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Allergen protease Der p1 engages protease-activated receptor 2 (PAR2) to program epithelial stress responses in lung inflammation

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Abstract

Background

Asthma is a chronic inflammatory airway disease in which house dust mite (HDM) allergens contribute to epithelial barrier disruption and airway inflammation through protease-mediated mechanisms, including protease-activated receptor 2 (PAR2) activation. SERPINB3, a cysteine protease inhibitor with reported protective functions in asthma, may modulate these responses. This study aimed to evaluate HDM and its major allergens, Der p1 and Der p2, in PAR2 signaling activation, PAR2-related pathways and SERPINB3 regulation.

Methods

Airway inflammation was induced in wild-type (WT) and *Par2* knockout (*Par2*KO) mice using HDM extract and Der p1 (high protease activity) and Der p2 (low protease activity) allergens, followed by transcriptomic and tissue-specific SERPINB3 analysis. Findings were validated in differentiated primary bronchial epithelial cells from healthy and asthmatic donors. Finally, *SERPINB3* was silenced using siRNA to evaluate its functional effects on caspase gene expression and activity.

Results

HDM, Der p1, and Der p2 induced pronounced lung inflammation and broad transcriptomic alterations, with Der p1 emerging as the predominant driver of SERPINB3 induction. Transcriptomic profiling revealed marked dysregulation of genes associated with immune responses, pulmonary vascular processes, and programmed cell death in WT mice. In contrast, these molecular changes were largely absent in *Par2*KO animals, indicating a critical role of PAR2 in shaping allergen-induced inflammatory and tissue-response programs. Notably, despite the PAR2-dependent nature of these transcriptomic alterations, SERPINB3 protein expression remained elevated in both *Par2*KO mice and in PAR2-blocked primary human epithelial cells, suggesting that SERPINB3 induction is regulated through a PAR2-independent mechanism. Functional silencing of SERPINB3 in primary human epithelial cells significantly increased Caspase-3 and Caspase-9 activity, particularly in cells from asthmatic donors, supporting its role in regulating apoptotic thresholds and epithelial survival.

Conclusion

In conclusion, our results reveal that proteolytic allergens elicit two functionally distinct epithelial programs in asthma, namely a PAR2-dependent inflammatory response and a PAR2-independent SERPINB3-mediated survival response. By limiting caspase-dependent epithelial cell death, SERPINB3 acts as a molecular checkpoint of epithelial resilience during allergen exposure. These findings redefine SERPINB3 as an active regulator of epithelial adaptation

rather than a passive marker of inflammation and highlight epithelial survival control as a potential therapeutic entry point in allergen-driven asthma.
Keywords: House dust mite, protease-activated receptor 2, SERPINB3, airway epithelial cells, asthma.

Introduction

Asthma is a complex, heterogeneous, chronic airway disease whose prevalence is gradually increasing worldwide [1-3]. It is characterized by epithelial barrier dysfunction, inflammatory cell recruitment, goblet cell hyperplasia and/or metaplasia, airway hyperresponsiveness, epithelial and smooth muscle cell hypertrophy and/or hyperplasia, as well as collagen deposition and mucus hypersecretion in the lung [4, 5]. Environmental factors and irritants, including cigarette smoke, diesel exhaust particles, respiratory viruses, bacteria, fungi, and aeroallergens, play critical roles in the onset, progression, and severity of asthma [6]. In addition, both endogenous and exogenous proteases are actively involved in asthma pathophysiology and warrant particular attention [7].

Environmental allergens such as house dust mite (HDM) induce epithelial damage in the lung in part through their proteolytic activity, which disrupts epithelial barrier integrity, cleaves junctional proteins, and activates protease-activated receptors (PARs). These events promote epithelial stress responses, alarmin release, and downstream inflammatory signaling, all of which contribute to airway inflammation and tissue remodeling [8, 9]. *Dermatophagoides pteronyssinus* allergen (Der p) 1 and Der p2 are the major immunodominant components of HDM, a ubiquitous environmental allergen. They have been shown to play a central role in initiating allergic sensitization, epithelial activation, and downstream type 2 inflammatory responses characteristic of allergic diseases, including allergic asthma [6]. In fact, Der p1, through its protease activity (specifically an endopeptidase), can modulate the immune system by activating specifically PAR2, disrupting epithelial tight junctions, and inducing the release of pro-inflammatory cytokines and chemokines [7, 10]. In response, the airway epithelium synthesizes protease inhibitors to counteract the allergen's potentially deleterious effects. Protease inhibitors have been shown to regulate inflammation, immune responses, mucus production, mucociliary clearance, extracellular matrix composition, and tissue remodeling,

among others [11]. A disruption in the balance of protease/protease-inhibitor can activate the immune system, triggering inflammatory responses through the production of cytokines, chemokines, and growth factors [12]. Serine protease inhibitors (SERPINs) constitute a superfamily comprising 37 members that regulate various physiological processes, including homeostasis, blood pressure, differentiation, and inflammation, as well as apoptosis [13].

SERPINB3, also known as squamous cell carcinoma antigen (SCCA1), is a member of the intracellular clade B SERPINs that can inhibit cysteine proteases [14]. Its expression level was found to be higher in neoplastic epithelial tissue and has been associated with differentiation of normal squamous epithelium [15]. In addition to its anti-proteolytic activity, SERPINB3 has also been shown to exert anti-apoptotic function [16, 17]. This activity was attributed to the interaction between SERPINB3 and the mitogen-activated protein (MAP) family of c-Jun terminal kinase 1 (JNK1) [18] or a reducing effect on the phosphorylation of proapoptotic p38 MAP kinase [19]. In addition, SERPINB3 has been shown to drive epithelial-mesenchymal transition (EMT) [20] and to increase transforming growth factor (TGF)-β expression, thereby promoting fibrogenesis [21, 22]. Besides, other studies have reported that SERPINB3 serum levels were higher in asthma and allergic diseases [23, 24], and it may exert a protective function in asthma [25, 26].

As HDM represents a major source of indoor allergens, sustained exposure to its proteolytic components may compromise airway epithelial barrier integrity, thereby perturbing pulmonary homeostasis. In this context, endogenous lung-derived protease inhibitors may serve as critical regulators of protease-driven inflammatory responses. Therefore, here we aimed to define the mechanistic contribution of PAR2 signaling to HDM-, Der p 1-, and Der p 2-induced lung inflammation and to SERPINB3 production *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animal Model

Experimental asthmatic airway inflammation

C57BL6/cmdb mice were exposed to HDM extract (Citeq Biologics; Figure 1A) or Der p1 or Der p2 allergens (both Cusabio; Figure 2A) varying in proteolytic activity [10, 27, 28]. Mice were challenged intranasally (i.n.) with 10 μg of HDM extract or with Der p1 or Der p2 allergens, each dissolved in 20 μl of saline, for five consecutive days in a two-week cycle, with a two-day break. The dose of Der p1 allergen (high proteolytic activity) was optimized based on its content in the HDM extract, and the same dose was applied for Der p2 (low proteolytic activity). Control mice received saline alone in the same final volume and according to the same schedule. Similarly, *Par2*KO (The Jackson Laboratories) mice with a C57BL/6 genetic background were challenged with Der p1 or Der p2 allergens in the experimental setup described above (Figure 3A). All mice were sacrificed 72 hours after the last challenge. Tissue samples were processed according to the optimized protocols. Procedures were performed with the approval of the Local Ethical Committee for Animal Experiments in Olsztyn, Poland (Approval number 31/2021; from April 21st, 2021, "Explanation of the role of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in the process of pulmonary remodeling in a chronic experimental model of asthma". Detailed information regarding the mouse model can be found in the Online Supplementary materials to this paper.

Total RNA isolation and transcriptome analysis

Total lung RNA was isolated as described previously [29, 30]. Lung lobes were stored in RNA later buffer (Invitrogen, USA) to preserve RNA integrity. Next, specimens were disrupted using TissueRuptor II (Qiagen) in RNA Lysis Buffer (RLT, Qiagen, USA) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich, USA). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. Only RNA with high integrity (RIN>8) was used for cDNA library preparation, following the TruSeq Stranded Total RNA protocol

(Illumina). The bioinformatic pipeline was detailed in the Online Supplementary Materials to this paper.

