) Phase contrast microscopy analysis of BEAS-2B after incubation with 1 μg/ml of Der f 3 for the indicated time period. The experiments were repeated three times independently with similar results. (B) Der f 3 decreases TEER (transepithelial electronic resistance) of the Calu-3 monolayers in Transwell plates. (C) Der f 3 increases transepithelial fluxes of FITC-dextran (4KD) across the Calu-3 monolayer. Values are mean standard errors (n=3). * $P<0.05$, ** $P<0.01$ versus control.

Supplementary Figure 3. BEAS-2B cells better respond to Der f 3 stimulation compared to Calu-3. BEAS-2B and Calu-3 cells were stimulated as indicated, and the expression level of IL-8 was measured by qPCR and ELISA. The data shown are the representatives of three independent experiments. Values are mean standard errors (n=3). * $P<0.05$, ** $P<0.01$, ***$P<0.001$ versus control.

Supplementary Figure 4. AECs fail to respond to LPS in the serum-free condition. ELISA quantitation of IL-8 production in supernatants of A549 and BEAS-2B cell lines after the overnight treatment. The data shown are the representatives of three independent experiments.

Supplementary Figure 5. Der f 3 induces IL-8 in a store-operated Ca^{2+}-dependent manner. The effect of Der f 3 on NFAT-1 nuclear translocation in the calcium-containing (A) and calcium free medium (B). BEAS-2B cells were incubated with Der f 3 (1 μg/ml) for various periods. Nuclear and cytosolic proteins were extracted and equal amounts of proteins were analyzed by western blotting. (C) qPCR analysis of IL-6 and IL-8 in BEAS-2B cells in the calcium-containing or calcium free medium after treatment with Der f 3 (1 μg/ml). (D) IL-6 and IL-8 induction in BEAS-2B cells by Der f 3 (1 μg/ml) in the calcium-containing or calcium free medium.

Supplementary Figure 6. The mRNA expression levels of PARs in BEAS-2B cells

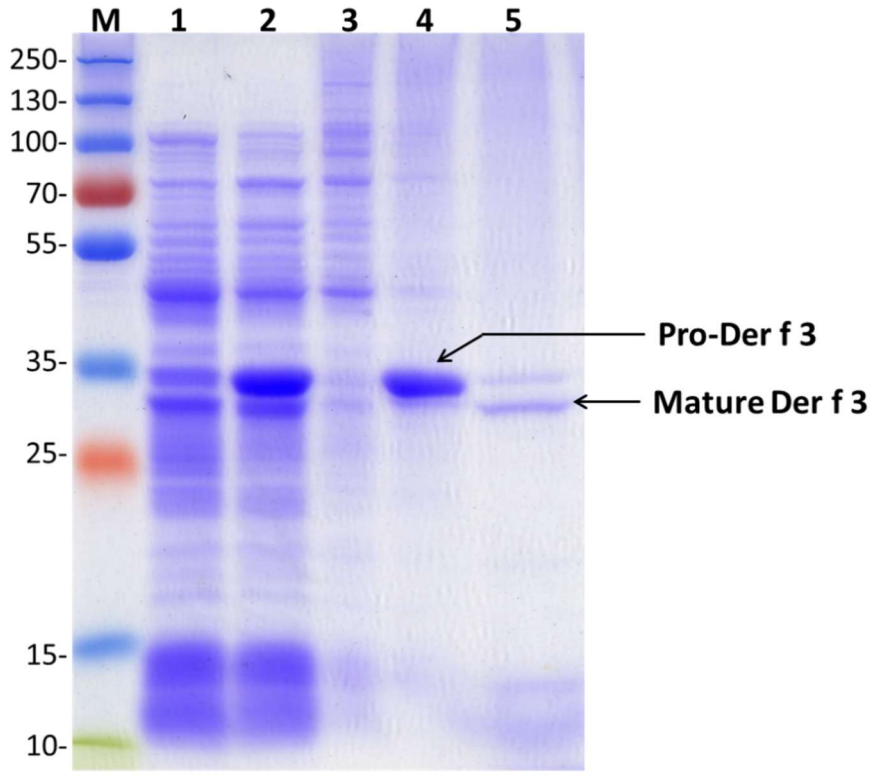
were analyzed by RT-PCR followed by agarose gel electrophoresis.

