

The Expression Profile of Protease Inhibitors in the Airway Epithelial Cells after Allergen (Der p 1) Stimulation

Cagatay Karaaslan^a Dilara Karaguzel^a Basak Ezgi Sarac^a Ceren Sucularli^b
Hayriye Akel Bilgic^a Omer Kalayci^c

^aDepartment of Biology, Molecular Biology Section, Faculty of Science, Hacettepe University, Ankara, Turkey;

^bDepartment of Bioinformatics, Institute of Health Sciences, Hacettepe University, Ankara, Turkey; ^cDepartment of Pediatric Allergy, Faculty of Medicine, Hacettepe University, Ankara, Turkey

Keywords

Der p 1 · Protease inhibitors · Airway epithelial cells

Abstract

Background: Airway epithelial cells are constantly exposed to intracellular and extracellular proteases that play a pivotal role in several airway diseases. *Dermatophagoides pteronyssinus* (Der p) 1 derived from house dust mite has protease activity that causes epithelial barrier defect and inflammatory response. Protease inhibitors released against proteases are involved in the maintenance of homeostasis. A disruption of the balance between proteases and protease inhibitors can lead to distortion of the cellular structures and cellular activities and thus culminate in disease processes. Although the effects of Der p 1 allergen on epithelial barrier integrity and inflammatory response are well-established, its contribution to protease inhibitor production is highly limited. **Objective:** This study aimed to determine the profile of the protease inhibitor response to Der p 1 allergen in human airway epithelial cells, A549 and BEAS-2B. **Methods:** Differentiated cells by the air-liquid interface were exposed to Der p 1 with or without Th2 type cytokines (IL-4 and IL-13). Gene expression of protease inhibitors was determined by qPCR

at 2 different time points. **Results:** We found that the effect of allergen exposure on the protease inhibitor profile can vary depending on the antigen concentration, treatment duration, and the presence or absence of type 2 cytokines. Gene expressions of serine protease inhibitor (*SERPIN*)B3 and *SERPIN*B4 were increased following Th2 cytokine stimulation in both cell types at both time points, whereas *SERPIN*B2 and *TFPI*-2 expressions were induced by 24-h Der p 1 stimulation in both cells. **Conclusions:** Our study suggests that Der p 1 exposure of the airway epithelium may have consequences related to its protease activity in the presence as well as in the absence of Th2 cytokines in the microenvironment.

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Introduction

The human airway is constantly exposed to noxious stimuli such as allergens, oxidants, and particulate matter, but in most individuals, owing to the counteracting protective mechanisms present in the organism, this exposure does not injure the airways. When the equilibrium between the offending forces and defense mechanisms is disturbed, various disease states promptly ensue. In this

regard, one of the most important systems in the airways is the balance between proteases and protease inhibitors.

Proteases are hydrolytic enzymes, which are classified according to the mechanism of their catalytic reaction. Cysteine, serine, aspartyl, and metalloproteases present in the human lung have significant roles in many pathological states such as airway remodeling, increased mucus production, and chemotaxis of neutrophils [1]. Extrinsic proteases such as house dust mite can contribute to hypersensitivity reactions and increased bronchial epithelial permeability, causing respiratory disorders [2].

HDM is the predominant source of most commonly observed aeroallergens associated with asthma [3]. The most common HDM species are *Dermatophagoides pteronyssinus* (Der p), *Dermatophagoides farinea* (Der f), *Euroglyphus maynei* (Eur m), and *Blomia tropicalis* (Blo t) [3]. Der p 1 is a protease derived from *Dermatophagoides pteronyssinus* and causes cytokine release and degradation of tight junction molecules and thus facilitates the passage through epithelial cell layers [4].

In this context, the production of protease inhibitors emerges as a defense mechanism to suppress the harmful effects of intrinsic and extrinsic proteases in the lung [1]. A balance is established between proteases and antiproteases to ensure respiratory homeostasis [5]. A disruption of this balance in the direction of proteases in the lung can lead to distortion of the cellular activities and structural damage in the airways and thus culminate in disease processes [1] [5]. Lung-based protease inhibitors such as tissue inhibitors of metalloproteinases, trappin-2/elafin, and serine protease inhibitors (SERPINs), can neutralize the effects of proteases and have immune regulatory functions in lung pathology [5].

SERPINS, expressed by a wide variety of tissues, play a regulatory role in various mechanisms such as clot formation, blood pressure regulation, tissue remodeling, immune and inflammatory responses in the lung, in addition to their involvement in apoptosis regulation [6]. There are altogether 37 different SERPINs, 30 of which have protease inhibitory activity [7]. Both increased expression and deficiency of various SERPINs have been implicated in various disease states. While protecting the cell from the harmful effects of proteases, their excessive activity can lead to pathological impacts [6].

Since the lung is constantly exposed to external proteases such as HDM antigens, the regulation of protease inhibitors in the lung may have a critical role in the pathogenesis of allergic airway diseases. In this study, we aimed to determine the profile of the protease inhibitor response against Der p 1 allergen protease in differentiated A549 and BEAS-2B cells using the air-liquid interface (ALI) culture. Considering the fact that SERPINs have an important role in many diseases, the data obtained can form a potential basis for new studies. The mechanistic information on this matter and particular changes in SERPINs detected in sensitized individuals in the presence of Th2 cytokine microenvironment may have significant implications not only for the pathogenesis but also for providing new targets for the individualized treatment of patients.

Materials and Methods

Cell Line, Culture Technique, and Differentiation of the Cells

In our experiments, we used 2 different cell lines. BEAS-2B (ATCC® CRL-9609™), a human bronchial epithelial cell line (ATCC, Manassas, VA, USA), and A549 cells, a human alveolar type II epithelium-like cell line (DSMZ, Braunschweig, Germany), were grown according to the manufacturers' instructions. Cell culture and differentiation methods are detailed in the online supplementary material (see www.karger.com/doi/10.1159/000518170 for all online suppl. material).