Immunofluorescence staining

Snap-frozen lung tissues were cryosectioned (Leica, Germany) at 8 μm and incubated in a blocking solution containing 10% goat serum, followed by overnight incubation with monoclonal rabbit anti-SERPINB3 antibody (clone 6J3U6; Invitrogen, cat. MA5-42597), at a dilution of 1:500 in 1% bovine serum albumin (BSA, Sigma) in PBS. SERPINB3 was detected using a goat anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (Invitrogen; cat. A-11035) at 1:2000 in 1%BSA in PBS. The nuclei were labeled with ProLong Gold Antifade Mountant with DNA Stain DAPI (Invitrogen). Slides were analyzed using a Leica Stellaris 5 confocal microscope (Leica). Subsequently, the level of SERPINB3 was quantified from TIFF files using ImageJ (NIH) according to the protocol detailed in the Online Supplementary materials to this paper [6, 12].

Cell culture

Primary human bronchial epithelial cells (HBECs) from healthy controls and asthmatic patients were purchased from Lonza (Switzerland) and Epithelix (Switzerland). Cells were grown in monolayers in bronchial epithelial basal medium (BEBM) supplemented with Bronchial Epithelial Cell Growth Medium Supplements and Growth Factors (Lonza). For the air-liquid interface (ALI) culture, HBECs were seeded onto transwell inserts with a pore size of 0.4 μm (Corning, USA) at a density of $1.5x10^5$ cells per insert and cells were differentiated as previously described [31]. Further information regarding cell culture experiments was given in the Online Supplementary Information file.

Low endotoxin (LoTox) Der p1 (Indoor Biotechnologies, USA) was initially used at concentrations of 2 and 10 μg/ml according to previous studies [28, 32], however 2 μg/ml concentration was chosen for *SERPINB3* knockdown experiments since at higher dose, cell

viability decreased below 40% in cytotoxicity assays and further cell death was observed during siRNA transfection and transwell experiments (Online Supplementary Figure 1).

Transient transfection

SERPINB3 gene expression was silenced by transfection with *SERPINB3* siRNA. Healthy and asthmatic primary HBECs were cultured in 24-well plates. After 70% confluency, the medium was discarded, and transfection medium (growth medium without antibiotics) was added to the wells. Cells were then transfected for 24 hours with a premix of *SERPINB3* siRNA and Lipofectamine 3000 reagent (Invitrogen) prepared in Opti-MEM (Gibco, USA) [33]. Negative control siRNA and Lipofectamine 3000 transfection agent controls were also included. Transfection efficiency was proven after each experiment by qPCR.

PAR2 Blocking

Healthy and asthmatic primary HBECs were maintained in ALI-culture for 21 days. On day 21, cells were incubated with PAR2 Antibody (SAM11, 20 µg/ml) (Santa Cruz Biotechnology, USA, cat. sc-13504) for 2 hours before stimulation with Der p1 allergen.

RNA isolation and gene expression analysis

Transfected and untransfected cells remained either unstimulated or were stimulated with 2 µg/ml of Der p1 for a duration of 24 hours. Total RNA was then isolated from the cells using RNeasy Mini Kit (Qiagen) according to the o manufacturer's instructions. RNA samples were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), and Real-time PCR was performed using the ABI 7500 Fast Real-time PCR system (Applied Biosystems, USA). mRNA levels were quantified using SYBR Green Master Mix (Thermo Fisher Scientific) [28, 33]. The primer list is given in Online Supplementary Table 1. Peptidylprolyl isomerase A (*PPIA*) was used as an internal reference to normalize the target transcripts using the $2^{-\Delta\Delta ct}$ method.

Western Blot analysis

Total protein was isolated using RIPA buffer (VWR, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by the Bradford method, and Western Blot analysis was performed as described previously [26, 33]. The mouse anti-SERPINB3 (1:1000, cat. sc-21767) and anti-PAR2 (1:1000 cat. sc-13504) antibodies were obtained from Santa Cruz Biotechnology. Antibody against β-actin (1:5000) (Sigma-Aldrich, clone: AC-15, cat. A5441) was used for normalization. HRP-conjugated goat anti-mouse IgG (1:10,000) secondary antibody was purchased from Jackson ImmunoResearch (UK, cat. 111-035-144). The membranes were visualized using an enhanced chemiluminescence kit on a ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Further details were given in the Online Supplementary Information file.

Caspase activity assay

Activity of caspases was measured by Caspase-3, Caspase-8, and Caspase-9 Multiplex Activity Assay Kit (Abcam, USA, cat. ab219915) according to the manufacturer's instructions. Briefly, healthy and asthmatic primary HBECs were seeded into a black, clear-bottom 96-well plate. After 70% confluency, cells were transfected with *SERPINB3*-siRNA and then stimulated with 2 μg/ml Der p1 for 24 hours. Caspase activities were measured as previously described [33, 34]. The protocol is detailed in the Online Supplementary Information file.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.10. Statistical significance among groups was evaluated using a one-way ANOVA followed by a post hoc Fisher test. A p-value of <0.05 was considered significant.

RESULTS

HDM induces PAR2 signaling and SERPINB3 expression in experimental asthmatic lung inflammation

Here, we used a previously described model of HDM-induced asthmatic lung inflammation, which mimics mixed granulocytic lung inflammation (Figure 1A) [12, 29, 35]. First, we confirmed lung inflammatory infiltrate and mucus overproduction in lung tissue (Figure 1B). Next, we aimed to explore the activation of specific molecular pathways involved in lung remodeling. In particular, we focused on the PAR2-associated signaling cascade, given its known role in protease-mediated responses in the asthmatic airway. Additionally, we sought to determine whether SERPINB3 exhibits differential expression in our model. This approach allowed us to assess the potential interplay between PAR2 activation and SERPINB3 expression in the context of allergic airway inflammation.

Activation of PAR2 initiates multiple intracellular signaling cascades, including NF-κB MAPK/ERK, as well as PI3K/Akt pathways, promote inflammation, epithelial barrier disruption, and tissue remodeling [36-40]. We observed that HDM exposure of mouse airways significantly upregulated the majority of genes associated with the canonical NF-κB signaling pathway (Figure 1C) and the ERK1/ERK2 cascade (Figure 1D). Moreover, we found significant dysregulation of genes associated with biological processes orchestrated by PAR2 activation, namely regulation of immune system processes (Figure 1E), lung vasculature development (Figure 1F), and programmed cell death (Figure 1G). Finally, we found that HDM-induced asthmatic lung inflammation significantly enhanced SERPINB3 expression in both epithelial and subepithelial regions (Figure 1H).

Airway exposure to Der p1 and Der p2 induces similar patterns of transcriptional changes

Having found alterations in PAR2-related pathways and increased SERPINB3 expression in the HDM-induced experimental asthma model, we next aimed to determine whether these effects were attributable to the complex nature of the HDM extract or specifically to its protease activity. Therefore, Der p1 and Der p2 recombinant allergens, with high and low protease activity, respectively, were used to stimulate WT mice (Figure 2A). First, we assessed

lung inflammatory infiltrate and mucus production (Figure 2B). We found a low inflammatory infiltrate after Der p1 exposure and relatively higher inflammation in Der p2-stimulated counterparts. Moreover, we observed increased mucus production in both allergen-exposed groups. To further investigate the underlying mechanisms, we next assessed changes in PAR2-related pathways. Surprisingly, transcriptomic analysis of lung tissue revealed similar alterations in gene expression profiles following allergen exposure, irrespective of the strength of proteolytic activity (Online Supplementary Figure 2A). This included canonical NF-κB signal transduction (Figure 2C, Online Supplementary Figure 2B), ERK1 and ERK2 cascade (Figure 2D, Online Supplementary Figure 2C), regulation of immune system processes (Figure 2E, Online Supplementary Figure 2D), lung vasculature development (Figure 2F, Online Supplementary Figure 2E), and programmed cell death (Figure 2G, Online Supplementary Figure 2F). Interestingly, however, protein expression of SERPINB3 in lung epithelium was higher after Der p1 exposure when compared to control and Der p2-exposed animals (Figure 2H). Our findings indicate that genes associated with PAR2-related signaling pathways may be regulated even by low levels of allergen protease activity. However, we demonstrated that high protease activity induces higher SERPINB3 expression at the protein level.