Supplementary Figure 7. Knockdown (KD) efficiency of siRNAs. BEAS-2B cells transfected with 40 pmol of siRNA for single KD (A) or with 60 pmol of siRNA (30 pmol siRNA for each) for double KD were harvested 48h after transfection and the level of indicated receptors were measured by qPCR.

Supplementary Figure 8. Der f 3-induced cell activation is PAR-2 dependent. A549 (left) or BEAS-2B (right) cells were prestimulated the indicated amount of PAR2-AP for 2h and washed out with PBS, and then re-stimulated with Der f 3 (1 μg/ml) for 2h. IL-8 mRNA levels were measured by qPCR.

Supplementary Figure 1. Recombinant Der f 3**.**

A



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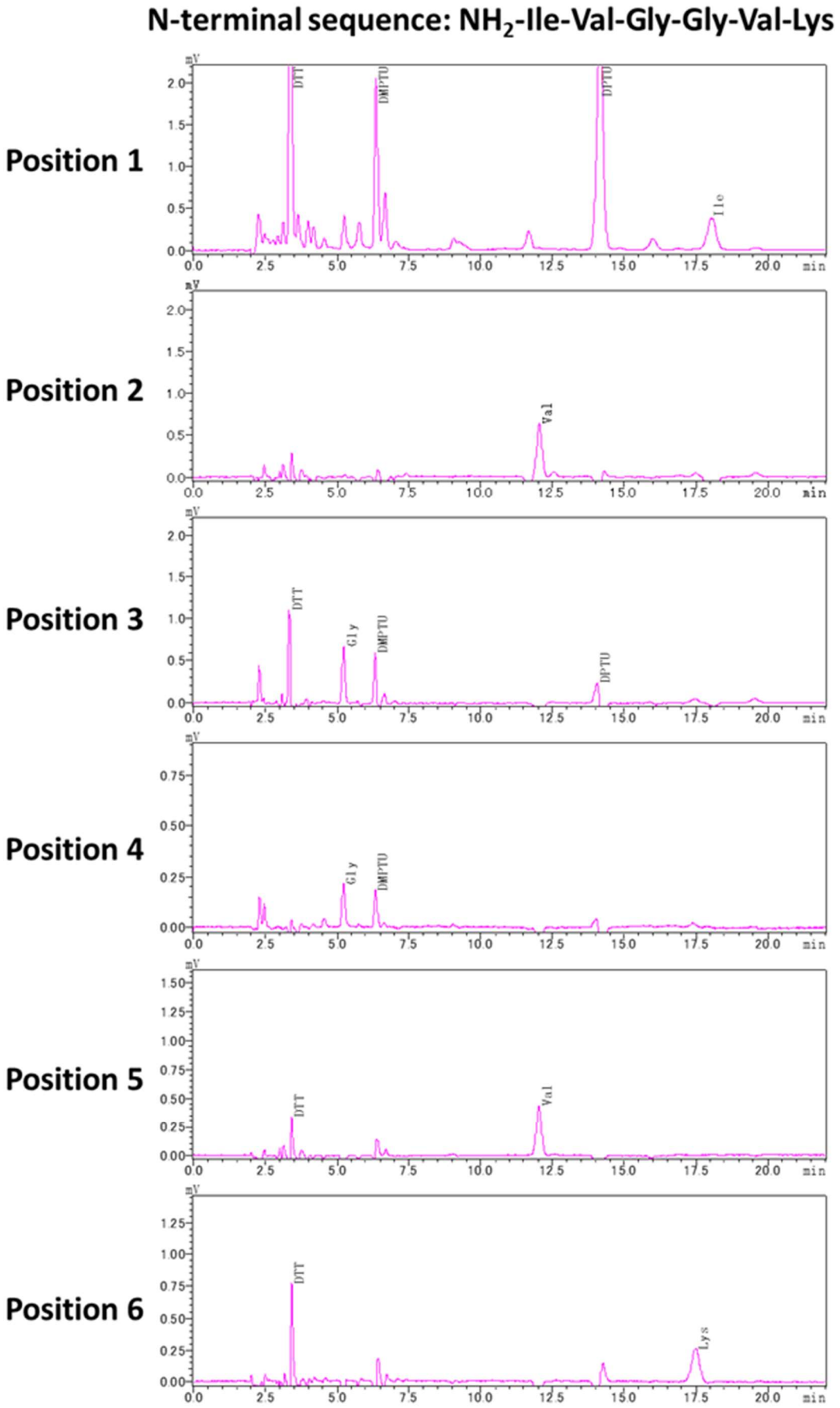
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Position 2
Position 3
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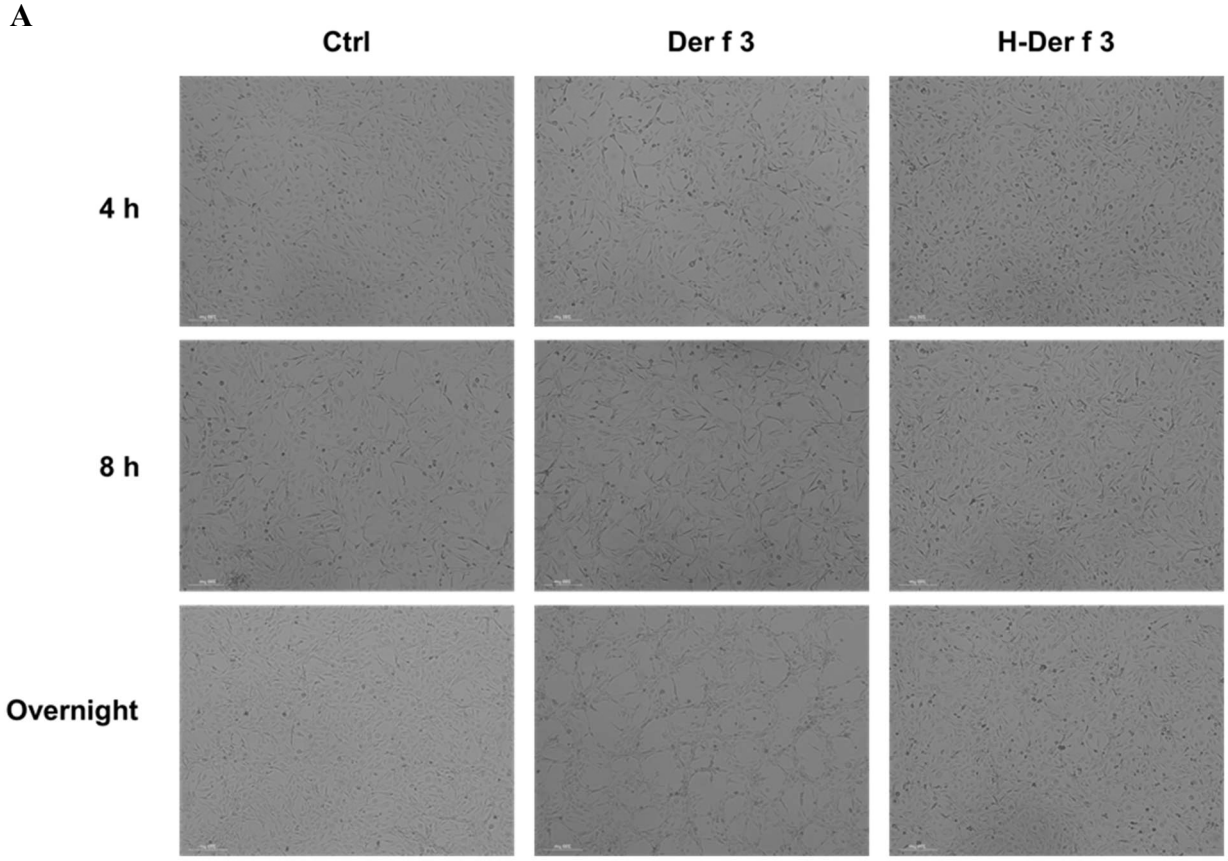
DMPTU
DPTU
Ile
Val
Gly
Lys
mV
min

Supplementary Figure 2. Der f 3 disrupts epithelial barrier.

