



The role of SPINK5 in asthma related physiological events in the airway epithelium

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Summary

Background: Genetic studies have shown that variants in SPINK5 may be associated with atopic diseases and asthma. However, the functional role of SPINK5 protein in asthma has not been elucidated.

Objectives: To determine the effects of SPINK5 on asthma related physiological events such as apoptosis, mucus and cytokine production by epithelial cells.

Methods: A549 cells were transfected with SPINK5 expression vector and stimulated with increasing doses of hydrogen peroxide and neutrophil elastase (NE) for measurement of cell viability or apoptosis and analysis of mucus production. Cell viability was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay and apoptosis by Annexin V/PI staining. Levels of IL-4, IL-6, IL-8, IL-12, IL-13, IFN γ , IL-1 β and RANTES were determined by ELISA in cell culture supernatants. Mucus production was determined by RT-PCR of the MUC5AC gene and PAS staining in NE treated cells.

Results: Epithelial cells transfected with SPINK5 expression vector produced more IL-6, IL-8 and RANTES compared to non-transfected cells ($p < 0.001$, $p = 0.003$, $p < 0.001$, respectively). Even though cells transfected with SPINK5 vector displayed significantly higher cell death, we have not observed any clear effect of SPINK5 on apoptosis. PAS staining showed that SPINK5 slightly decreased the mucin production induced by neutrophil elastase in A549 cells. However, SPINK5 had no effect on MUC5AC transcription.

Conclusion: SPINK5 is an important molecule in asthma. Its role extends beyond its well known protease inhibitor properties.

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Introduction

The physiopathology of allergic diseases including asthma is incompletely understood. It is generally believed that asthma is the result of a complex network of various genetic, immunological and environmental factors. Among the many molecules that have been shown to play significant roles in the pathogenesis of asthma, some have emerged as dichotomous pairs, the net action of which may determine the course of the pathological events. Within this framework, in addition to some of the well known Th1/Th2 molecules¹ and oxidant/antioxidant forces,² recent studies have shown that the effects of stimulation by proteases acting through membrane receptors and the counteraction by anti-proteases may be important in the pathogenesis of asthma.^{3,4}

Proteases can be exogenous or endogenous. Well known endogenous proteases include thrombin, plasmin, cathepsin G and kallikrein. Central to the allergic inflammation, elastase released by neutrophils and tryptase and chymase released from mast cells have protease activity, as well.⁵ As exogenous sources, almost all major allergens such as pollens, fungi, mites, cockroaches and hymenoptera contain proteases and various microorganisms such bacteria, rhinovirus and influenza have protease activities.^{3,6–8}

Proteases act through specific cell membrane receptors called Protease-Activated Receptors (PARs). These receptors are expressed on almost all cell types and therefore proteases have a wide spectrum of action. In addition to their roles in coagulation and homeostasis, they have the ability to stimulate inflammatory cells and resident cells of the airways, which make them critical for the inflammatory diseases of the airways such as asthma. Various observations have, in fact, supported this notion. In a mouse model of asthma, lack of PAR2 signaling was shown to decrease the eosinophilic infiltration and airway hyper-reactivity.⁹ Stimulation of the airway epithelial by proteases was shown to result in increased synthesis of interleukin (IL)-6, IL-8, GM-CSF, eotaxin and platelet derived growth factor.¹⁰ Mast cell tryptase increases the expression of PARs on epithelial cells and disturbs tight junctions between cells and stimulates collagen production by fibroblasts.¹¹ Neutrophilic proteases cathepsin G and elastase as well as mast cell chymase increases the mucus production from epithelial cells and mucus glands. Expression of adhesion molecules such as P and E selectins, Inter-Cellular Adhesion Molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) increased in endothelial cell stimulated with PAR agonists.¹²

The human body is equipped with a number of protease inhibitors and activation of proteases counter-activates protease inhibitors which balance the noxious effects of proteases from endogenous and exogenous sources. The best known clinical example is alpha-1 anti-tyrpsin and neutrophil elastase. A genetic deficiency in the protease inhibitor alpha-1 anti-tyrpsin leads to unopposed action of the protease leading to emphysema.¹³ In a mouse model of asthma it was shown that removal of proteases from allergens results in decreased airway responsiveness and inflammation.¹⁴ Furthermore, administration of a serine protease inhibitor during sensitization to house dust mite was shown to prevent the development of allergic inflammation and airway hyper-

reactivity, and the production of IL-8 and GM-CSF production by airway epithelial cells stimulated with mite extracts.³