Cytotoxicity Analysis

Both A549 and BEAS-2B cells were stimulated with 2, 5, and 10 µg/mL of Der p 1 allergen (low endotoxin 0.03 EU/µg Indoor Biotechnologies, Cardiff, UK) with or without IL-4 + IL-13 (25 ng/mL) cytokines (Peprotech, London, UK). MTT (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed after 12-h and 24-h incubations, as previously described [8]. The optical density of soluble formazan crystals was determined by an EnSight™ Multimode Microplate Reader (Perkin Elmer, MA, USA) on 570 nm.

Transepithelial Electrical Resistance Measurement and Epithelial Permeability

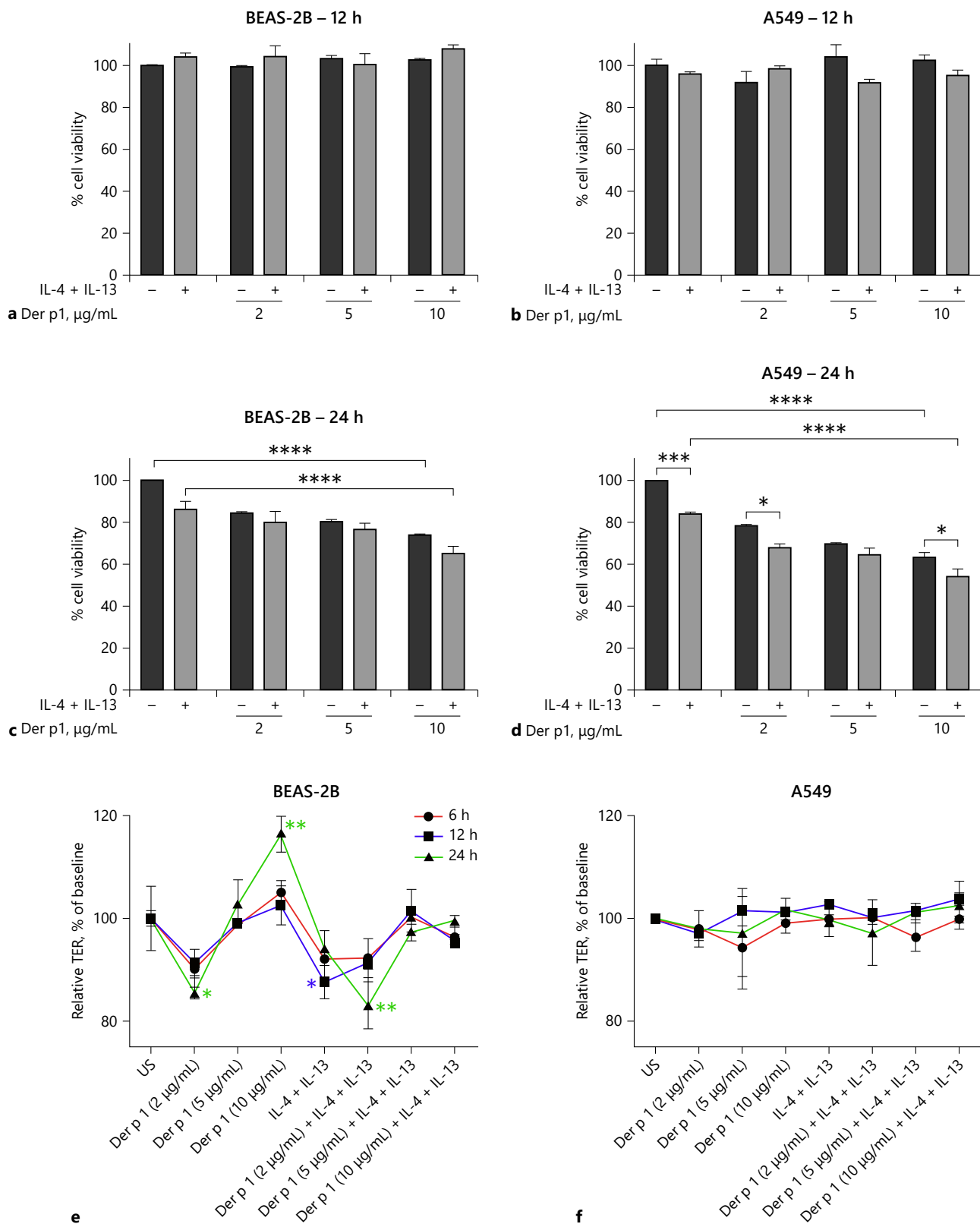
Transepithelial electrical resistance (TER) was measured after 6-h, 12-h, and 24-h Der p 1 stimulations using a Millicell ERS-2 epithelial volt-ohm meter (Millipore, Billerica, MA, USA). TER measurements were performed in triplicates, as described [9].

RNA Isolation and qPCR Analysis

Total RNA was extracted with a RNeasy Mini Kit (Qiagen, Germantown, MD, USA) after 12-h and 24-h stimulations. Samples

Fig. 1. MTT analysis results of BEAS-2B cells (a, c) and A549 cells (b, d). Cells were treated with Der p 1 allergen without or with IL-4 and IL-13 cytokines for 12 h and 24 h. Der p 1 with Th2 cytokines (IL-4 and IL-13) did not change cell viability after 12 h (a, b) but reduced cell viability of both BEAS-2B and A549 cells after 24-h

stimulation (c, d). TER was measured after 6-h, 12-h and 24-h stimulation of BEAS-2B (e) and A549 (f). Results are representative of 3 separate assays. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Der p 1, *Dermatophagoides pteronyssinus*; TER, transepithelial electrical resistance. (For figure see next page.)



were reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and real-time PCR was performed using the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Details were given in the online supplementary file. The primer list is given in online supplementary Table 1.

Protein-Protein Interaction Networks

The online Search Tool for the Retrieval of Interacting Genes (STRING) database (<https://string-db.org/cgi/input.pl>) was employed to obtain the direct or indirect relationships among proteins. The organism was limited to "*Homo sapiens*," and the confidence interaction score was set as 0.150 to construct the protein-protein interaction (PPI) networks.