A



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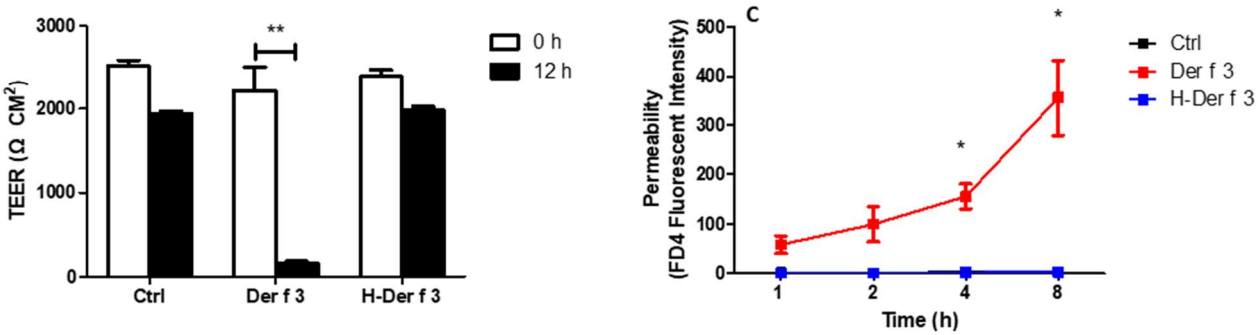
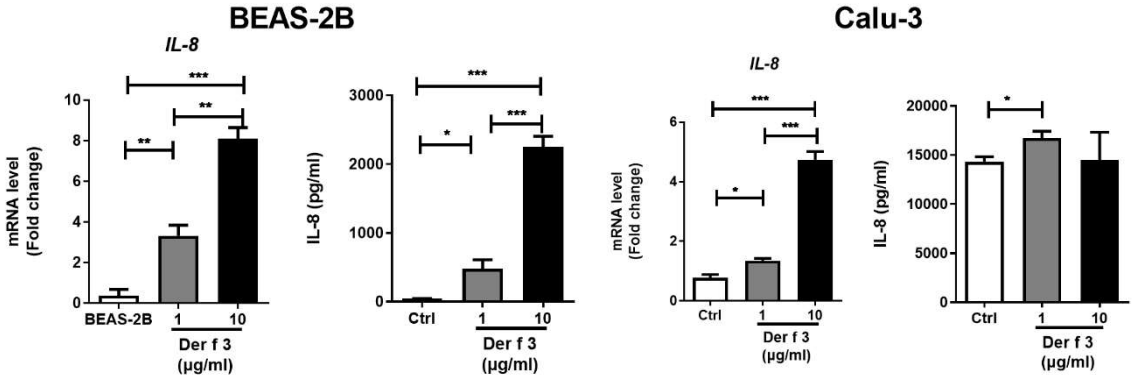
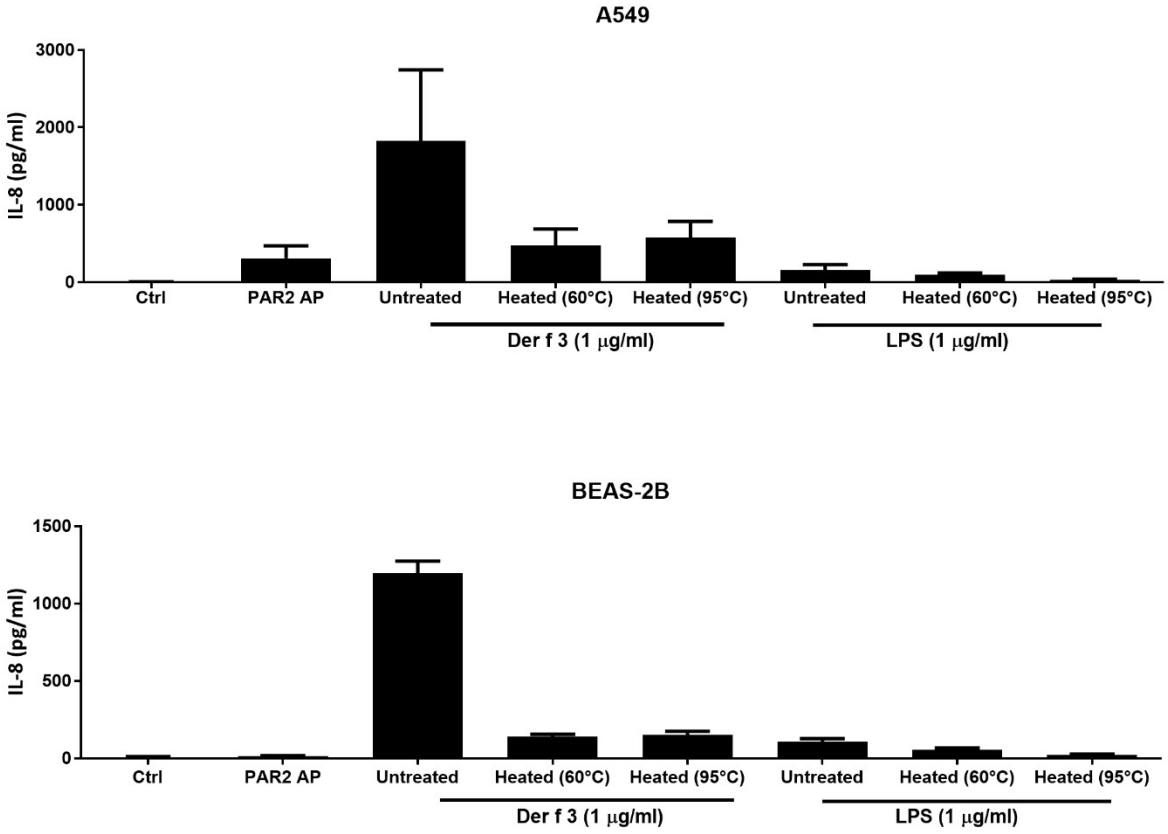