One of the serine protease inhibitors, lymphoepithelial Kazal type 5 (LEKTI) encoded by SPINK5 gene on chromosome 5q31-32 within the cytokine cluster region, was shown to be related with asthma in several studies. SPINK5 contains 33 exons and encodes a precursor protein of 1064 amino acids.¹⁵ SPINK5 plays critical role in the epidermal barrier function by regulating protease activity.¹⁶ Dysfunction of this gene leads to atopic manifestations in skin due to unbalanced protease activity. In addition, mutations in this gene have been found to be related to Netherton syndrome characterized by abnormal desquamation of skin, elevated IgE levels, and atopic manifestations.¹⁶

Because of its biological properties and the localization of the gene, a number of studies have focused on searching for associations between the genetic variants of the SPINK5 gene and atopy, atopic dermatitis and asthma.^{17–19} Even though many studies have investigated the role of genetic variants that belong to the SPINK5 gene, the functional role of SPINK5 protein in asthma is not well elucidated. We hypothesized that, similar to various proteases and protease inhibitors, participation of SPINK5 protein in asthma extend beyond its protease inhibition properties and we aimed to determine the physiological role of SPINK5 on asthma related physiological events such as apoptosis, mucus and cytokine production by epithelial cells.

Materials and methods

Cell culture and cytokine measurements

A549 cells (DSMZ, Braunschweig, Germany) were grown in 1640 RPMI (GIBCO BRL, Grand Island, NY) supplemented with 10% Fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO₂ atmosphere at 37 °C. A549 cells were seeded in 24 well culture dishes and 24 h later cells were transiently transfected with SPINK5 full length expression vector (a kind gift by Dr. Marina D'alesio, Instituto Dermopatico Dell'immacolata, Roma, Italy) using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. In order to determine the effect of SPINK5 on protease induced cytokine production by epithelium, transfected and untransfected cells were stimulated with Der p1 at 1 µg, an antigen with known protease activity. 48 h after transfection, supernatants were collected and stored at –80 °C until analysis. Efficiency of transfection with SPINK5 expression vector was checked by a PCR based method. RNA was extracted from mock transfected and SPINK5 transfected cells. SPINK5 gene was amplified by primer pairs designed according to the RNA sequence of the gene. Transfection efficiency was proven before proceeding to each set of experiments.

We have chosen representatives from a wide spectrum of cytokines and chemokines that may have a strong effect on the eosinophilic airway inflammation that is a characteristic of asthma and determined the concentrations of IL-1β, INF-γ, IL-4, IL-6, IL-8, IL-12, IL-13 and RANTES (regulated upon activation, normal T cell expressed and secreted) using commercial enzyme-linked immunosorbent assay (ELISA)

kits (PIERCE, Thermo Fisher Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions.

Measurement of cell viability

A549 epithelial cells were transfected with SPINK5 full length expression vector as outlined above. Cells were transferred to serum free media and treated with 125, 250, or 500 μM H_2O_2 for 24 h. 500 μM H_2O_2 was chosen as the highest physiological concentration on the basis of preliminary experiments which showed that higher concentrations have resulted in exclusive cell death. A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay was used to determine the cell viability. Briefly, 65 μg of MTT (Sigma–Aldrich Corp, St Louis, MO) was added to each well and cells were incubated in 5% CO_2 for 4 h. Subsequent to aspiration of cell culture medium, cells were lysed with 100 μl isopropanol and absorbance was measured at 550 and 630 nm.

Measurement of apoptosis

Transfected A549 cells were treated with 125, 250, or 500 μM H_2O_2 for 24 h as outlined above. After 24 h, cell pellets were obtained by trypsinization and washed with ice cold PBS twice. Cells were resuspended in ice cold $1 \times$ binding buffer at a concentration of 1×10^6 cells/ml. 200 μl of cell suspension were stained with annexin FITC antibody and PI dye using ApopNexin™ Apoptosis Kit (Chemicon, MA, USA) according to manufacturer's instructions and read by flow cytometry (Facs Aria, BD Biosciences, San Jose, CA, USA). Annexin positive cells were considered as early while annexin and PI double positive cells were considered as late apoptotic. Cells that stained positively with PI only were regarded as necrotic.