Statistical Analysis

Statistical analysis was performed with Prism 9 (GraphPad Software Inc., San Diego, CA, USA), and $p < 0.05$ was considered statistically significant. Two-way ANOVA was used to compare data among the groups. The data shown are the mean of 3 independent experiments $\pm$ SEM.

Results

Th2 Cytokines (IL-4 and IL-13) and Der p 1 Decrease Cell Viability after 24-h Incubation

The effects of Der p 1 and IL-4 + IL-13 on cell viability were determined by MTT assay. BEAS-2B and A549 cells were stimulated with protease for 12 h and 24 h. Although 12 h of Der p 1 stimulation did not affect the viability (Fig. 1a, b), 24-h stimulation caused a decrease in both BEAS-2B (Fig. 1c) and A549 (Fig. 1d) cells in a dose-dependent manner. IL-4 + IL-13 decreased cell viability only after 24-h incubation but did not show an additive effect with Der p 1 on cell survival in BEAS-2B cells. However, IL-4 + IL-13 displayed a synergistic effect with Der p 1 stimulation on cell death in A549 cells. Microscopic images showed similar patterns in both cell types in terms of morphology, and cell staining images corresponded to the results measured by the MTT assay (pictures not shown).

Effects of Der p 1 and IL-4 and IL-13 on TER

In BEAS-2B cells, 6-h stimulations did not significantly change TER values. However, IL-4 and IL-13 decreased TER values after 12 h (Fig. 1e); 2 μ g/mL Der p 1 decreased TER values at all time points, but it reached significance

at only 24-h stimulation. However, it showed an increasing trend at 10 μ g/mL concentration. A similar pattern was obtained in the presence of Th2 cytokines. On the other hand, 6 h, 12 h, and 24 h of stimulations did not change TER values in A549 cells (Fig. 1f).

Effect of Der p 1 and IL-4 and IL-13 on the Expression of Protease Inhibitors

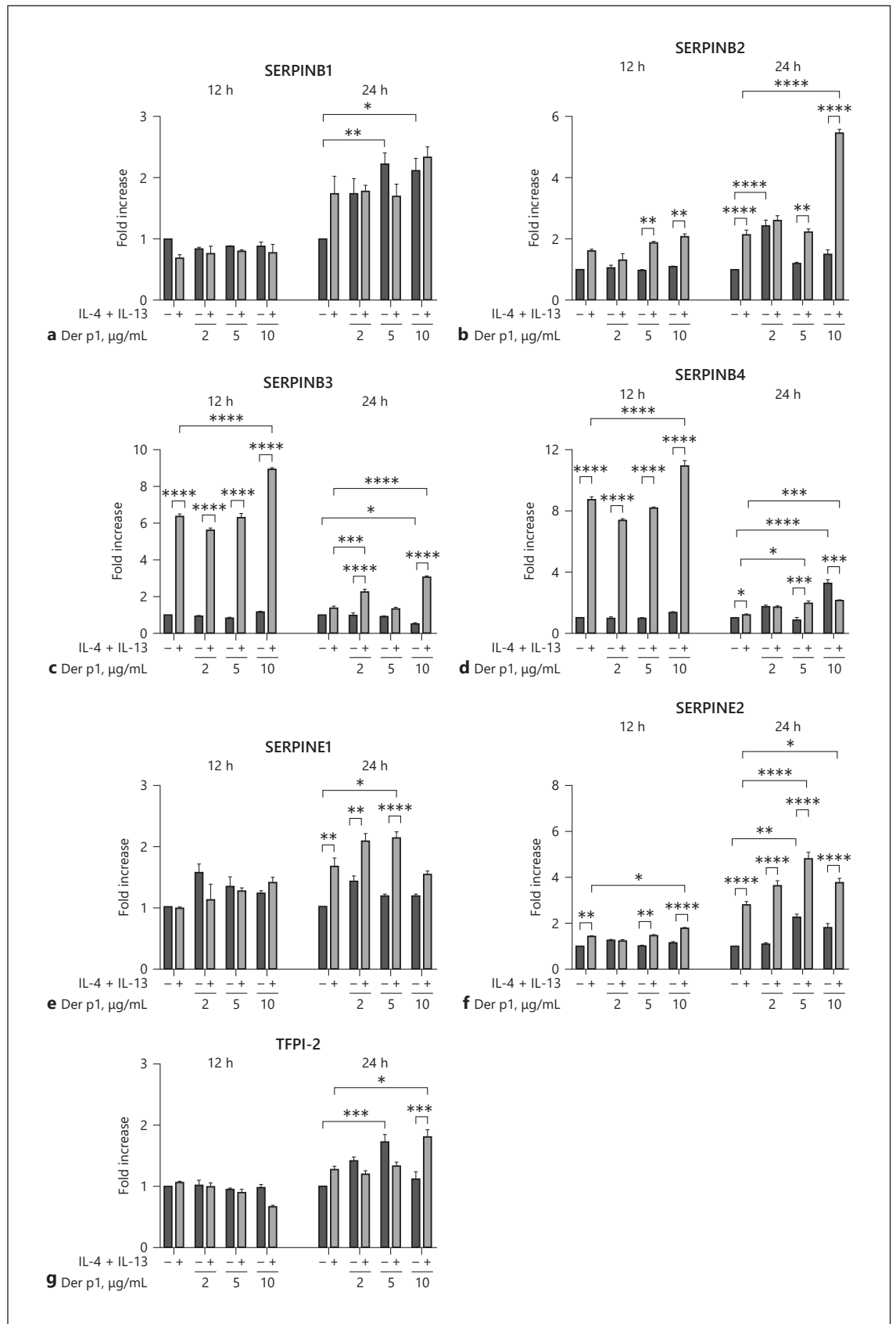
In BEAS-2B cells, *SERPINB1* expression increased in 24-h stimulation with Der p 1 but not in 12-h stimulation (Fig. 2a). The expression of *SERPINB2* was induced only by co-stimulation of Der p 1 and Th2 cytokines after 12 h, while this increment was further induced by 24-h stimulation (Fig. 2b); 12-h stimulation showed similar patterns in both *SERPINB3* and *SERPINB4* expression levels, which are elevated up to 10-fold in all conditions with Th2 cytokines. Although this induction was reduced after 24-h stimulation, the statistical significance was still preserved (Fig. 2c, d). *SERPINE1* expression, on the other hand, was mainly induced by co-stimulation with Der p 1 and IL-4 + IL-13 only after 24 h (Fig. 2e). *SERPINE2* expression was mainly induced in the case of stimulation with Th2 cytokines after both 12 h and 24 h (Fig. 2f). *TFPI-2* expression was not changed after 12-h stimulation, while 24-h incubation induced its expression after 5 μ g/mL Der p 1 alone and co-stimulation with Der p 1 and Th2 cytokines (Fig. 2g).