PAR2 signaling regulates SERPINB3 expression in bronchial epithelium

Once we revealed the transcriptomic profile of PAR2-related pathways following exposure to major HDM allergens with varying proteolytic activities, we next investigated whether PAR2 signaling is essential for SERPINB3 expression by employing *Par2*KO mice (Figure 3A). First, we evaluated lower airway inflammation and found low-grade inflammatory infiltrate in the lung tissue, mostly localized around the blood vessels (Figure 3B). As expected, the previously observed robust dysregulation of genes associated with PAR2-related processes in WT mice after Der p1 and Der p2 exposure was not detected in *Par2*KO mice (Figure 3C-

E). However, dysregulation of genes associated with angiogenesis (Figure 3F and Online Supplementary Figure 3A, B) and apoptotic processes (Figure 3G and Online Supplementary Figure 3C, D) was still observed with significant differences among the used stimulators. More specifically, exposure of mice to Der p2 (an allergen with low protease activity) significantly downregulated a higher number of genes associated with angiogenesis compared to Der p1-treated counterparts (Figure 3F and Online Supplementary Figure 3A, B). Moreover, lung exposure to allergen with high protease activity (Der p1) induced upregulation of genes associated with apoptosis compared to Der p2 (Figure 3G and Online Supplementary Figure 3C, D).

Finally, and surprisingly, we observed that knocking out the PAR2 gene significantly affected SERPINB3 protein expression in unstimulated mice (Der p1 or Der p2), suggesting that PAR2 signaling may regulate basal SERPINB3 production in the bronchial epithelium (Figure 3H). Specifically, the absence of PAR2 was associated with increased SERPINB3 expression under NaCl treatment (control), suggesting that PAR2 signaling may limit SERPINB3 expression in the absence of allergen exposure. However, following allergen stimulation, the percentage of SERPINB3-positive epithelial cells appeared comparable between WT and *Par2*KO mice, indicating that allergen-induced SERPINB3 protein expression is largely independent of PAR2 signaling. Notably, exposure to Der p1 resulted in higher SERPINB3 expression compared with Der p2-exposed counterparts, suggesting that SERPINB3 protein production may be more strongly associated with the high proteolytic activity of Der p1 rather than PAR2-dependent signaling alone. Thus, although HDM allergen proteases can activate PAR2 and modulate PAR2-associated gene expression profiles, our *in vivo* findings suggest that SERPINB3 protein expression is not strictly dependent on PAR2 signaling and may be regulated by additional protease-driven mechanisms.

Der p1-mediated *SERPINB3* upregulation occurs via a PAR2-independent pathway in human asthmatic airway epithelial cells

Having found that Der p1 was the major HDM allergen causing SERPINB3 overproduction, next we wished to analyze the responses of fully differentiated HBECs derived from healthy (non-asthmatic, non-atopic) and asthmatic individuals to Der p1 protease activation *in vitro*. First, we assessed *SERPINB3* gene expression in response to Der p1 and found that both healthy and asthmatic cells showed a significant increase in *SERPINB3* expression (Figure 4A). However, at the protein level, we observed that only asthmatic HBECs had upregulated SERPINB3 expression (Figure 4B). More importantly, we found that cells treated with a PAR2-blocking antibody (SAM11) showed elevated SERPINB3 protein expression compared with unblocked counterparts (Figure 4B). Notably, even low levels of proteolytic activity from allergens are sufficient to activate PAR2. These observations are consistent with our observations in *Par2*KO mice. Antibody-mediated PAR2 blockade in cell culture experiments demonstrated significant efficacy, as it suppressed Der p1-mediated ERK1/2 phosphorylation (Online Supplementary Figure 4A, B) and IL-8 production (Online Supplementary Figure 4C). Moreover, our findings from the *in vivo* knockout model provide evidence that *Par2*KO is compensated by a significant increase in SERPINB3 protein expression in pulmonary tissues (Figure 4C). Based on these results, we found that PAR2 blockade did not modulate *SERPINB3* gene expression in human primary epithelial cells.

Derp1-mediated caspase gene expression profiles differed in healthy and asthmatic HBECs after *SERPINB3* knockdown

Based on transcriptomic analysis and cell culture results, we sought to determine whether the known anti-apoptotic effect of PAR2 may result from PAR2-mediated *SERPINB3* overexpression and its associated overactivation. Therefore, we analyzed the effect of Der p1 stimulation on the expression of *CASP3*, *CASP8*, and *CASP9* in healthy and asthmatic primary

HBECs. We first confirmed that siRNA-mediated knockdown of *SERPINB3* expression is effective after Der p1 activation in both asthmatic and healthy HBECs (Figure 5A). Der p1 exposure increased *CASP3* and *CASP8* in fully differentiated healthy primary HBECs compared to unstimulated cells ($p<0.05$) (Figure 5B). Surprisingly, this induction was reduced in *CASP8* but not in *CASP3*. *SERPINB3* gene silencing caused an induction in C*ASP3* and *CASP9* transcription ($p<0.05$), however, Der p1 stimulation in *SERPINB3*-silenced cells reversed the effect observed in *CASP9*. In asthmatic primary cells, Der p1 stimulation showed a similar increase in *CASP8* expression ($p<0.001$). Surprisingly, *SERPINB3* gene silencing increased the expression of all caspase genes, but this effect was reduced by Der p1 stimulation, with only *CASP3* expression showing a significant increase. Notably, no changes in caspase gene expression were observed in cells transfected with scrambled siRNA (Online Supplementary Figure 5).

We found that Der p1 stimulation elevated Caspase-3 activity in both healthy and asthmatic HBECs (Figure 5C). In fully differentiated cells isolated from healthy donors, *SERPINB3* gene silencing alone increased Caspase-3 activity by up to 3-fold, but this change did not reach statistical significance. *SERPINB3* knockdown suppressed the alterations in Caspase-3 activation mediated by Der p1 stimulations in both groups. We did not observe any change in Caspase-8 activity in both healthy and asthmatic groups, although the trend in activity was similar to the gene expression profile. Caspase-9 activity did not change in the healthy group after stimulations, but it increased in *SERPINB3* knockdown-asthmatic cells when stimulated with Der p1 allergen.

DISCUSSION

In this study, we explored SERPINB3 involvement in an airway inflammation mouse model induced by HDM or its major allergens, Der p1 or Der p2, as well as the role of the PAR2 receptor in these responses. We showed that HDM and its major allergens effectively modulated PAR2-related pathways, including ERK1/2 and NF-κB signaling cascades, lung vasculature

development, immune system regulation, and programmed cell death. HDM significantly induced SERPINB3 protein production in bronchial epithelium. A similar response was observed in the Der p1-induced mouse model but not in the Der p2-induced model, suggesting that increased protease activity may contribute to SERPINB3 overproduction in the lung. We also found that lung inflammation and the inflammatory transcriptomic profile induced by Der p1 and Der p2 allergens were attenuated by PAR2 genetic deletion, whereas *Par2*KO did not completely reverse the allergens' effects on cell death pathways. Then, since our *in vivo* findings represent whole lung tissue involving various cell types, we next focused on the HBECs point of view and investigated the potential apoptotic effect of *SERPINB3* on Der p1-stimulated healthy and asthmatic primary cells. We indicated that *SERPINB3* knockdown alone induced caspase gene expression in our *in vitro* experimental setting, supporting the established protective role against cell death; however, interestingly, it can also suppress executioner caspase-3 activation induced by Der p1 in both groups.