Measurement of MUC5AC expression

Transfected A549 cells were stimulated with neutrophil elastase for 24 h. Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). MUC5AC gene was amplified using the following primers: sense 5'TATGGGACTT CTCCTACCAAT 3' and antisense 5' AGCGTGGTGGACGGACAGT 3'. Cyclophilin was used as an intrinsic control. Sequence of cyclophilin primers were as follows: sense 5-AGGTCCCAAAGACAGCAGAA-3' and antisense, reverse 5'-TGTCACAGTCAGCAATGGT-3'. Intensity of amplification bands was measured by image analyzer (Syngene Gene Genius, Cambridge, UK) by using Gene Tools Analysis Software 3.02.00 (Synoptecs software). Expression of MUC5AC gene in unstimulated samples was arbitrarily defined as 1 and expression index of other conditions calculated relative to unstimulated cells.

Quantification of mucus secretion by PAS staining

A549 cells were transfected with SPINK5 expression vector and 24 h after transfection, cells were treated with 200 nM of neutrophil elastase (NE) and incubated for another 24 h.

Mucus secretion was determined by Periodic acid-Schiff (PAS) Staining (Sigma–Aldrich Corp, St Louis, MO) by following manufacturer instructions. Hematoxylin solution was used for counterstaining of slides. Slides were rinsed in running tap water, and mounted with xylene-based mounting medium. Mucin positive cells are stained in red with PAS staining.

Statistical analyses

Statistical analyses were done with Prism 5 for Windows (GraphPad Software, Inc. CA, USA). Repeated measures of ANOVA was used to compare cytokine levels, cell viability and apoptosis in cell culture experiments. For all analyses a p value < 0.05 was considered significant.

Results

Efficiency of transfection

PCR studies using mock transfection as negative control and SPINK5 vector itself as positive control have shown that transfection successfully induces expression of SPINK5 in A549 cells (Fig. 1).

The effect of the SPINK5 on cytokine release by A549 cell

Epithelial cells transfected with SPINK5 expression vector produced more IL-6, IL-8 and RANTES compared to non-transfected cells ($p < 0.001$, $p = 0.003$, $p < 0.001$, respectively, Fig. 2A–C). The background levels of RANTES in unstimulated cells varied between 10 and 40 pg/ml as reported before.²⁰ There was no difference among the two groups in IFN- γ and IL-1 β levels. Levels of IL-12, IL-4 and IL-13 were mostly undetectable. Stimulation of the cells with Der p1 failed to induce any further increase in the cytokine and chemokine levels over that produced by unstimulated cells or SPINK5 overexpression (data not shown).

The effect of the SPINK5 on cell viability and apoptosis

A549 cells were stimulated with increasing doses of hydrogen peroxide and cell viability was measured by MTT assay and apoptosis by Annexin V/PI staining. A549 cells transfected with the SPINK5 expression vector showed decreased cell viability compared to non-transfected cells



Figure 1 Control of transfection efficacy by RT-PCR (A = Non-transfected A549 cells, B = Cells transfected with SPINK5 expression vector (unstimulated), C = Cells transfected with SPINK5 expression vector and stimulated with NE (200 μM) D = SPINK5 vector DNA as a positive control of PCR reaction).