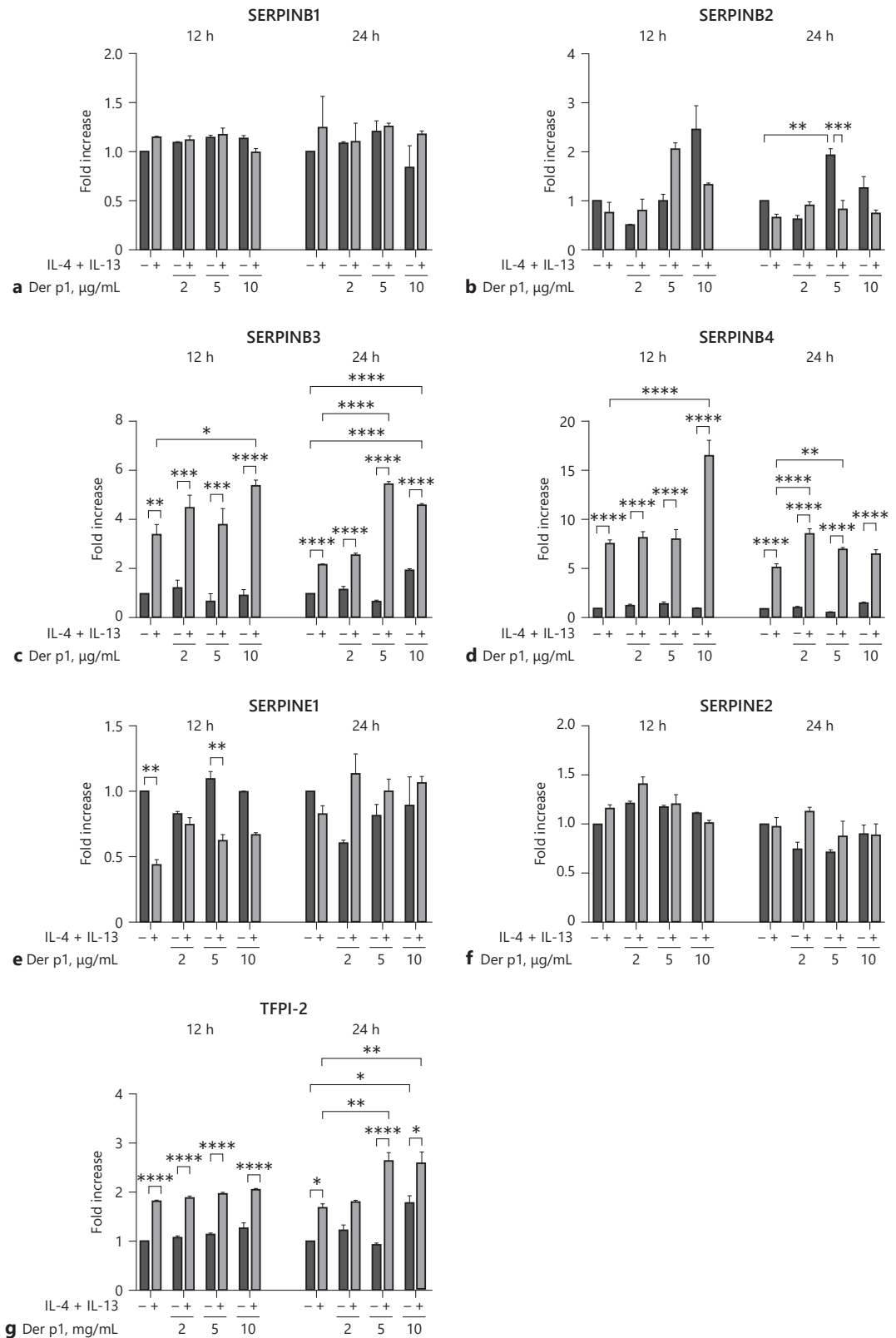
In A549 cells, *SERPINB1* and *SERPINE2* expressions did not change after allergen or IL-4 + IL-13 stimulation (Fig. 3a, f) in both 12-h and 24-h stimulations; 12-h stimulation did not change the *SERPINB2* expression level significantly (Fig. 3b). Unlike BEAS-2B cells, 24-h incubation with only Der p 1 increased *SERPINB2* gene expression in A549 cells, but the presence of IL-4 + IL-13 failed to amplify the effect. Similar to BEAS-2B cells, *SERPINB3*, and *SERPINB4* gene expression levels were elevated at both time intervals by IL-4 and IL-13. However, only expression of *SERPINB3* but not *SERPINB4* was induced by the high concentration of Der p 1 after 24 h (Fig. 3c, d). Whereas the *SERPINE1* expression level was lowered in the presence of Th2 cytokines after 12 h, the statistical significance was lost after 24 h (Fig. 3e). *TFPI-2* expression was induced in conditions involving Th2 cytokines after both 12 h and 24 h, while the higher concentration of Der p 1 stimulation also had an inducing effect after 24 h (Fig. 3g).