HDM exposure in mice has repeatedly been shown to result in aberrant cytokine and chemokine production, immune cell infiltration, mucus overproduction, and subepithelial collagen deposition in the lung tissue [12, 41, 42]. HDM-induced inflammation has been shown to occur through activation of various inflammatory pathways and signaling cascades, including NF-κB, ERK1/ERK2, JNK, and MAPK, all of which can be triggered by PAR2 activation. Similarly, our findings indicated that HDM exposure established a unique signature, characterized by modulation, especially of canonical NF-κB and ERK1/ERK2 signaling. We observed that HDM also regulated lung vasculature development, which represents a hallmark of lung remodeling [43]. Given the alterations in PAR2-related pathways and the upregulation of SERPINB3 observed in the HDM-induced asthma model, we aimed to determine whether these responses were driven by the multifaceted composition of the HDM extract or specifically by its protease activity. For this purpose, we used major HDM allergens, namely Der p1 (high

proteolytic activity) and Der p2 (low proteolytic activity) for inflammation, employing the same experimental design of the HDM-induced asthma model. Although both allergens provoked immune cell infiltration in the lung, interestingly, low-proteolytic Der p2 caused relatively more robust inflammation compared to Der p1. This suggests that the magnitude of cellular inflammation is not solely determined by allergen protease activity. While Der p1 may predominantly act through protease-dependent mechanisms, including epithelial barrier disruption and PAR2 activation, Der p2 may promote inflammation through protease-independent pathways. In fact, Der p2 has been reported to mimic myeloid differentiation factor-2 (MD-2) and facilitate Toll-like receptor 4 (TLR4) signaling. Thus, Der p2-driven inflammation may be mediated, at least in part, by innate immune receptor activation rather than direct proteolytic activity [44-46]. Consistent with the literature, PAS staining in our experimental setup also revealed similar patterns of elevated mucus production both in Der p1 and Der p2 groups compared to controls, mirroring the phenotype observed in the HDM-induced model. Collectively, these findings indicated that both allergens contributed to airway inflammation and mucus production regardless of their protease activity, which was reversed by *Par2*KO.

Interestingly, transcriptomic analysis further supported this convergence; airway exposure to Der p1 and Der p2 allergens established similar patterns in gene expression profiles in canonical NF-κB signal transduction, regulation of immune system processes, lung vasculature development, and programmed cell death. Despite the similar inflammatory profiles, only exposure to high proteolytic Der p1 allergen induced SERPINB3 protein production, which was notably absent in Der p2 group, suggesting that SERPINB3 induction is a specific signature of proteolytic injury. *Par2*KO in the control group induced SERPINB3 protein production up to 6-fold in the bronchial epithelium compared to WT mice. A possible explanation is that PAR2 signaling exerts a homeostatic restraining effect on *SERPINB3*

expression in the human bronchial epithelium. In this scenario, *Par2*KO would remove this stable inhibitory signal, leading to spontaneous activation of epithelial protective or stress-response pathways and increased SERPINB3 production in mice. This does not necessarily contradict the Der p1-induced increase in SERPINB3, as allergen-driven induction may occur via PAR2-independent mechanisms associated with proteolytic epithelial injury. Consistent with our mouse results, *in vitro* experiments showed that Der p1 stimulation increases *SERPINB3* transcription in both healthy and asthmatic HBECs. Therefore, our findings are consistent with previous reports showing that Der p1 can modulate the expression of epithelial protease inhibitors, including SERPINB3/SERPINB4 [47]. However, although antibody-mediated PAR2 blockade in cell culture experiments demonstrated significant efficacy, our *in vitro* findings with fully differentiated HBEC cultures revealed that antibody-mediated PAR2 blockade did not affect Der p1-mediated SERPINB3 protein production in the asthmatic group, similar to the results obtained in mouse experiments. Der p1 may induce SERPINB3 through additional PAR2-independent epithelial stress pathways, which could compensate for complete or partial PAR2 blockade. Given the potent cysteine protease activity of Der p1 [47, 48], the observed upregulation of SERPINB3, a cysteine protease inhibitor, may represent a protective cellular response aimed at counteracting its detrimental proteolytic effects of Der p1.

The contribution of HDM in cell death pathways is well studied [49-51]. In line with the literature, our transcriptomic analysis reveals distinct regulated cell death (RCD) landscapes shaped by the high- or low- proteolytic components of HDM. In our experimental design, HDM, Der p1, and Der p2 exposure shared a consistent transcriptional signature marked by strong upregulation in *Gsdme*, *Nlrp1a*, *Relt*, *Spata2,* and the DNA damage sensor *Trp53*. These findings suggest that GSDME-dependent pyroptosis, triggered downstream of caspase-3 activation, a key mechanism of airway epithelial barrier dysfunction and inflammation, potentially linking apoptotic and lytic cell death pathways [52-55]. The consistent reduction in

Rnf130 gene expression across all groups further implies a common impairment in membrane protein homeostasis [56]. However, the divergence in downstream effectors highlights the distinct pathogenic mechanisms of the allergens. Interestingly, Der p1 exposure led to the selective downregulation of key necroptotic mediators, namely *Ripk1* and *Mlkl* [57, 58]. This finding implies that highly proteolytic Der p1 may attenuate canonical necroptosis, potentially by cleaving key upstream components of the necroptotic pathway, thereby redirecting epithelial cell death towards GSDME-dependent lytic cell death [12]. This interpretation may be further supported by our previous observations showing that necroptosis of structural cells becomes detectable only after prolonged HDM exposure, whereas it is absent in short-term exposure models [12]. Thus, Der p1-driven suppression or delay of canonical necroptotic signaling could represent an early, context-dependent mechanism that precedes the emergence of necroptosis during chronic allergen-induced airway remodeling. However, this remains speculative, and the hypothesis proposed here requires further experimental validation.

In contrast, Der p2, acting as an MD-2 mimetic/TLR4 agonist with lower protease activity [59], may induce a broader RCD response marked by upregulation of *Ninj1*, a critical mediator of plasma membrane rupture [60]. Surprisingly, although there was still a concomitant differential gene expression associated with RCD pathways in *Par2*KO mice after Der p1 and Der p2 exposure, *Par2*KO markedly altered the lung transcriptomic responses to both Der p1 and Der p2, which were broadly redirected toward a tissue-protective phenotype, despite the distinct biochemical properties of these allergens. PAR2 deficiency attenuated cell death programs linked to lytic cell death and tissue remodeling, which are central drivers of allergen-induced airway pathology rather than mere bystanders of inflammation. In the context of Der p1 exposure, PAR2 deficiency favored induction of epithelial defense and immune regulatory signatures, suggesting uncoupling of protease-driven epithelial stress from downstream inflammatory and remodeling cascades, a process previously implicated in allergic sensitization

and chronic airway disease [61]. Similarly, following Der p2 challenge, PAR2 knockout dampened immune effector and remodeling-associated transcriptional programs, consistent with the notion that Der p2 can initiate innate sensing through TLR4-dependent mechanisms, while PAR2 is required for full pathological propagation of these signals [44]. The emergence of damage-limiting and regulatory transcriptional profiles in the absence of PAR2 further supports that PAR2 may function as an amplifier rather than a primary initiator of allergen-induced lung inflammation. Collectively, all these findings position PAR2 as a central player in allergen-associated pathobiology and underscore its critical role in driving tissue-damaging immune responses in the airway. Although Der p2 is previously regarded as non-proteolytic, its ability to function as an MD-2 mimic and to facilitate TLR4 signaling provides a potent mechanism for innate immune activation independent of classical protease activity. Notably, the low but detectable proteolytic activity observed in our study may potentially contribute to epithelial stress and PAR2 engagement, thereby triggering downstream inflammatory and tissue-damaging events beyond those driven by TLR4 signaling alone.

Having established that the HDM-derived allergens exerted a unique function in cell death pathways in mice, which could not be significantly suppressed by *Par2*KO, we next sought to investigate the potential involvement of *SERPINB3* in the expression and activity of apoptotic markers in HBECs. Our *in vitro* findings in primary HBECs indicated that Der p1 modulates apoptotic pathways through a complex interplay between caspase regulation and *SERPINB3* expression, rather than through uniform suppression of cell death. Der p1 stimulation increased *CASP3* and *CASP8* expression and Caspase-3 activity in both healthy and asthmatic epithelial cells, suggesting a role in apoptosis-related signaling in response to allergen-induced epithelial stress. Notably, *SERPINB3* gene silencing alone led to broad induction of caspase gene expressions, supporting the previous findings of SERPINB3 as a negative regulator of apoptosis and caspase-mediated cell death and a mediator of epithelial

cell survival under stress conditions in epithelial cells [17, 18, 62]. In contrast, the observation that Der p1 stimulation partially reversed specific caspase responses in *SERPINB3*-silenced cells suggests that Der p1 may activate compensatory survival or stress pathways that can take place independently of SERPINB3. This is further supported by the observation that *SERPINB3* knockdown suppressed Der p1-induced Caspase-3 activity despite increased basal caspase transcription, supporting the notion that epithelial responses to Der p1 can favor modulation of upstream events of the apoptotic process rather than executing full apoptotic pathways. Together, these results support a model in which Der p1 induces epithelial stress responses that engage apoptotic signaling while potentially activating SERPINB3-dependent and -independent mechanisms to limit excessive caspase activation, thereby favoring epithelial survival over robust apoptosis in the airway barrier. Moreover, Der p1–induced modulation of apoptotic and non-apoptotic cell death pathways in epithelial cells may reflect a balance between protease-driven injury and compensatory survival mechanisms aimed at preserving barrier integrity [63].