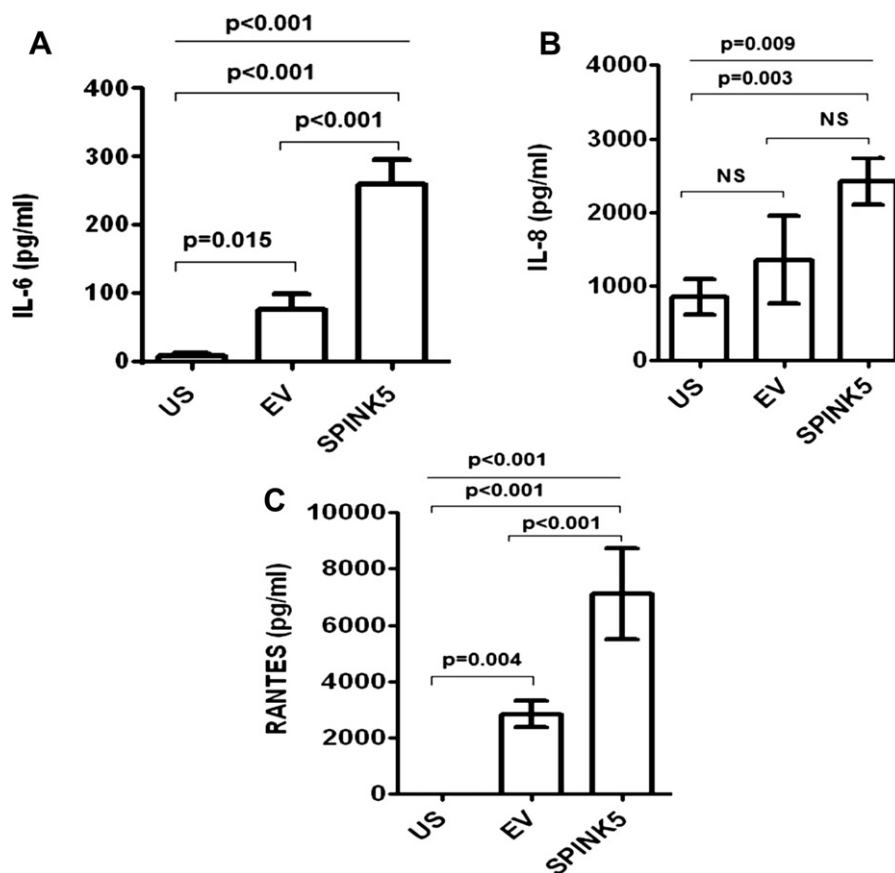


Figure 2 A–C. IL-6, IL-8 and RANTES levels released from A549 cells transfected with SPINK5 vector (US = unstimulated cells, EV = empty vector, SPINK5 = SPINK5 expression vector). Columns and error bars represent mean + SEM, $N = 3$.

as measured by the MTT assay (Fig. 3A). SPINK5 did not have the same affect on apoptosis of A549 cells as shown by Annexin V/PI staining (Fig. 3B).

The effect of the SPINK5 on mucus production by epithelial cells

Mucus production by A549 cells stimulated with a serine protease, neutrophil elastase, was measured by reverse transcriptase PCR of MUC5AC gene at mRNA level and by PAS staining of mucins at protein level. Although SPINK5 had

no effect on mucus production induced by NE at the transcriptional level (Fig. 4), PAS staining showed that SPINK5 slightly decreased the neutrophil elastase induced mucin production by A549 cells compared to non-transfected cells (Fig. 5).

Discussion

Our study shows that SPINK5 has biological actions other than protease inhibition that may be relevant in the pathogenesis of asthma. These include increased production of

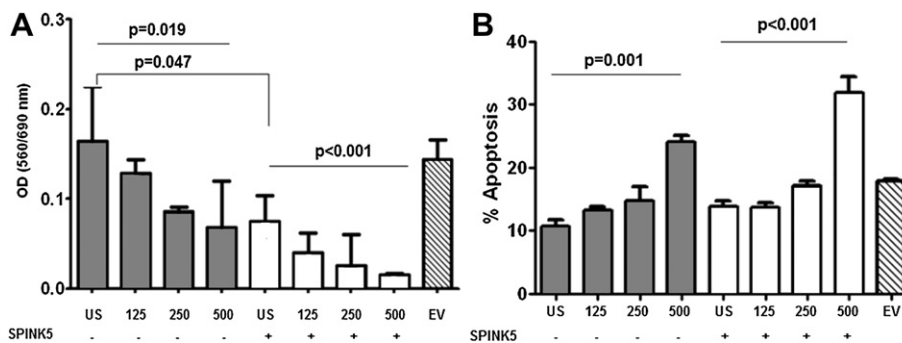


Figure 3 (A) MTT assay and (B) Annexin V/PI staining shows the viability and apoptosis of A549 cells following stimulation with increasing doses of H₂O₂, respectively. (US = unstimulated cells, EV = empty vector, SPINK5 = SPINK5 expression vector). Columns and error bars represent mean + SEM, $N = 3$.