Fig. 2. Gene expression results of protease inhibitor genes in BEAS-2B cells following Der p 1 and IL-4 + IL-13 cytokine stimulation for 12 h and 24 h. *SERPINB1* (a), *SERPINB2* (b), *SERPINB3* (c), *SERPINB4* (d), *SERPINE1* (e), *SERPINE2* (f), and

TFPI2 (g); $n = 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Der p 1, *Dermatophagoides pteronyssinus*; SERPIN, serine protease inhibitor.

(For figure see next page.)





IL-4 and IL-13 Have the Potential to Stimulate Protease Inhibitor Synthesis

The 10 genes investigated in this study were entered the STRING database for PPI network analysis and a PPI network of 10 nodes and 31 edges with PPI enrichment p value $<1.0\text{e-}16$ (online suppl. Fig. 1). PPI network analysis revealed that *SERPINE1* is co-expressed with *SERPINE2*, *SERPINB2*, and *TFPI2*; *SERPINB13* is co-expressed with *SERPINB2*, *SERPINB3*, and *SERPINB4*; and *SERPINB3* is co-expressed with *SERPINB2* and *SERPINB13*. In addition, *SERPINB3* is also co-expressed with *SERPINB4* with relatively higher strength.

Discussion/Conclusion

The balance between protease and protease inhibitors is essential in maintaining lung homeostasis. Disruption of this balance is one of the key factors triggering blood coagulation, fibrinolytic pathways, programmed cell death, inflammation, and airway remodeling [10, 11]. However, the effect of protease allergens on the protease inhibitor profile in lung epithelial cells and their role in maintaining this particular balance is not fully understood. Here, we addressed the alteration in the protease inhibitor profile in response to HDM-derived Der p 1 allergen with and without Th2 cytokines (IL-4 and IL-13) in human epithelial cell lines. We found that Der p 1 increased the transcription of *SERPINB2* and *TFPI-2* in both cell types after 24-h stimulation, while Th2 cytokines induced the expression of *SERPINB3* and *SERPINB4* in both cell types at both time points. We also observed similar trends in the gene expression profiles of *SERPINB3* and *SERPINB4* in A549 cells and *SERPINE1* and *SERPINE2* in each cell line, in accordance with the STRING interaction network. Although the STRING interaction network showed that *SERPINB13* is co-expressed with *SERPINB2*, *SERPINB3*, and *SERPINB4*, we could not detect the *SERPINB13* mRNA level in either cell line by qPCR.

Although there are contradicting results in the literature concerning the effects of IL-4 and IL-13 on cell viability depending on the cell type, various studies showed that, IL-4, and IL-13 stimulation decreased cell viability in A549 and BEAS-2B cells [12, 13]. Studies suggest that

IL-4 and IL-13 may exert their apoptotic effect by decreasing the antiapoptotic molecule Bcl-xl, which is followed by the activation of pro-apoptotic molecules such as Bax. The final results are caspase-3 activation [12], downregulation of ERK-dependent signaling cascade [14], and activation of STAT-6-mediated cell death [15]. IL-4, together with IL-13, has also been shown to cause epithelial barrier dysfunction in a JAK-dependent manner [16]. Decreasing cell viability with increasing Der p 1 concentration in BEAS-2B and A549 cells indicates that exposure to Der p 1 alone without the presence of Th2 cytokines may be sufficient to injure the healthy bronchial epithelium. The additive effect of Th2 type cytokines suggests that the effect of allergen proteases is amplified in the presence of Th2 type inflammation. These observations may have strong implications regarding the effect of the antigen in the inception of the airway diseases by damaging the airway as well as augmentation of already present Th2-driven airway diseases independent of their immunological properties.

In our study, Der p 1 and Th2 cytokine (IL-4 +IL-13) stimulation did not change epithelial integrity in alveolar A549 cells. Interestingly, in BEAS-2B cell with a bronchial origin, while low concentration of Der p 1 reduced transepithelial resistance, TER values showed an increasing trend at high concentration (10 $\mu\text{g/mL}$). Although literature presents contradictory data in other cell types [17, 18], Lee et al. [19] also showed that Der p 1 at 10 $\mu\text{g/mL}$ concentration caused a sustained increase in TER values after 4 h, 8 h, and 24 h in primary normal human bronchial epithelial cells. We showed that IL-4 and IL-13 stimulation reduced TER values after 12 h and 24 h in accordance with the literature [16, 20].

SERPINs constitute a superfamily of protease inhibitors that are involved in many cellular events such as homeostasis, inflammation, wound healing, airway remodeling, and apoptosis [21]. They have pleiotropic effects on apoptosis in that they may have both apoptotic and anti-apoptotic effects [22, 23]. The Der p 1 allergen has a destructive impact on tight junction proteins and causes epithelial disruption, which may initiate apoptosis in the epithelial cell [4]. It has been shown that protease activity of Der p 1 can be blocked by *SERPINB4* but not *SERPINB3* [24]. Although they are 91% identical at the amino acid level, they show a different inhibition pattern against

Fig. 3. Gene expression results of protease inhibitor genes in A549 cells following Der p 1 and IL-4 + IL-13 cytokine stimulation for 12 h and 24 h. *SERPINB1* (a), *SERPINB2* (b), *SERPINB3* (c), *SERPINB4* (d), *SERPINE1* (e), *SERPINE2* (f), and *TFPI-2* (g); $n = 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Der p 1, *Dermaphagoides pteronyssinus*; SERPIN, serine protease inhibitor.

Der p 1 protease activity because of the difference in substrate specificity [25]. In our study, *SERPINB4*, *SERPINB1*, and to a lesser extent *SERPINB2* and *SERPINE2* but not *SERPINB3* and *SERPINE1* expression increased following Der p 1 stimulation. Interestingly, gene expression levels of protease inhibitors actively involved in antiapoptotic signaling [22, 23, 26, 27] showed different trends against cell viability levels that occur with increasing Der p 1 concentration. Remarkably, the protease inhibitor profile seen in BEAS-2B cells is different from that observed in A549 cells. These results may suggest that the profile protease inhibitor is also a function of the responding cell type.