Several limitations should be considered when interpreting the inflammatory outcomes of this study. First, transcriptomic analyses performed in mouse lung tissue reflect the integrated response of multiple resident and infiltrating cell populations, whereas gene expression analyses in primary cell experiments were restricted to HBECs, limiting direct comparability between *in vivo* and *in vitro* inflammatory signatures. Moreover, *SERPINB3* in humans has four orthologs in mice; *Serpinb3a*, *Serpinb3b*, *Serpinb3c*, and *Serpinb3d*. While the antibody used in this study was chosen to selectively detect *Serpinb3a*, the murine homolog of human *SERPINB3*, the high degree of sequence similarity within this serpin subfamily may permit cross-reactivity with other closely related orthologs [64]. In addition, although primary cells were selected based on parameters including age, sex, race, history of atopy/asthma, and smoking status, their underlying genetic and disease background was unknown, introducing

donor-dependent variability that could not be fully controlled. Despite the inclusion of multiple donors per group, inter-individual heterogeneity resulted in variability across experiments, complicating the interpretation of the alteration in gene expression. Finally, the differences between murine and human systems, particularly in inflammatory signaling and receptor regulation, should be considered when extrapolating these findings across models.

In conclusion, HDM and its major allergens Der p1 and Der p2 can potentiate inflammatory and transcriptomic alterations in the lung, accompanied by increased mucus production and epithelial stress responses. While both major allergens promoted airway inflammation, Der p1 was identified as the primary driver of SERPINB3 protein induction in the bronchial epithelium in mice. *Par2*KO substantially reduced allergen-induced inflammatory changes and attenuated modulation of multiple inflammatory pathways, indicating a prominent role for PAR2 in modulating allergen-mediated lung inflammation. Although alterations in programmed cell death pathways were still detectable in PAR2-deficient mice, the affected pathways were consistently shifted towards epithelial defense and controlled stress adaptation rather than lytic cell death and mitochondrial stress, which is observed in WT mice. Notably, modulation of SERPINB3 expression was not modulated by PAR2 status in both *in vivo* and *in vitro* settings, suggesting that SERPINB3 protein expression is not solely dependent on PAR2 signaling and may instead be regulated through additional protease-mediated mechanisms.

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Author contributions

Cell culture experiments, data analysis and validation - BES, DK, HAB; mice experiments preparation, data collection, data analysis and validation - AW, AT, MT, MS, JR-G, AE; bioinformatic analysis, data presentation - AJ, KB; NGS analysis - MN, AK; data analysis, funds acquisition, data validation - CK, MM, AE; conceptualization and study design - CK, AE, MM; manuscript preparation - BES, DK, AE, CK. All authors read, edited, and approved the final version of the manuscript.

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Figure Legends

Figure 1. HDM exposure altered overall airway inflammatory status and PAR2-related pathways and SERPINB3 protein production in the mouse lung tissue. (A) Graphical summary

of the HDM-induced lung inflammation mice model used in the study. The representative (B) hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) stainings of lung tissue. (C-G) Summary of transcriptomic profiles of the lung. (H) The representative immunofluorescence stainings of SERPINB3 in bronchial epithelium. The heatmap shows all differentially expressed genes (DEGs) among the analyzed groups. The changes in the gene expression for log2FC > 0.5 or log2FC < −0.5 were considered as upregulated (marked as gray) or down-regulated (marked as yellow), while the changes below the cut- off were recognized as not- changed (marked in white). Wald test with Benjamini-Hochberg correction was used ($n = 4$ mice for control and n=5 mice for HDM-exposed group). ***padj < 0.001;**padj < 0.01, *padj < 0.05.

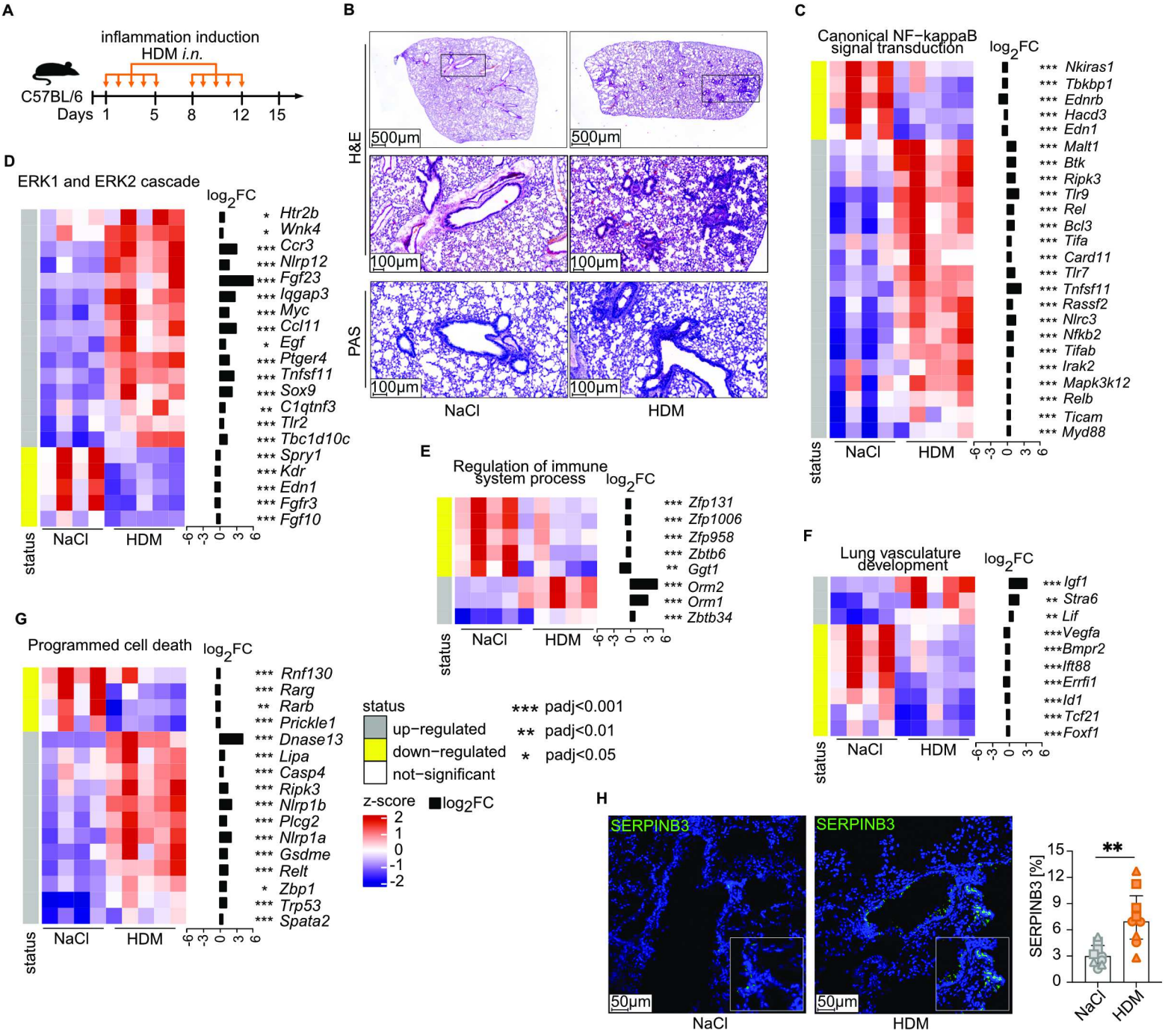
Figure 2. Der p1 and Der p2 allergens established similar inflammatory profiles regardless of their proteolytic ability, while only Der p1 with high protease activity induced SERPINB3 production in bronchial epithelium. (A) Graphical summary of Der p1 or Der p2-induced lung inflammation mice models used in the study. The representative (B) hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) stainings of lung tissue. (C-G) Summary of transcriptomic profiles of the lung. (H) The representative immunofluorescence stainings of SERPINB3 in bronchial epithelium in response to Der p1 or Der p2 exposure. The heatmap shows all differentially expressed genes (DEGs) among the analyzed groups. The changes in the gene expression for log2FC > 0.5 or log2FC < −0.5 were considered as upregulated (marked as gray) or down-regulated (marked as yellow), while the changes below the cut- off were recognized as not- changed (marked in white). Wald test with Benjamini-Hochberg correction was used ($n = 4$ mice for control and n=5 mice for allergen-exposed groups). ***padj < 0.001;**padj < 0.01, *padj < 0.05.