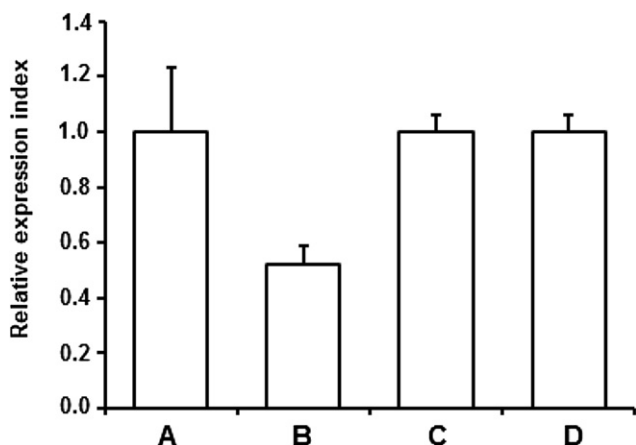


Figure 4 Effects of SPINK5 on MUC5AC expression by A549 cells stimulated with neutrophil elastase (NE). (A) Unstimulated cells, (B) Cells transfected with SPINK5 expression vector (unstimulated), (C) Cells stimulated with NE (200 μ M) (no transfection), (D) Cells transfected with SPINK5 expression vector and stimulated with NE (200 μ M). (Representative of three independent experiments).

IL-6, IL-8 and RANTES by airway epithelial cells; decreased viability of airway epithelial cells following an oxidant stimulus and slightly decreased mucus production in airway epithelial cells.

IL-6, IL-8 and RANTES are all produced at higher levels by the resident and inflammatory cells of the asthmatic airways^{21–23} and they have important roles in the inflammation underlying the pathogenesis of asthma. IL-6 is an important growth factor for T and B cells and induces antibody production²¹; IL-8 is a very potent chemotactic factor for neutrophils²⁴ and RANTES is a powerful chemoattractant for eosinophils.²³ Judging from the effects of IL-6, IL-8 and RANTES, there is reason to believe that SPINK5 is capable of stimulating the involvement of a variety of different cells such as B and T lymphocytes, neutrophils and eosinophils that are active in the inflammatory process of the airways through its stimulatory effects on pro-inflammatory cytokines and chemokines.

Our findings seem to be somewhat different from previous observations. Several studies have shown that activation of protease-activated receptors on airway epithelium by proteases causes the releases of IL-6 and IL-8.^{7,11} In our experiments, however, Der p1, an antigen with known protease activity, failed to induce the release of cytokines from transfected and untransfected A549 cells. However, the possibility to observe an effect with higher Der p1 doses cannot be excluded.

Protease inhibitors, such as E-64, α 1-AP (α 1-antiprotease), SBTI (soybean trypsin inhibitor), AEBSF, FK-706, SLPI (secretory leukocyte protease inhibitor) were shown to inhibit protease-activated cytokine release from airway epithelium and other cells such as lung fibroblasts