IL-4 and IL-13 increased the gene expression of SERPINs in both cell lines in our study. It has been shown that IL-4 and IL-13 cytokines play a role in apoptosis and protease inhibitor synthesis [28]. Furthermore, increased *SERPINB3* and *SERPINB4* expressions were determined in both healthy and asthmatic epithelial cells after IL-4 and IL-13 cytokines stimulation [29].

Our study shows that Der p 1 and Th2 cytokines significantly affect the expression of protease inhibitors that are important players in apoptotic events. Specifically, a proapoptotic molecule TFPI-2 and SERPINs with dual activity on apoptosis *SERPINB1* and *SERPINE2* are stimulated [23, 30, 31]. These results suggest that the effect of antigens and cytokines extends beyond their immunologic properties and may indirectly influence apoptotic events.

Our study has some limitations. First, using primary cells, instead of cell lines, would more closely mimic the physiologic events. Second, even though our experiments show a distinct pattern of protease inhibitors in different cell lines, the consequences of the altered expression of various proteins await further studies.

To conclude, our study shows that antigenic stimulation of bronchial epithelial cells may have significant effects on the integrity of the epithelium as well as on the

protease inhibitors produced by these cells. These effects may vary depending on the presence or absence of Th2 cytokines in the microenvironment. Therefore, our results suggest that antigens may play a role in the inception of airway diseases without the presence of Th2 inflammation and may also augment the existing Th2 type diseases.

Statement of Ethics

BEAS-2B and A549 cell lines used in this study were commercially purchased from ATCC and DSMZ, respectively, and their use does not require ethical approval.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

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Author Contributions

C.K. and H.A.B. designed the study. C.K., D.K., B.E.S., and H.A.B. performed experiments. C.K., D.K., and C.S. analyzed data. D.K. and B.E.S. drafted the manuscript. C.K. and O.K. critically revised the article. All authors have read and approved the final version of the article.

Data Availability Statement

All the data obtained and analyzed during the current study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

References

- Greene CM, McElvaney NG. Proteases and antiproteases in chronic neutrophilic lung disease: relevance to drug discovery. *Br J Pharmacol*. 2009;158(4):1048–58.
- Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C, et al. House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol*. 2003 Sep;29(3 Pt 1):381–9.
- Milian E, Diaz AM. Allergy to house dust mites and asthma. *P R Health Sci J*. 2004 Mar; 23(1):47–57.
- Chevigne A, Jacquet A. Emerging roles of the protease allergen Der p 1 in house dust mite-induced airway inflammation. *J Allergy Clin Immun*. 2018 Aug;142(2):398–400.
- Meyer M, Jaspers I. Respiratory protease/antiprotease balance determines susceptibility to viral infection and can be modified by nutritional antioxidants. *Am J Physiol Lung Cell Mol Physiol*. 2015;308(12):L1189–201.
- Lucas A, Yaron JR, Zhang L, Ambadapadi S. Overview of serpins and their roles in biological systems. *Methods Mol Biol*. 2018;1826: 1–7.
- Kellici TF, Pilka ES, Bodkin MJ. Small-molecule modulators of serine protease inhibitor proteins (serpins). *Drug Discov Today*. 2020; 26:44254.