Figure 3. *Par2* genetic deletion can attenuate Der p1 or Der p2-mediated lung inflammation and epithelial response related to the PAR2 signaling pathway, while SERPINB3 protein production is not strictly dependent on PAR2 signaling. (A) Graphical summary of Der p1 or Der p2-induced lung inflammation in *Par2*KO mice models used in the study. The representative (B) hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) stainings of lung tissue. (C-G) Summary of transcriptomic profiles of the lung. (H) The representative immunofluorescence stainings of SERPINB3 in bronchial epithelium in response to Der p1 or Der p2 exposure. The heatmap shows all differentially expressed genes (DEGs) among the analyzed groups. The changes in the gene expression for log2FC > 0.5 or log2FC < −0.5 were considered as upregulated (marked as gray) or down-regulated (marked as yellow), while the changes below the cut- off were recognized as not- changed (marked in white). Wald test with Benjamini-Hochberg correction was used ($n = 5$ mice per group). ***padj < 0.001;**padj < 0.01, *padj < 0.05.

Figure 4. Der p1 allergen induces SERPINB3 gene and protein expression in human primary bronchial epithelial cells via PAR2-independent mechanisms. (A) SERPINB3 gene expression levels after 24-hour Der p1 stimulation at 2 and 10 μg/ml concentration in healthy (blue) and asthmatic (orange) HBECs. (B) Representative Western Blot membrane of SERPINB3, PAR2, and housekeeping protein β-actin, and summary of quantification results (Red bar represents SERPINB3, while blue bar represents PAR2 protein). SAM11 (monoclonal PAR2 antibody, 1/10) was used to block PAR2 in HBECs. (C) The percentage of immunofluorescence stainings of SERPINB3 in lung tissues of wild-type (WT) and *Par2*KO mouse after Der p1 stimulation.

One-way ANOVA with post hoc Fisher's test was used to analyze differences among groups (n=3). ****$p < 0.0001$;***$p < 0.001$

Figure 5. Der p1 stimulation (2 μg/ml) and *SERPINB3* knockdown established similarly patterns in caspase gene expression and activity levels in healthy (blue) and asthmatic (orange) HBECs after 24 hours. (A) *SERPINB3* gene expression levels after Der p1 stimulation and/or *SERPINB3* siRNA transfection. (B) Gene expression of *CASP3*, *CASP8*, and *CASP9* and (C) caspase activity levels after Der p1 stimulation and/or *SERPINB3* siRNA transfection. One-way ANOVA with post hoc Fisher's test was used to analyze differences among groups (n=3). ****$p < 0.0001$;***$p < 0.001$;**$p < 0.01$; *$p < 0.05$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Days 1 5 8 12 15
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Canonical NF−kappaB signal transduction
log2FC
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*** Tbkbp1
*** Ednrb
*** Hacd3
*** Edn1
*** Malt1
*** Btk
*** Ripk3
*** Tlr9
*** Rel
*** Bcl3
*** Tifa
*** Card11
*** Tlr7
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*** Rassf2
*** Nlrc3
*** Nfkb2
*** Tifab
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*** Tlr2
*** Tbc1d10c
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*** Zfp1006
*** Zfp958
*** Zbtb6
** Ggt1
*** Orm2
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** Stra6
** Lif
***Vegfa
***Bmpr2
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***Errfi1
***Id1
***Tcf21
***Foxf1
status
NaCl
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Programmed cell death
log2FC
*** Rnf130
*** Rarg
** Rarb
*** Prickle1
*** Dnase13
*** Lipa
*** Casp4
*** Ripk3
*** Nlrp1b
*** Plcg2
*** Nlrp1a
*** Gsdme
*** Relt
* Zbp1
*** Trp53
*** Spata2
status
NaCl
HDM
-6 -3 0 3 6
status
up−regulated
down−regulated
not−significant
*** padj<0.001
** padj<0.01
* padj<0.05
z-score
log2FC
2
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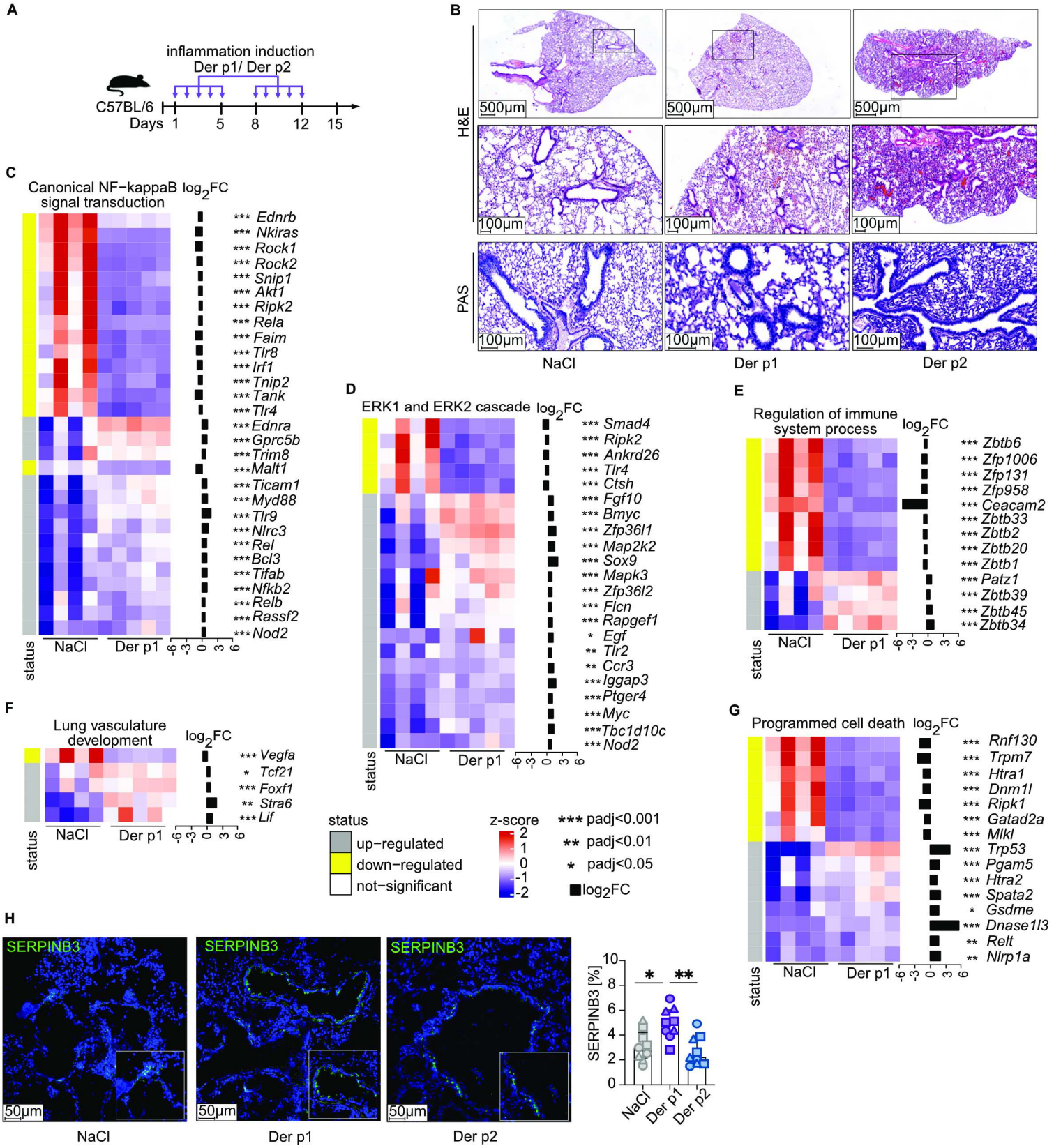
HDM
SERPINB3 [%]
0 3 6 9 12 15
**
NaCl
HDM