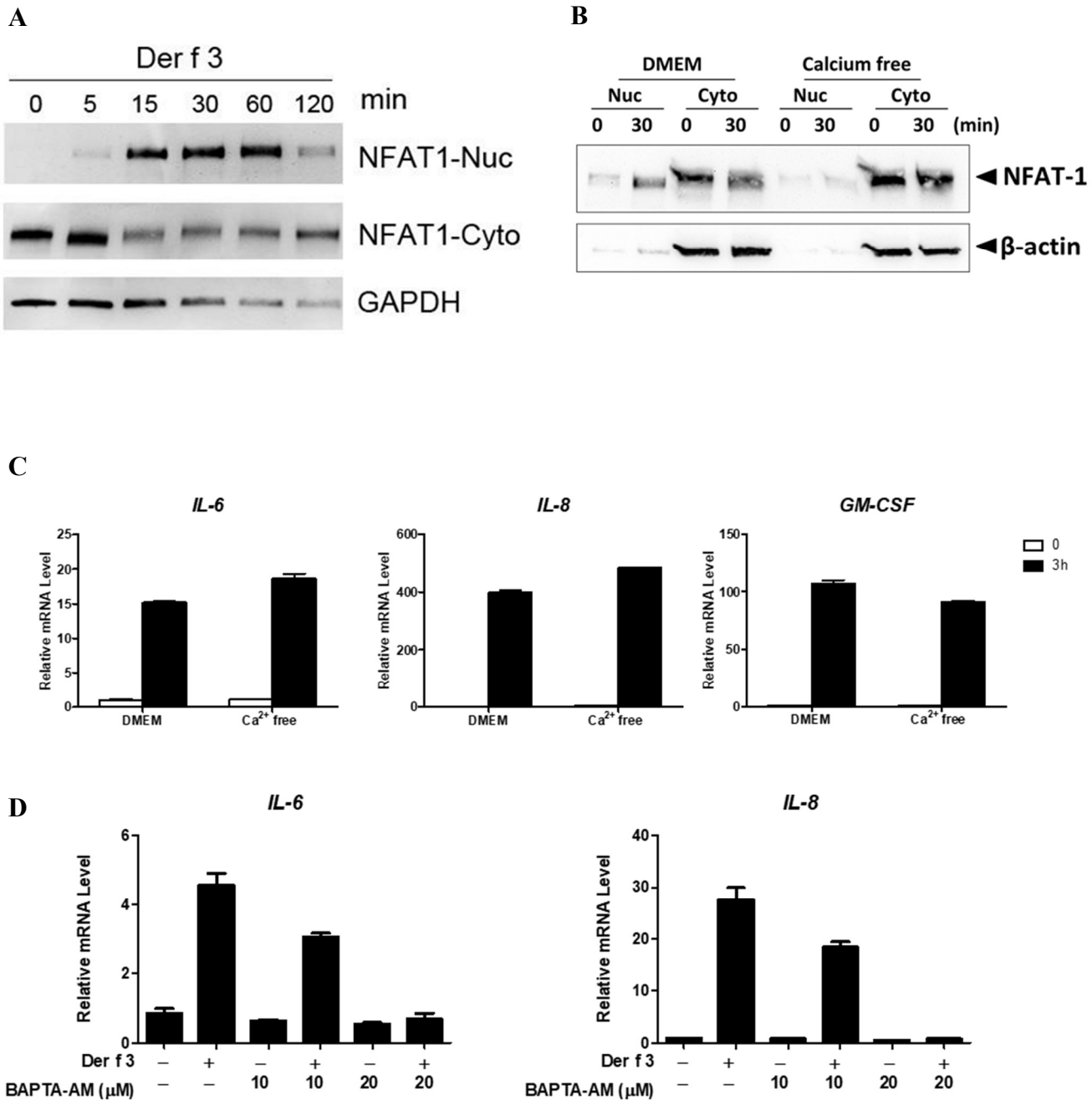
Supplementary Figure 3. BEAS-2B cells respond better to Der f 3 stimulation compared to Calu-3 cells.