- 8 Karaaslan C, Birben E, Keskin O, Sahiner U, Sackesen C, Kalayci O. The role of SCCA1 in asthma related physiological events in the airway epithelium and the effect of promoter variants on asthma and gene function. *Respir Med*. 2013 Mar;107(3):368–79.
- 9 Xian M, Ma S, Wang K, Lou H, Wang Y, Zhang L, et al. Particulate matter 2.5 causes deficiency in barrier integrity in human nasal epithelial cells. *Allergy Asthma Immunol Res*. 2020;12(1):56–71.
- 10 Jacquet A. Interactions of airway epithelium with protease allergens in the allergic response. *Clin Exp Allergy*. 2011;41(3):305–11.
- 11 Khan MS, Singh P, Azhar A, Naseem A, Rashid Q, Kabir MA, et al. Serpin inhibition mechanism: a delicate balance between native metastable state and polymerization. *J Amino Acids*. 2011;2011:606797–7.
- 12 Shankaranarayanan P, Nigam S. IL-4 induces apoptosis in A549 lung adenocarcinoma cells: evidence for the pivotal role of 15-hydroxyicosatetraenoic acid binding to activated peroxisome proliferator-activated receptor γ transcription factor. *J Immunol*. 2003;170(2):887–94.
- 13 Borowski A, Kuepper M, Horn U, Knüpper U, Zissel G, Höhne K, et al. Interleukin-13 acts as an apoptotic effector on lung epithelial cells and induces pro-fibrotic gene expression in lung fibroblasts. *Clin Exp Allergy*. 2008;38(4):619–28.
- 14 Doseff AI, Baker JH Jr, Bourgeois TA, Wewers MD. Interleukin-4-induced apoptosis entails caspase activation and suppression of extracellular signal-regulated kinase phosphorylation. *Am J Respir Cell Mol Biol*. 2003 Sep;29(3 Pt 1):367–74.
- 15 Nishimura Y, Nitto T, Inoue T, Node K. STAT6 mediates apoptosis of human coronary arterial endothelial cells by interleukin-13. *Hypertens Res*. 2008 Mar;31(3):535–41.
- 16 Saatian B, Rezaee F, Desando S, Emo J, Chapman T, Knowlden S, et al. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. *Tissue Barriers*. 2013 Apr 1;1(2):e24333.
- 17 Henriquez OA, Den Beste K, Hoddeson EK, Parkos CA, Nusrat A, Wise SK. House dust mite allergen Der p 1 effects on sinonasal epithelial tight junctions. *Int Forum Allergy Rhinol*. 2013 Aug;3(8):630–5.
- 18 Kortekaas Krohn I, Seys SF, Lund G, Jonckheere AC, Dierckx de Casterlé I, Ceuppens JL, et al. Nasal epithelial barrier dysfunction increases sensitization and mast cell degranulation in the absence of allergic inflammation. *Allergy*. 2020 May;75(5):1155–64.
- 19 Lee PH, Kim BG, Lee SH, Lee JH, Park SW, Kim DJ, et al. Alteration in claudin-4 contributes to airway inflammation and responsiveness in asthma. *Allergy Asthma Immunol Res*. 2018 Jan;10(1):25–33.
- 20 Schmidt H, Braubach P, Schilpp C, Lochbaum R, Neuland K, Thompson K, et al. IL-13 impairs tight junctions in airway epithelia. *Int J Mol Sci*. 2019 Jun 30;20(13):3222.
- 21 Almonte AG, Sweatt JD. Serine proteases, serine protease inhibitors, and protease-activated receptors: roles in synaptic function and behavior. *Brain Res*. 2011 Aug 17;1407:107–22.
- 22 Song KJ, Ahn HJ, Nam HW. Anti-apoptotic effects of SERPIN B3 and B4 via STAT6 activation in macrophages after infection with *Toxoplasma gondii*. *Korean J Parasitol*. 2012 Mar;50(1):1–6.
- 23 Torriglia A, Martin E, Jaadane I. The hidden side of SERPINB1/Leukocyte Elastase Inhibitor. *Semin Cell Dev Biol*. 2017 Feb;62:178–86.
- 24 Sakata Y, Arima K, Takai T, Sakurai W, Masumoto K, Yuyama N, et al. The squamous cell carcinoma antigen 2 inhibits the cysteine proteinase activity of a major mite allergen, Der p 1. *J Biol Chem*. 2004 Feb 13;279(7):5081–7.
- 25 Izuhara K, Yamaguchi Y, Ohta S, Nunomura S, Nanri Y, Azuma Y, et al. Squamous cell carcinoma antigen 2 (SCCA2, SERPINB4): an emerging biomarker for skin inflammatory diseases. *Int J Mol Sci*. 2018 Apr 6;19(4):1102.
- 26 Pavon MA, Arroyo-Solera I, Cespedes MV, Casanova I, Leon X, Mangués R. uPA/uPAR and SERPINE1 in head and neck cancer: role in tumor resistance, metastasis, prognosis and therapy. *Oncotarget*. 2016 Aug 30;7(35):57351–66.
- 27 Hsieh HH, Chen YC, Jhan JR, Lin JJ. The serine protease inhibitor serpinB2 binds and stabilizes p21 in senescent cells. *J Cell Sci*. 2017 Oct 1;130(19):3272–81.
- 28 Sun Y, Sheshadri N, Zong WX. SERPINB3 and B4: from biochemistry to biology. *Semin Cell Dev Biol*. 2017 Feb;62:170–7.
- 29 Yuyama N, Davies DE, Akaiwa M, Matsui K, Hamasaki Y, Suminami Y, et al. Analysis of novel disease-related genes in bronchial asthma. *Cytokine*. 2002 Sep 21;19(6):287–96.
- 30 George J, Gondi CS, Dinh DH, Gujrati M, Rao JS. Restoration of tissue factor pathway inhibitor-2 in a human glioblastoma cell line triggers caspase-mediated pathway and apoptosis. *Clin Cancer Res*. 2007 Jun 15;13(12):3507–17.
- 31 McKee CM, Ding Y, Zhou J, Li C, Huang L, Xin X, et al. Protease nexin 1 induces apoptosis of prostate tumor cells through inhibition of X-chromosome-linked inhibitor of apoptosis protein. *Oncotarget*. 2015 Feb 28;6(6):3784–96.

1Online Supplement

2Cell Line, Culture Technique, and Differentiation of the Cells

3BEAS-2B cells (ATCC® CRL9609™), a human bronchial epithelial cell line (ATCC, Virginia, USA) (DSMZ,
4Braunschweig, Germany) were in Bronchial Epithelial Basal Medium (BEBM) containing BEGM Bullet
5Kit (Lonza, Basel, Switzerland). For differentiation of BEAS-2B cells in ALI culture, BEGM without
6retinoic acid (RA) and triiodothyronine (T3) and DMEM were mixed in 1:1 containing fresh 15 ng/mL
7RA [1].

8A549 cells, a human alveolar type II epithelium-like cell line (DSMZ, Braunschweig, Germany), were
9grown in RPMI 1640 medium (Sigma-Aldrich, Missouri, USA) containing 10% heat-inactivated fetal
10bovine serum and 1% Penicillin/Streptomycin. In addition, RPMI 1640 containing 1% ITS+ (human
11recombinant insulin, human transferrin, selenous acid, BSA, and linoleic acid) (BD Biosciences,
12California, USA,) 0.1% Gentamicin/Amphotericin, Hydrocortisone, Bovine Pituitary Extract (BPE),
13retinoic acid, and human epithelial growth factor was used for differentiation of the A549 cell line [2].

14Cells were seeded at 7×10^4 per 6.5 mm polyester Transwell inserts with a pore size of 0.4 μm
15(Corning, New York, USA) for differentiation. After 3-4 days (100% confluency), the apical medium
16was discarded, and the basal medium was replaced with a differentiation medium. Both cells were
17differentiated for 21 days. After differentiation, cells were stimulated with different concentrations of
18low endotoxin Der p1 allergen (low endotoxin 0.03 EU/ μg , Indoor Biotechnologies, Cardiff, UK) in the
19presence or absence of Th2 cytokines, IL-4 and IL-13 (25 ng/mL) (Peprotech, London, UK) for 24
20hours. The purity of Der p1 on silver-stained SDS-PAGE was detected as >95% by the company. In
21addition, we did not detect any RNA or DNA contamination by measurement with Qubit.