Figure 1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inflammation induction
Der p1/ Der p2
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Canonical NF−kappaB signal transduction
log2FC
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*** Irf1
*** Tnip2
*** Tank
*** Tlr4
*** Ednra
*** Gprc5b
*** Trim8
*** Malt1
*** Ticam1
*** Myd88
*** Tlr9
*** Nlrc3
*** Rel
*** Bcl3
*** Tifab
*** Nfkb2
*** Relb
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log2FC
*** Smad4
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*** Ctsh
*** Fgf10
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*** Zfp36l1
*** Map2k2
*** Sox9
*** Mapk3
*** Zfp36l2
*** Flcn
*** Rapgef1
* Egf
** Tlr2
** Ccr3
*** Iggap3
*** Ptger4
*** Myc
*** Tbc1d10c
*** Nod2
E
Regulation of immune system process
log2FC
*** Zbtb6
*** Zfp1006
*** Zfp131
*** Zfp958
*** Ceacam2
*** Zbtb33
*** Zbtb2
*** Zbtb20
*** Zbtb1
*** Patz1
*** Zbtb39
*** Zbtb45
*** Zbtb34
F
Lung vasculature development
log2FC
*** Vegfa
* Tcf21
*** Foxf1
** Stra6
*** Lif
G
Programmed cell death
log2FC
*** Rnf130
*** Trpm7
*** Htra1
*** Dnm1l
*** Ripk1
*** Gatad2a
*** Mlkl
*** Trp53
*** Pgam5
*** Htra2
*** Spata2
* Gsdme
*** Dnase1l3
** Relt
** Nlrp1a
status
up−regulated
down−regulated
not−significant
z-score
2 1 0 -1 -2
*** padj<0.001
** padj<0.01
* padj<0.05
log2FC
H
SERPINB3
50µm
NaCl
Der p1
Der p2
SERPINB3 [%]
0 2 4 6 8 10
*
**

Figure 2

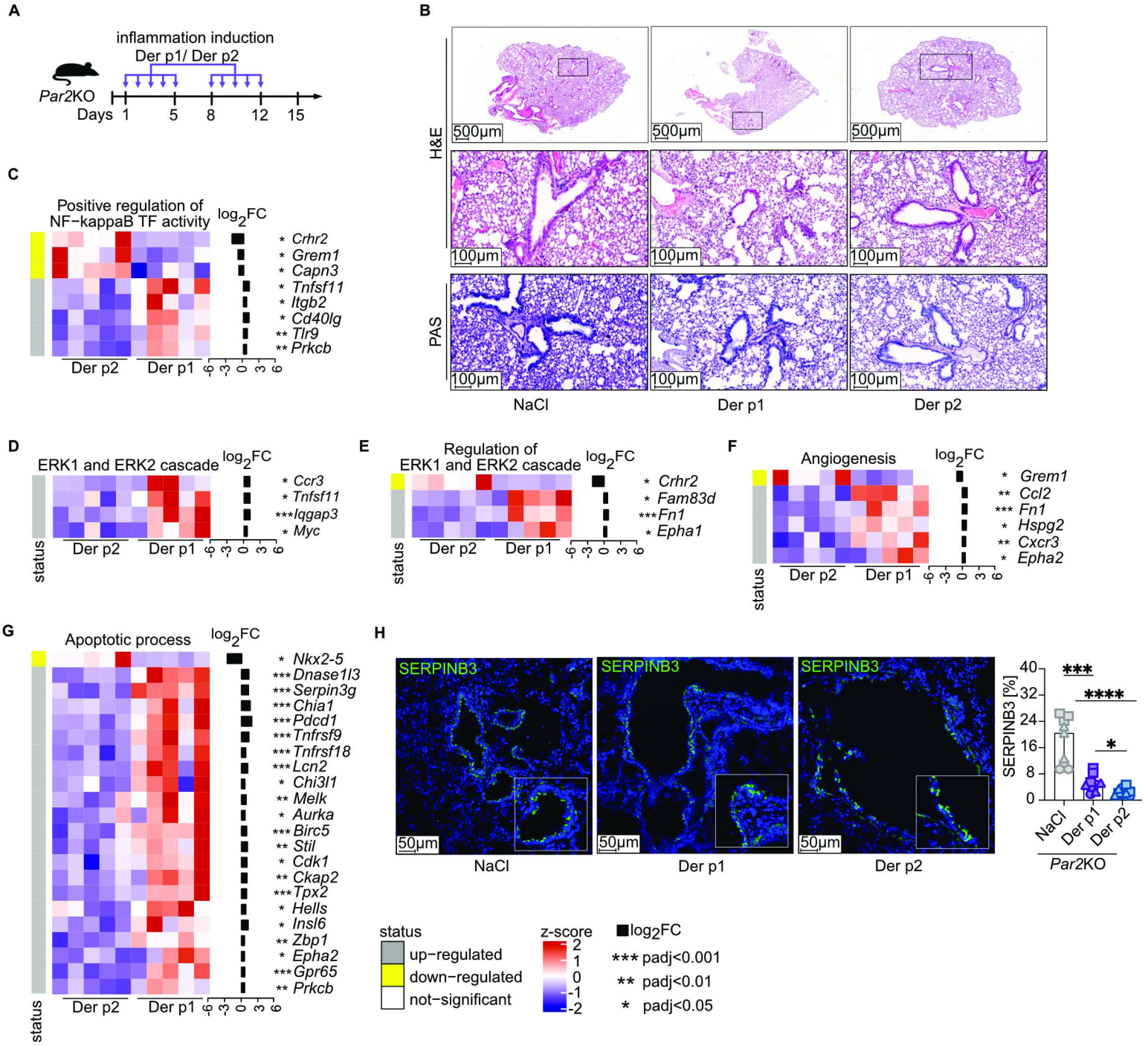
A
inflammation induction
Der p1/ Der p2
Par2KO
Days 1 5 8 12 15
B
H&E
PAS
500µm
100µm
NaCl
Der p1
Der p2
C
Positive regulation of NF−kappaB TF activity
log2FC
* Crhr2
* Grem1
* Capn3
* Tnfsf11
* Itgb2
* Cd40lg
** Tlr9
** Prkcb
Der p2
Der p1
D
ERK1 and ERK2 cascade
log2FC
* Ccr3
* Tnfsf11
*** Iqgap3
* Myc
status
E
Regulation of ERK1 and ERK2 cascade
log2FC
* Crhr2
* Fam83d
*** Fn1
* Epha1
F
Angiogenesis
log2FC
* Grem1
** Ccl2
*** Fn1
* Hspg2
** Cxcr3
* Epha2
G
Apoptotic process
log2FC
* Nkx2-5
*** Dnase1l3
*** Serpin3g
*** Chia1
*** Pdcd1
*** Tnfrsf9
*** Tnfrsf18
*** Lcn2
* Chi3l1
** Melk
* Aurka
*** Birc5
** Stil
* Cdk1
** Ckap2
*** Tpx2
* Hells
* Insl6
** Zbp1
* Epha2
*** Gpr65
** Prkcb
H
SERPINB3
50µm
NaCl
Der p1
Der p2
SERPINB3 [%]
***
****
*
Par2KO
status
up−regulated
down−regulated
not−significant
z-score
log2FC
*** padj<0.001
** padj<0.01
* padj<0.05