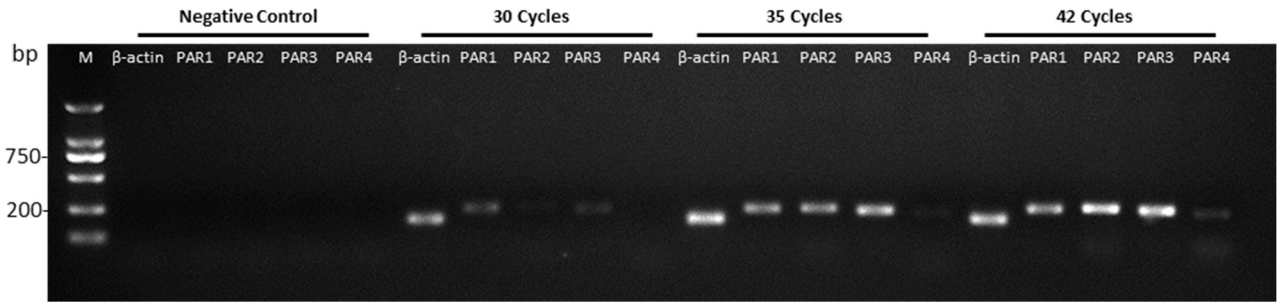
Supplementary Figure 4. AECs fail to respond to LPS in the serum-free condition.