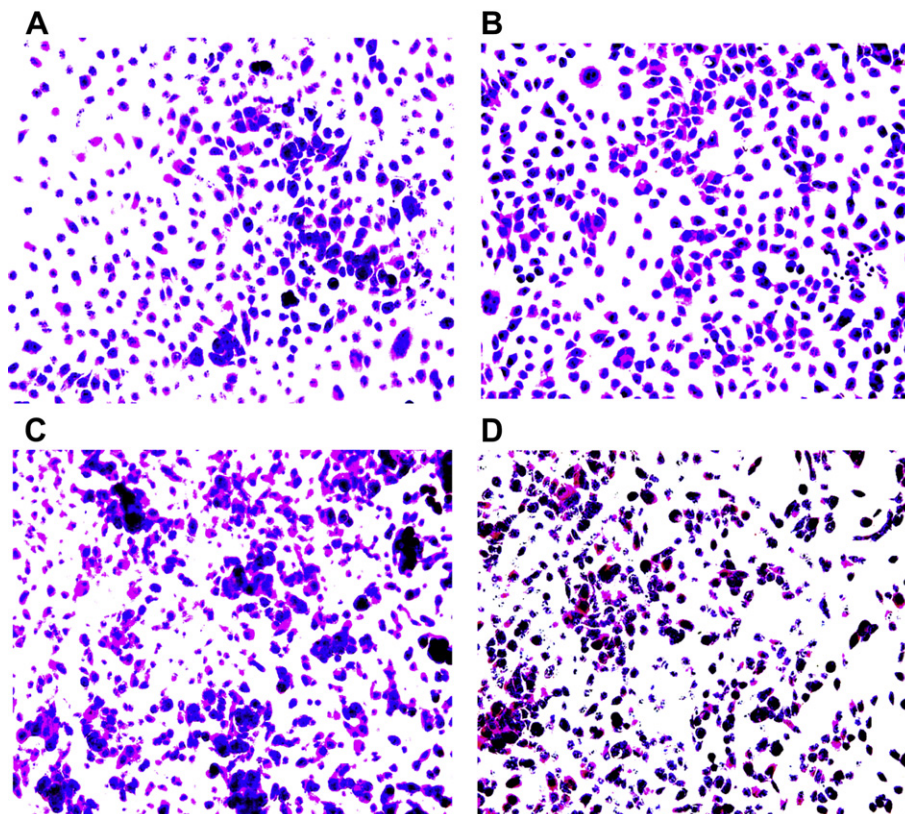


Figure 5 Effects of SPINK5 on mucin production by A549 cells stimulated with neutrophil elastase (NE) showing by PAS staining. (A) Unstimulated cells, (B) Cells transfected with SPINK5 expression vector (unstimulated) (C) Cells stimulated with NE (200 μ M) (no transfection), (D) Cells transfected with SPINK5 expression vector and stimulated with NE (200 μ M). (Representative of three independent experiments).

and conjunctival epithelial cells.²⁵ Our results show that biological action of SPINK5 differs from other protease inhibitors in this regard as it stimulates the release of IL-6 and IL-8. This observation strongly suggests that even though protease inhibitors may have some common class-defined actions and inhibit proteases, some biological properties may be significantly different among specific molecules.

Mucus production by airway epithelial cells protects the respiratory tract against pathogenic and environmental challenges such as pollens, dusts, particles and chemicals. Secreted mucin traps particles, toxins, bacteria and viruses in inhaled air. Mucin is generally cleared by ciliary motion and by cough. Overproduction or decreased clearance of mucin causes occlusion of airways with mucus plugs and is one of the most important factors in the morbidity and mortality of chronic airway diseases including asthma.²⁶ Our results suggest that SPINK5 may slightly decrease mucus production but this is not mediated by its effect on MUC5AC expression. PAS staining is not specific for MUC5AC protein but it stains all mucin products in the cells. Therefore, it is possible that SPINK5 exerts its effect on secretory mucin genes other than MUC5AC such as MUC5B, MUC2, MUC8 and MUC19.

We have not observed any clear effect of SPINK5 on apoptosis even though cells transfected with SPINK5 vector displayed significantly higher cell death. Proteases such as granzymes were shown to stimulate apoptosis by activating several caspases such as caspase 3 and 9. Accordingly protease inhibitors were shown to have inhibitory actions on apoptosis.^{27,28} However, another protease inhibitor, tissue factor pathway inhibitor-2 (TFPI-2), a member of the Kunitz-type family of serine protease inhibitors, strongly inhibits the proliferation of VSMCs without any effects on apoptosis.²⁹ In addition, *Medicago scutellata* trypsin inhibitor (MsTI), a serine protease inhibitor, decreased the clonogenic survival of breast and cervical cancer cells.³⁰ Therefore, proteases and protease inhibitors may have a variety of effects on cell proliferation, cell death and apoptosis and these effects may in fact be class specific. Another possible reason for the lack of any effect on apoptosis by SPINK5 may be that apoptosis induced by H₂O₂ may not be caspase dependent. It seems that unlike several protease inhibitors SPINK5 does not have any effect on apoptosis but similar to some others it decreases cell viability. However, it must be pointed out that our results with H₂O₂ cannot be directly extrapolated to other stimuli including air pollutants, viruses such as RSV and bacteria such as *Leigoneilla pneumonia*, diphtheria toxin and CO even though their effect may be similar to that caused by H₂O₂. The exact mechanism as well as the reason for different effects of various proteases and protease inhibitors remains to be determined.

Our study has apparent weaknesses. First of all, even though our study defines new properties of SPINK5 other than protease inhibition, it does not provide information concerning its biological activity *in-vivo*. Secondly, even though our result suggests that SPINK5 has certain effects on cell viability and mucus production it does not provide definitive information on the exact mechanisms. Finally, other important and relevant questions such as the effect of SPINK5 on the epithelial integrity provided by ZO1 and

other tight junction molecules remain to be investigated. These questions require further studies.

To conclude, our study suggests that SPINK5 is an important molecule in asthma. Its role extends beyond its well known protease inhibitor properties.

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Conflict of interest statement

The authors have no potential conflict of interest.

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