Figure 3

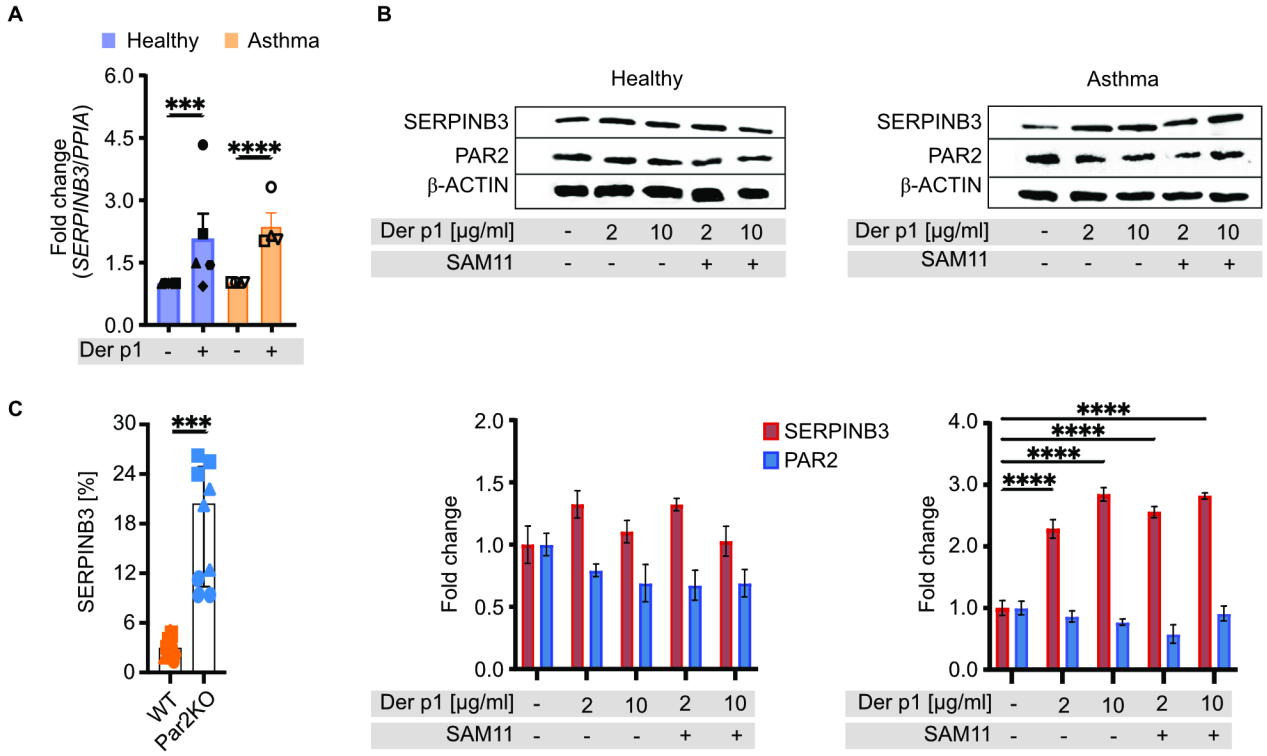
A
Healthy
Asthma
Fold change (SERPINB3/PPIA)
6.0
4.5
3.0
1.5
0.0
***
****
Der p1 - + - +
B
Healthy
Asthma
SERPINB3
PAR2
β-ACTIN
Der p1 [µg/ml] - 2 10 2 10
SAM11 - - - + +
C
SERPINB3 [%]
30
24
18
12
6
0
***
WT
Par2KO
Fold change
2.0
1.5
1.0
0.5
0.0
SERPINB3
PAR2
4.0
3.0
2.0
1.0
0.0
****

Figure 4

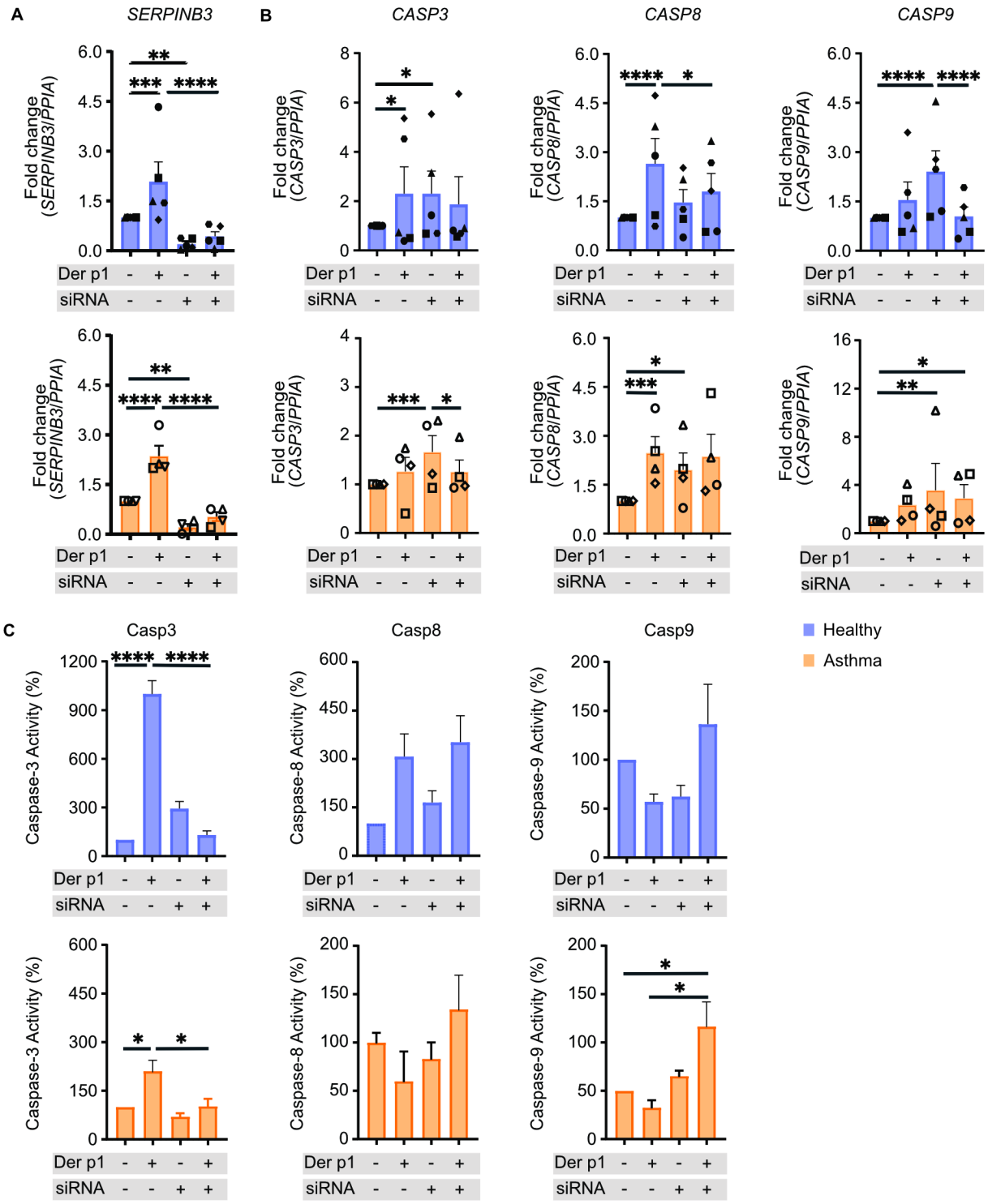
A
SERPINB3
B
CASP3
CASP8
CASP9
Fold change (SERPINB3/PPIA)
Fold change (CASP3/PPIA)
Fold change (CASP8/PPIA)
Fold change (CASP9/PPIA)
Der p1
siRNA
C
Casp3
Casp8
Casp9
Caspase-3 Activity (%)
Caspase-8 Activity (%)
Caspase-9 Activity (%)
Healthy
Asthma

Figure 5

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- BasakSuplementaryFigure4.tif
- BasakSuplementaryFigure3.tif
- BasakSuplementaryFigure5.tif
- UncroppedWBusedforSupplementaryFigure4.pdf
- BasakSuplementaryFigure1.tif
- BasakSuplementaryFigure2.tif
- UncroppedWBusedforFigure4.pdf
- SupplementaryMaterialsFinal.docx