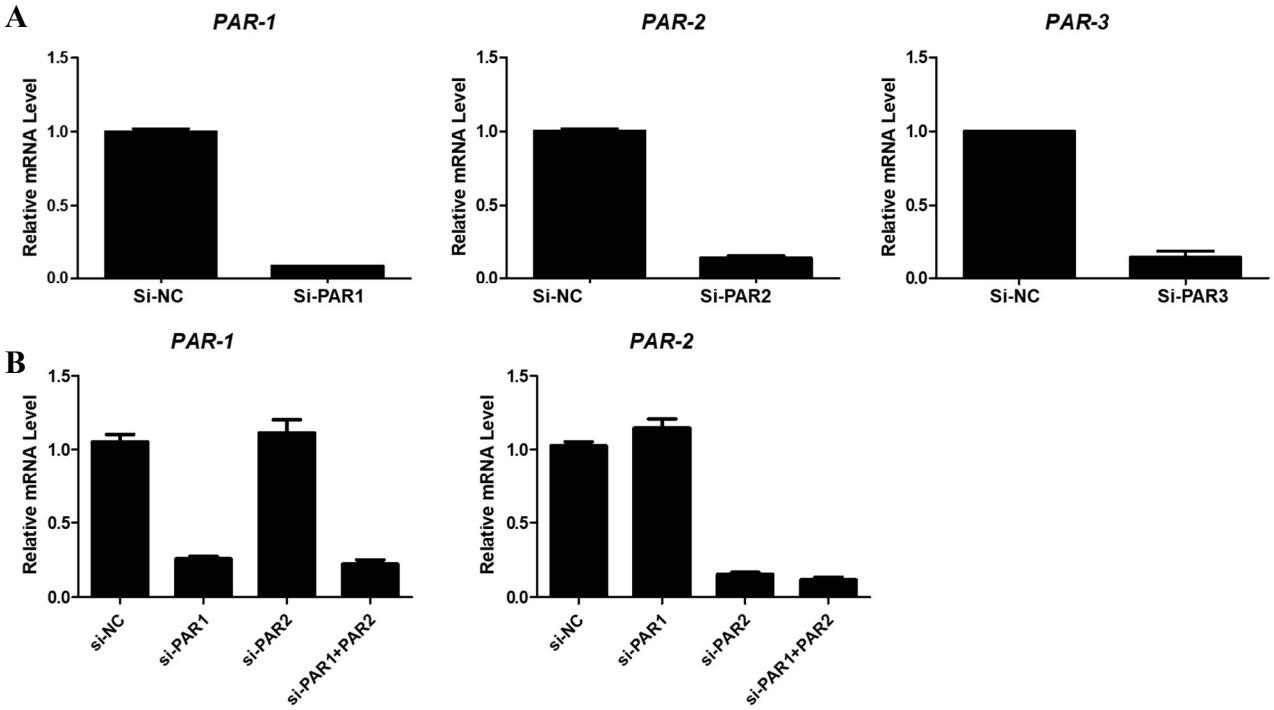
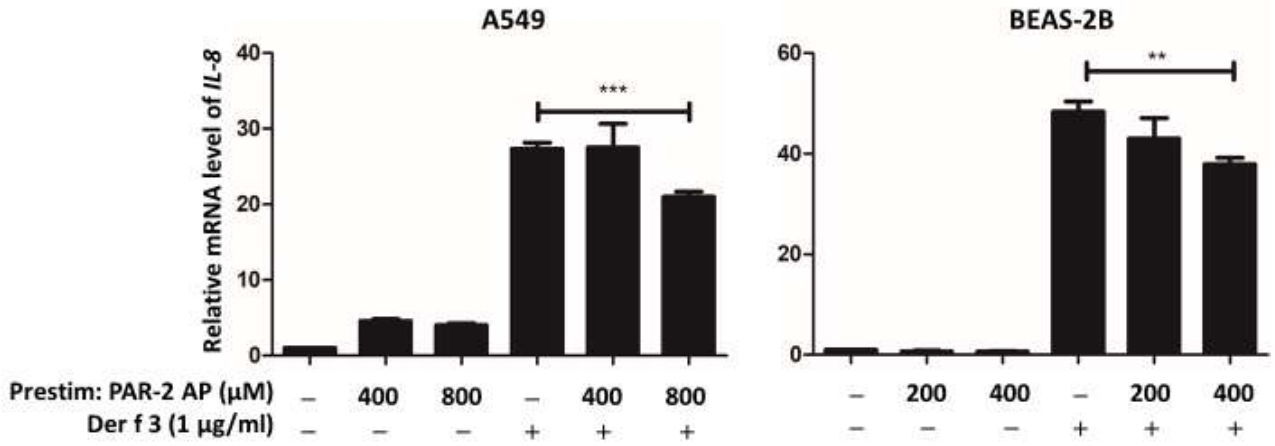
Supplementary Figure 5. Der f 3 induces IL-8 in a store-operated Ca^{2+}-dependent manner.



Supplementary Figure 6. The mRNA expression levels of PARs in BEAS-2B cells.



Supplementary Figure 7. Knockdown (KD) efficiency of siRNAs.



Supplementary Figure 8. Der f 3-induced cell activation is PAR-2 dependent.