22RNA Isolation and qPCR Analysis

23After cDNA synthesis performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher
24Scientific, Massachusetts, USA), mRNA levels of *SERPINB1* (MNEI-monocyte-neutrophil elastase

25inhibitor), *SERPINB2* (PAI-2, plasminogen activator inhibitor-2), *SERPINB3* (SCCA1, squamous cell
 26carcinoma antigen 1), *SERPINB4* (SCCA2, squamous cell carcinoma antigen 2), *SERPINB13* (Serp
 27Family B Member 13), *SERPINE1* (PAI-1, plasminogen activator inhibitor-1), *SERPINE2* (PN-1, protease
 28nexin-1), and *TFPI2* (Tissue Factor Pathway Inhibitor 2) were quantified using SYBR Green Master Mix
 29(Thermo Fisher Scientific, Massachusetts, USA) by the ABI 7500 Fast Real-time PCR system (Applied
 30Biosystems, California, USA). *PPIA* (Peptidylprolyl isomerase A) was used as an internal reference to
 31normalize the target transcripts using the $2^{-\Delta\Delta Ct}$ method.

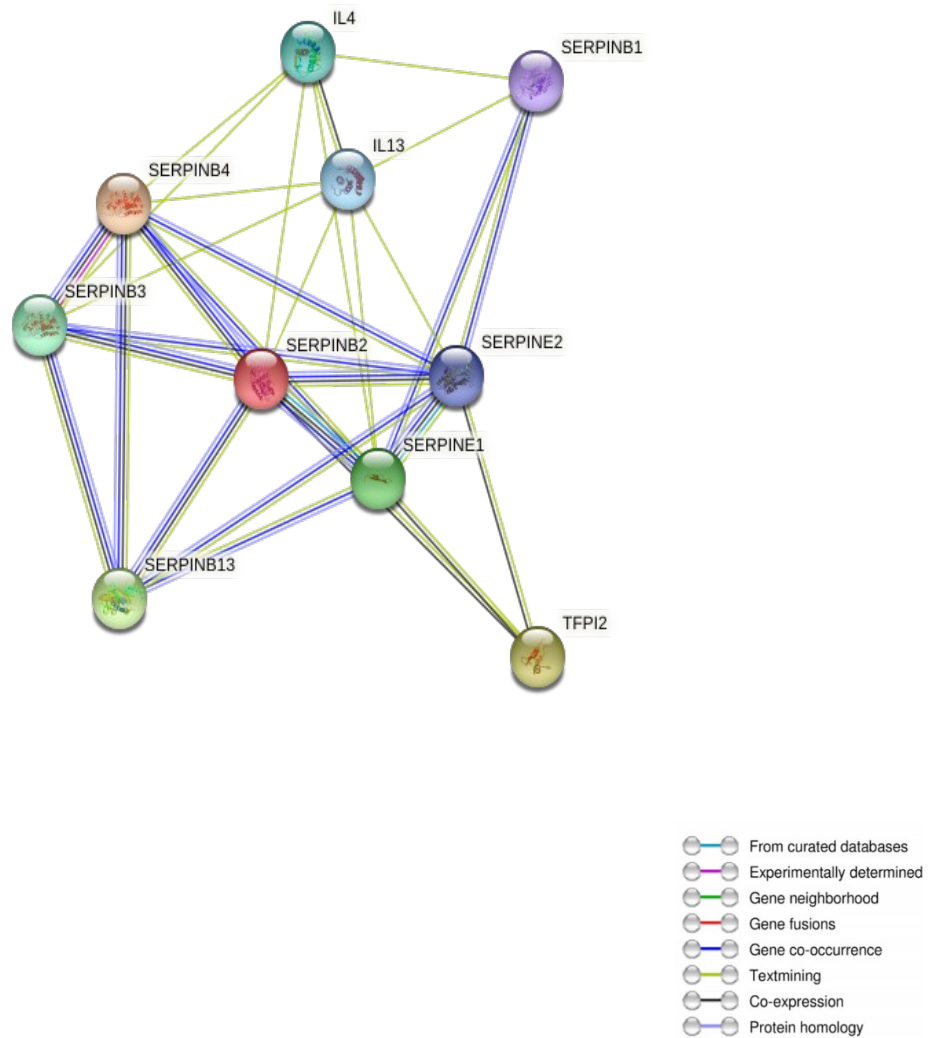
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33REFERENCES

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351. Heijink, I.H., et al., *Characterisation of cell adhesion in airway epithelial cell types using*
 36 *electric cell-substrate impedance sensing*. Eur Respir J, 2010. **35**(4): p. 894-903.
372. Hermanns, M.I., et al., *Lung epithelial cell lines in coculture with human pulmonary*
 38 *microvascular endothelial cells: development of an alveolo-capillary barrier in vitro*.
 39 Laboratory Investigation, 2004. **84**(6): p. 736-752.

Online Suppl. Fig. 1. The protein-protein interaction network of the IL-4 and IL-13 cytokines with protease inhibitors.



Online Suppl. Table 1. Primer sequences used for real-time PCR.

Gene	Reference Sequence	Forward (5'-3')	Reverse (5'-3')
<i>SERPINB1</i>	NM_030666.4	TCCTTCCTGAGTTCTTGGTTTC	ACCCACTGGTTTATGGTCTTC
<i>SERPINB2</i>	NM_001143818.1	TCCAGAAGGGTAGTTATCCTGA	TTGATTGCAGAGCTGAGAGAG
<i>SERPINB3</i>	NM_006919.3	TCCTGAAGGTAATATTGGCAGCA	TGTATTGCCTCATCATCTGTATG
<i>SERPINB4</i>	NM_002974.4	CAGACCAGTGTGGAATCTAC	CAAGAACCAGTGTCTGTATCAT
<i>SERPINB13</i>	NM_012397.4	TGTGCTTCTGCCCAACGACATC	ACCGTCCTCCACCTCAAACCG
<i>SERPINE1</i>	NM_000602.5	TGACAACAGGAGGAGAAACC	GAGCTCCTTGTACAGATGCC
<i>SERPINE2</i>	NM_001136528.2	CACATCAGCACCAAGACCATA	TTCAGCGGCTCCTTCAAATC
<i>TFPI2</i>	NM_001271003.2	GCTGTGGAGGGAATGACAATA	TTCCGGATTCTACTGGCAAAG
<i>PPIA</i>	NM_001300981.2	CATCCTAAAGCATACGGGTCC	TCTTTCACCTTGCCAAACACC